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266 Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects

SERIE UNIVERSITARIA

Fundación Juan March

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Organized by

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I N D E X

PROGRAMME

INNOVATIONS ON PROTEASES AND THEIR INHIBITORS: FUNDAMENTAL AND APPLIED ASPECTS.

1. Monday 30th:

Chairmen: F.X. Avilés and T.L. Blundell

H. Neurath - The Regulation of protease action.

A. Puigserver - Control of the expression of pancreatic proteases.

H. Fritz - Protease inhibitors as physiological regulators and pharmacological protectors.

Chairmen: W. Bode and V. Turk.

T.L. Blundell - New insights into cellular and viral proteinase structures and the design ofinhibitors.

C.S. Craik - Structure-function analysis of serine proteases using mutagenesis.

E.W. Davie - Proteases of blood coagulation.

2. Tuesday 1st:

Chairmen: P. Carbonero and J. Kenny

K. Suzuki - Molecular biology of calpain and calpastatin.

V. Turk - Properties and structure of lysosomal cysteine proteinases and their comparison with plant enzymes.

J. Kenny - Plasma membrane metallo-endopeptidases.

S. Shaltiel - KSMP - A membranal metalloendopeptidase that splits off the tails of receptor kinases.

Chairmen: H. Neurath and A. Puigserver.

L.D. Fricker - Carboxypeptidase E/H and the processing of bioactive peptides.

R.L. Stevens - Mast cell carboxypeptidases and serine proteases

J. Vendrell - Activation of pancreatic procarboxypeptidases.

3. Wednesday 2nd:

Chairmen: R. Huber and K. Wüthrich

W. Bode - The crystal structure of thrombin as a starting point for designing inhibitors.

R.W. Carrell - A new latent (L) conformation of serpins: evidence for a mobile reactive centre.

V. Turk - Current status on cystatins.

4. Thursday 3rd:

Chairmen: T.E. Creighton and C.A. Ryan

C.A. Ryan - Signal transduction pathways for wound-inducible proteinase inhibitor genes in plants.

P. Carbonero - Cereal inhibitors of serine proteases active against phytophagous insects.

J.J. Sánchez-Serrano - Wound-induced expression of proteinase inhibitors in plants.

B. San Segundo - Expression and hormonal regulation of proteolytic activities in germination of cereal seeds.

Chairmen: E.W. Davie and H. Fritz

T.E. Creighton - Bovine pancreatic trypsin inhibitor (BPTI) as a model for studies of protein evolution and protein folding.

K. Wüthrich - NMR structures of protein proteinase inhibitors in solution.

R. Huber - Structural studies of proteases and their protein inhibitors.

H. Neurath
A. Puigserver
H. Fritz

The Regulation of Protease Action

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Research on proteases and their regulation has a long and distinguished history that goes back to the crystallization of pancreatic proteases and protease inhibitors by Northrop, Kunitz and Herriott (1939) who demonstrated that these proteins obey the thermodynamic criteria of constant solubility and hence are chemically pure entities. The seeds to a molecular description of protease regulation were sown by Linderstrom-Lang and Ottesen's perspicacious observation that the conversion of ovalvumin to plakalbumin was a process of *limited proteolysis* catalyzed by a microbial enzyme, subtilisin (1947). The first quantitative description of the primary events of zymogen activation, i.e. the cleavage of a specific peptide bond and the elucidation of the chemical structure of the activation peptide of trypsinogen and chymotrypsinogen, respectively, by Desnuelle et al. and by Neurath and Davie (1955) had to await Sanger's method of protein sequence analysis. The elucidation of the structural and mechanistic consequences of this primary chemical event has depended on the determination of the x-ray structure of these proteases and their zymogens. As in other areas of protein science, research on proteolytic regulation was driven by advances in methodology and instrumentation. The more recent advances are the results of the application of the most advanced techniques of protein chemistry and molecular biology which have confirmed as well as revised some of the earlier views. Having had the good fortune of witnessing and contributing to some of these developments, I shall discuss in a historical perspective the current status of the field, with particular emphasis on the topics of the present conference.

The two principal methods of protease regulation are zymogen activation and protease-inhibitor interaction. They are mechanistically different and distinct processes which conceivably have diverged from a common ancestral prototype. In general, protein protease inhibitor have a less restricted specificity than their cognate proteases and in specific instances (e.g. serpines), they undergo significant structural alterations when forming inactive protease-inhibitor complexes (R. Huber).

Some of the most fundamental and yet incompletely understood aspects of zymogen activation can be summarized by the following questions:

1. What structural features of the zymogen determine its proteolytic cleavage sites? What is the significance of C-terminal processing in specific cases?

2. What is the nature of the processing enzymes in different systems, particularly in those which are extrinsic to the cells in which the zymogens are segregated?

3. How does the primary event of peptide bond cleavage relate to the conformational transition that generates enzymatic activity? Does the activation peptide (domain) per se influence the structural rearrangement? Are there alternative methods of initiating zymogen activation?

4. How have the zymogens of the "simple" digestive proteases acquired the more complex mosaic of domains seen in the regulatory proteases?

A certain parallelism exists between the physiological regulation of protease action and the regulation of other types of enzymes, notably those that are regulated by ligand interaction (allostery) and by post-translational modification of side chains by phosphorylation. In all cases regulation can proceed through transduction of a signal that is amplified by linked, consecutive enzymatic reactions (cascades). The primary difference is that regulation by proteolytic peptide bond cleavage is essentially irreversible, whereas regulation by ligand binding or by phosphorylation is reversible. A parallelism exists also between the phenomenon of *autoinhibition* exhibited by certain calcium/calmodulin regulated protein kinases and zymogen activation in that removal of the calcium/calmodulin docking domain of kinases causes activation (albeit with loss of calcium sensitivity) (T. R. Soderling et al., K. A. Walsh et al.), just as the removal of the amino-terminal activation peptide generates protease activity. These and related evolutionary considerations will underlay the present discussion. In this respect, the emergence of regulatory proteases endowed with domains that fulfill intrinsic physiological functions and specify recognition sites with cell surface receptors, has elevated the study of proteases to a more sophisticated level than was assigned to them initially. Their *in vivo* interaction with other cell constituents invites caution in reaching conclusions from studies of purified proteases, removed from their physiological environment.

CONTROL OF THE EXPRESSION OF PANCREATIC PROTEASES.

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About 20 proteins are synthesized and secreted from the exocrine pancreas of higher vertebrates, a number of them being proteases responsible for the digestion of dietary proteins and peptides. It is recognized for a long time that their levels are regulated by the composition of the diet since increased intakes of proteins led to increased levels of proteolytic enzymes as a result of modifications in the rate of enzyme synthesis. Besides peptides or amino acids, a number of gastro-intestinal hormones were also found to modulate the biosynthesis of pancreatic endopeptidases.

In an attempt to determine the genetic mechanisms which mediate such changes it was necessary to find out whether the responses of proteases to dietary manipulations involved transcriptional and/or translational control of gene expression. This was achieved by measuring the relative levels of specific mRNAs in the rat pancreatic tissue, first by using an in vitro cell-free translation system and later on with cDNA probes through hybridization experiments. This experimental approach indicated that the synthesis of serine proteases was mostly regulated at the transcriptional level. However, the half-life of the messengers encoding some serine proteases was also changed in response to increased protein intakes, indicating that the stability of the corresponding mRNAs might also be involved in the control of the levels of the active proteases responsible for the hydrolysis of protein substrates.

Since a cationic, a neutral and two anionic forms of trypsin as well as two chymotrypsins, two elastases and several carboxypeptidases are known to be present in the rat pancreatic secretion, it was interesting to find out whether isoenzymes may respond to similar signals when rats are adaptated either to a high-protein diet or to a protein-free diet. By use of synthetic oligonucleotides complementary to the 3' non-coding regions of the mRNAs specific for trypsin isozymic forms it was found that the mechanism by which

the biosynthesis of cationic and anionic trypsins is regulated was not unique. By contrast, trypsin synthesis was coordinately regulated with the synthesis of the pancreatic secretory trypsin inhibitors strongly supporting the idea that the molecular mechanisms controlling the enzyme and its specific inhibitor are quite similar.

All the above-mentioned studies led us to clone a specific trypsin gene in order to study its upstream region and the corresponding interacting proteins involved in its transcriptional regulation in response to dietary manipulations.

Protease inhibitors a physiological regulators and pharmacological protectors

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The (primary) so-called inflammatory response of the organism to an irritating stimulus (tissue damage due to multiple trauma or major surgery, invasive microbae, etc.) is characterized by complex mutual interactions of cellular and humoral systems, directed physiologically towards repair and healing. The relation of stimuli, mediators, effectors, and inhibitors to each other finally determines the effectiveness or failure of the inflammatory response. Concerning the latter, excessive release of cellular enzymes (e.g. PMN elastase, macrophage cathepsin B, mast cell tryptase) as well as uncontrolled activity of the plasma cascade proteinases (plasma kallikrein, thrombin, plasmin, complement esterases, etc.) seem to play a prominent role in the ongoing biochemical destruction of vital tissue and soluble proteins following blunt major injury (1). Such proteolysis-induced pathomechanisms are enabled by a concurrently arising imbalance - either locally or systemically - between the proteinases and their regulatory antagonists (e.g. $\alpha_1$proteinase inhibitor = α_1PI, $\alpha_2$macroglobulin, antithrombin III, $\alpha_2$antiplasmin, C1 inactivator). In this respect among the lysosomal or granular constituents of inflammatory cells, the extracellularly released neutral serine proteinase elastase from PMN granulocytes turned out to be especially significant because of its ability to inactivate also inhibitory proteins effectively by proteolytic cleavage, partly even in the presence of its main antagonist, the α_1PI. Such a proteolytic degradation may be enhanced by oxidative denaturation of the proteins in the surroundings of activated inflammatory cells (2).

Over the last few years, we have been able to show a clear correlation between the amount of PMN elastase released into the circulating blood and the severity of organ failure in patients suffering from blunt major injury (3, 4). Determination of ela-stase in consecutive plasma samples turned out to be a helpful tool for early diagnosis and prognosis of organ failure. In addition, in pilot studies with a newly developed assay for an elastase-induced fibrinogen split product we could demonstrate for the first time that elastase has been active extracellularly in the posttraumatic course (manuscript in preparation). Similar to PMN elastase, high plasma levels of the macrophage-derived cysteine proteinase cathepsin B indicated already a few hours after the traumatic event a forthcoming organ failure (5). In contrast, plasma levels of neopterin, an end product of the macrophage GTP metabolism, increased only gradually in the early posttraumatic phase allowing, however, from the 4th post-traumatic day onwards a significant prognosis of the patient's outcome (3). Measurement of the phagocyte proteinases and elastase-derived split product in consecutive bronchoalveolar lavage fluids of traumatized patients clearly confirmed the hypothesis of proteolysis-induced pathomechanisms contributing to the development of organ failure following blunt major injury.

In several clinical studies with patients suffering from peritonitis and/or septicemia we could show that excessive local or systemic consumption of proteinase inhibitors, like α_1PI and antithrombin III, during severe inflammatory reactions is a most critical event which may contribute to the propagation of (multiple) organ failure (6, 7, 8). Therefore, supplementation of the body's inhibitor potential by exogenous proteinase inhibitors (isolated from human material or produced by gene technology) seems to be a most promising therapeutic approach. In a clinical pilot study antithrombin III was administered in a high dosage to septic patients. The results show a clear therapeutic effect of such a proteinase inhibition therapy: organ failure and late lethality was significantly decreased in these critically ill patients (9). To prove this hypothesis further, we have used recombinant proteinase inhibitors for PMN elastase and thrombin in an experimental animal shock model. We studied the effectiveness of each inhibitor to diminish the inflammatory response. Again, a significant reduction of the endotoxin-induced organ dysfunctions in septicemia by such a proteinase inhibition therapy was observed (10).

The given data show clearly that during acute inflammation the inhibitor levels have to be kept well above the normal plasma values to achieve a significant improvement of the clinical situation. As the natural sources for the isolation of proteinase inhibitors from human material are very restricted, the design of highly effective inhibitory proteins on the basis of human inhibitor molecules by molecular modelling and their production by recombinant DNA technology is the most promising approach at present to get the quantities necessary for proteinase inhibition therapy in future (11).

References

1. Jochum M. Fritz H. (1989): Pathobiochemical mechanisms in inflammation. In: Immune Consequences of Trauma, Shock and Sepsis. Faist E., Ninnemann J.L., Green D.R. (eds.), Sprin-ger-Verlag, Berlin-Heidelberg, 165-172.
2. Jochum M., Fritz H. (1990): Elastase and its inhibitors in intensive care medicine: Biomed-Progress 3, 55-59.
3. Jochum M., Dwenger A., Joka T., Sturm J.A. (1989): Posttrau-matic plasma levels of mediators of organ failure. In: Progress in Clinical and Biological Research, Vol. 308: Second Vienna Shock Forum. Schlag G., Redl H. (eds.) A.R. Liss Inc., New York, 673-681.
4. Nast-Kolb D., Waydhas Ch., Jochum M., Spannagl M., Duswald K.-H., Schweiberer L. (1990): Günstigster Operations-zeitpunkt für die Versorgung von Femurschaftfrakturen bei Polytrauma. Der Chirurg 61, 259-265.
5. Assfalg-Machleidt I., Jochum M., Nast-Kolb D., Siebeck M., Billing A., Joka T., Rothe G., Valet G., Zauner R., Scheuber H.P., Machleidt W. (1990): Cathepsin B - indicator for the release of lysosomal cysteine proteinases in severe trauma and inflammation. Biol. Chem Hoppe-Seyler 371, Suppl., 211-222.
6. Billing A., Kortmann H., Fröhlich D., Jochum M. (1989): Breakdown of C3 complement and IgG in peritonitis exudate - pathophysiological aspects and therapeutical approach. In: Progress in Biological Research, Vol. 308: Second Vienna Shock Forum. Schlag G., Redl H. (eds.), Alan R. Liss Inc., New York, 527-533.

7. Duswald K.-H., Jochum M., Schramm W., Fritz H. (1985): Re-leased granulocytic elastase: An indicator of pathobio-chemical alterations in septicemia after abdominal surgery. Surgery, 98: 892-899.
8. Jochum M., Assfalg-Machleidt I., Inthorn D., Nast-Kolb D., Waydhas Ch., Fritz H. (1990): Leukozytäre Proteinasen und Hämostasestörung bei Sepsis. In: XXXII. Hamburger Symposion über Blutgerinnung 1989: Infektion, Entzündung und Blutge-rinnung. V. Tilsner, R. Matthias (Hrsg.), Editiones <Roche>, Basel, 241-254.
9. Jochum M., Inthorn D., Nast-Kolb D., Fritz H. (1991): AT III - ein neues therapeutisches Konzept bei der Behandlung der Sepsis und beim Organversagen? In: Blut, Blutkomponenten und Blutersatzstoffe in der Intensivmedizin. Bericht über das 10. Bremer Interdisziplinäre Intensivtherapie-Colloquium. W.F. Henschel (Hrsg.), W. Zuckschwerdt Verlag München, im Druck.
10. Siebeck M., Hoffmann H., Weipert J., Spannagl M. (1989): Therapeutic effects of the combination of two proteinase inhibitors in the endotoxin shock pig. In: Progress in Clinical and Biological Research, Vol 308: Second Vienna Shock Forum. Schlag G., Redl H. (eds.) Alan R. Liss, Inc., New York, 937-943.
11. Fritz H., Collins J., Jochum M. (1991): Proteinase inhibitor candidates for therapy of enzyme-inhibitor imbalances. In: Current Concepts in the Biochemistry of Pulmonary Emphysema. C. Grassei, J. Travis, L. Casali, M. Luisetti (eds.), Sprin-ger-Verlag, London/Bi & Gi, Verona Publishers, im Druck.

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New insights into cellular and viral proteinase structures and the design of inhibitors

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We shall describe the 3-D structures of aspartic proteinases and their complexes defined by X-ray analysis at Birkbeck. These include not only cellular enzymes such as human and mouse renins, pepsin, chymosin, endothiapepsin and mucorpepsin, but also the retroviral enzyme, HIV proteinase. The high resolution studies in our and other laboratories have shown that both classes of aspartic proteinases are characterized by two catalytic aspartic acid residues at the centre of an extended active site cleft.

More than twenty X-ray structural studies of inhibitors complexed with renins and other aspartic proteinases indicate an extended conformation in the active site cleft on either side of the catalytic aspartates and complementary interactions in well defined specificity pockets. Of particular interest are the interactions at subsites S1, S1', S3' and S4' in renin which are responsible for the specificity of this enzyme.

Biochemical studies suggest that the mechanism is a non-covalent general acid-base catalysis with water playing the role of nucleophile. In the complex with a potent morpholine-containing tripeptide (CP-81,282, D.Hoover, Pfizer), the scissile bond surrogate, a difluorostatone moiety. is hydrated on the enzyme. The statine-like hydroxyl of the tetrahedral carbonyl hydrate is hydrogen-bonded to both active site aspartates 32 and 215 in the position occupied by a water in the free enzyme. The second hydroxyl oxygen of the hydrate is hydrogen-bonded only to the outer oxygen of Asp 32, whilst the $-CF_2-$group occupies an equivalent position within hydrogen bonding distance of the outer oxygen of Asp 215. We have used these experimental data as a basis for a model of the tetrahedral intermediate in aspartic proteinase-mediated cleavage of the amide bond. It is consistent with a mechanism in which Asp 32 is the proton donor and Asp 215 carboxylate polarizes bound water for nucleophilic attack. We shall describe the application of these crystallographic data to the design of molecules that seem certain to be of clinical value.

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Structure-Function Analysis of Serine Proteases Using Mutagenesis

The ability to engineer proteins for specific purposes is of enormous importance for biotechnology. However, before this achievement can be fully accomplished the fundamentals of protein design and redesign must be established. A rigorous test of one's understanding of structure-activity relationships in proteins is the use of those relationships in altering the function of a protein in a prescribed fashion. In recent years, the field of protein engineering has progressed to the point where the rational design of proteins is now providing an avenue for the development of proteins as reagents with a practical use. This is especially true of proteins whose activities and/or structures are dependent upon coordinating metal ions. The database of information on metalloproteins is developed to the extent that basic rules governing protein/metal interactions can be formulated and applied in engineering metalloproteins for specific purposes.

An essential requirement for any system involving a proteolytic enzyme is the regulation of hydrolytic activity. Laboratory methods for manipulating the activity of proteases have used synthetic inhibitors that involve affinity labeling and mechanism-based inactivation. A novel approach to regulating the activity of a protease in a metal-dependent fashion is by introducing a neighboring amino acid residue that assists in reversibly repositioning an essential active-site amino acid residue. We have engineered such a site in trypsin. A recombinant trypsin was designed whose catalytic activity can be regulated by varying the concentration of Cu^{+2} in solution. Substitution of Arg 96 with a His in rat trypsin (trypsinR96H) places a new imidazole group on the surface of the enzyme near the essential active site His 57. The unique spatial orientation of these His side chains results in the formation of a stable, metal-binding site that chelates divalent first row transition metal ions. Occupancy of this site by a metal ion prevents the imidazole group of His 57 from participating as a general base in catalysis. As a consequence, the primary effect of the transition metal ion is to inhibit the esterase and amidase activities of trypsin R96H in a velocity modulated fashion. As expected from the initial design, copper ions do not inhibit the activity of trypsin R96H in a competitive fashion. The apparent K_i for this inhibition is in the micromolar range for copper, nickel and zinc, the tightest binding being to Cu^{+2} at 21μM. The apparent K_i for Ni^{+2} is 49μM and for Zn^{+2} is 128μM. This is the same order predicted based on experimentally determined association constants for metal binding to imidazole. Trypsin R96H activity can be fully restored by removing the bound Cu^{+2} ion with EDTA. Multiple cycles of inhibition by Cu^{+2} ions and reactivation by EDTA demonstrate that reversible regulatory control has been introduced into the enzyme. Crystals of trypsin R96H were grown in $MgSO_4$ and Tris at pH 8 and then treated with 20mM $CuCl_2$, 20mM Tris pH 8. X-ray diffraction data on several crystals was collected to 2.5 Å. Structure refinement is still in progress

and currently R = 0.22. The 2Fo-Fc map shows His 57 rotated out of the active site coordinated to the bridging metal which is in turn coordinated to His 96. This structure provides evidence for the inhibition of the enzyme by Cu and verifies the design principles. Efforts are currently directed at creating tridentate binding sites with greater affinity and selectivity for the transition metals. The introduction of a coordination complex into trypsin involving the active-site His 57 illustrates how a metal switch can be engineered into a protease for the purpose of regulating its function. This is a useful step in protein design which may not be limited to proteases since histidine is one of the most common amino acids at the active site of a protein. It may be feasible to introduce a metal-dependent regulatory switch into many histidine-dependent proteins, several of which are of significant therapeutic and biotechnological importance.

Critical components of substrate specificity in several enzymes have been studied by making amino acid substitutions in the structure and characterizing the functional consequences of the alterations. Though powerful, this approach generally requires a prior assessment of the importance of the substituted amino acid. A genetic selection can significantly expedite analysis as it provides a means to search a large number of protein structures for those that satisfy a functional requirement, making it unnecessary to economize on the number of mutants that may be sampled. Asp189 and Ser190 are located at the base of the substrate binding pocket in trypsin and are major components in conferring the basic amino acid substrate specificity on the enzyme. We have replaced Asp189 and Ser190 (D189, S190) with all other amino acid combinations. This library of variants was analyzed using a genetic selection which uses trypsin expression to confer a nutritional advantage on bacteria. The selection relies on Arg auxotrophic *E. coli* secreting active trypsin into the periplasm and converting a non-nutritionally active Arg derivative into an anabolic source of Arg. The selection was shown to span 5 orders of magnitude of proteolytic activity by using plasmids encoding mutant trypsins of known activity to confer selective advantage to *E. coli*. 90,000 transformants of the library were searched resulting in 68 clones expressing active trypsin mutants. The isolates were screened for arginyl amidolysis activity using a microplate reader and arginyl esterolysis activity using an Arg-ester-overlay assay. The nucleotide sequence of trypsin was determined for 55 of 68 isolates in which the trypsin activity had been confirmed; trypsin and 15 trypsin mutants were identified. These enzymes with partially preserved function were characterized kinetically on arginyl and lysyl peptide substrates. Alternate arrangements of amino acids in the substrate binding pocket sustained efficient catalysis. A negative charge at amino acid position 189 or 190 was shown to be essential for high-level catalysis. With the favored aspartic acid residue at position 189, several amino acids could replace serine at position 190. Modulation of the specificity for arginine and lysine substrates was shown to depend on the amino acid at position 190. The regulatory effect of the amino acid side chain at position 190 on substrate specificity was also reflected in substrate binding pockets of naturally occurring trypsin homologs such as thrombin, kallikrein and urokinase. We are currently overexpressing a subset of the binding pocket mutants and purifying them to crystallinity for structural analysis.

Proteases of Blood Coagulation

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The generation of fibrin during the blood coagulation cascade results from the stepwise interaction of many proteins that circulate in blood in a precursor or inactive form. Each of the plasma proteins are activated by minor proteolysis and are converted to an active enzyme or cofactor in the process. The initiation of blood coagulation is triggered by tissue factor, an integral membrane glycoprotein that comes in contact with factor VII in the blood after vascular injury. Factor VII and tissue factor form a complex at the site of injury during which the factor VII is converted to an active serine protease (factor VIIa). These reactions initiate the extrinsic pathway of blood coagulation. The complex of factor VIIa-tissue factor then converts factor X to a serine protease (factor Xa) and this enzyme in turn converts prothrombin to thrombin in the presence of factor Va.

Factor VII, factor X and prothrombin as well as factor IX, protein C, and protein S are vitamin-K dependent proteins synthesized in the liver and secreted into the blood. Each contains γ-carboxyglutamic acid and five of these proteins are converted to serine proteases. The vitamin K-dependent proteins are synthesized with a preproleader sequence that is removed during protein biosynthesis by signal peptidase and a processing protease. The latter enzyme recognizes Arg residues in P1 and P4 positions in the substrate. A potential candidate for this processing has been purified in our laboratory employing rabbit liver microsomes.

When thrombin is generated in the final stages of the coagulation cascade, it converts fibrinogen to fibrin and also activates factor XIII. The latter protein when activated (factor XIIIa) crosslinks fibrin monomers by forming ϵ-(γ-glutamyl) lysine bonds between two adjacent molecules. This leads to the formation of a strong fibrin clot. Thrombin can also activate factor XI, converting it to a serine protease (factor XIa). The latter enzyme then activates factor X in the presence of factor VIIIa. These reactions constitute the intrinsic pathway of blood coagulation.

The coagulation cascade is regulated in part by a number of plasma serine protease inhibitors such as LACI and antithrombin III. It is also regulated by activated protein C which inactivates factor Va and factor VIIIa.

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Molecular Biology of Calpain and Calpastatin

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Calpain partcipates in various cellular functions mediated by calcium ions as one of the receptors but its precise physiological function is still not clear (1,2). Two isozymes with distinct calcium sensitivities are known, μ- and m-calpains which require μM and mM levels of calcim ions, respectively, for activity in vitro. Both are heterodimers composed of a catalytic 80kDa and a regulatory 30kDa subunits.

Various lines of evidence indicate that calpain which had been believed to be active is actually an inactive proenzyme (1,2). Inactive calpain translocates from the cytosol to the membrane upon binding calcium, where autocatalytic activation occurs in the presence of phospholipids such as PIP2 and calcium. During the activation, the N-terminal regions of both subunits are modified but the modification of the 80kDa subunit is essential for the appearance of activity. When cells are stimulated by various outer stimuli, very rapid conversion of inactive calpain to the active form is observed. The active form disappears rapidly in a few minutes. This instatbility of the active form is a main reason why nobody has yet isolated the active form.

Since calpain is activated at the membrane, membrane proteins and membrane associated proteins are presumed primary targets of calpain (1,2). In particular, protein kinase C (PKC) is translocated and activated under conditions similar to those for calpain. Both enzymes are activated at the membrane simultaneously. Active calpain hydrolyzes only the active form of PKC (3). Further, the calpain gene is a member of phorbol ester responsive genes and the transcription level is significantly increased by treatment of cells with phorbol ester. This is a feed forward regulation, because PKC eventually increases the level of calpain which is responsible for its down regulation. Recently, we found that transcription factors, c-Jun and Fos are good substrates of calpain(4). The expression of calpastatin cDNA in cultured cells enhances the c-Jun induced transcriptional activation of the TRE-CAT gene (5), which is lowered below the control level by expression of the antisense-calpastatin cDNA. These results together with others suggest that calpain participates in the regulation of signal transduction mediated by calcium, especially in the diminution of the signal. Our recent molecular and cell biological studies on the structure and physiological function of calpain are discussed.

References

1) K. Suzuki, S. Ohno, Cell Structure Function, 12, 1-5 (1990).
2) K. Suzuki, S. Imajoh, Y. Emori, H. Kawasaki, Y. Minami, S. Ohno, Adv. Enzyme Regul., 27, 153-169 (1988)
3) S. Ohno, Y. Akita, A. Yano, K. Suzuki, J. Biol. Chem., 265, 6296-6300 (1990)
4) S.-I. Hirai, H. Kawasaki, M. Yaniv, K. Suzuki, FEBS Lett., 287, 57-61 (1991)
5) A. Hata, S. Ohno, Y. Akita, K. Suzuki, J. Biol. Chem., 264, 6404-6411 (1989)

PROPERTIES AND STRUCTURE OF LYSOSOMAL CYSTEINE PROTEINASES AND THEIR COMPARISON WITH PLANT ENZYMES

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The cysteine proteinases (EC 3.4.22) are found in animals, plants and microorganisms. Recent studies indicate the existance of several gene families as papain calpain, streptococcal, clostripain, picorna virus family and others. The most studied cysteine proteinase is the plant enzyme papain which represent a focal point for mechanistic and structural studies.

Papain exhibits considerable structural homology to mammalian cysteine proteinases, lysosomal cathepsins B,H,L and S. These enzymes play an important role in the intracellular protein degradation under normal and pathological conditions (inflammation, diseases of the central nervous system, muscle dystrophy and malignancy). They are single polypeptides with Mr of about 25000, sometimes in a two-chain form (cathepsin B,H,L) or single-chain form (cathepsin S). Lysosomal cathepsins are synthesized as larger precursor proteins that are then processed to the mature forms. These mature forms are glycosilated, exept cathepsin S. Whereas cathepsins B,H and L have been extensively studied and their primary structures are known, there was only recently shown by the amino acid sequence analysis that cathepsins L and S are structurally different enzymes. All four cathepsins are inhibited by tight and reversibly binding protein inhibitors cystatins.

Very recently the crystal structure of human cathepsin B was determined indicating that its overall folding patern and the arrangement of the active site residues are similar to sequentially related cysteine proteinases papain and actinidin of plant origin. From the structure the well known dipeptidyl-carboxypeptidase activity of cathepsin B might be explained.

Beside well known plant cysteine proteinases papain and actinidin several other enzymes were also isolated and characterized. The amino acid sequences of chymopapain, papaya proteinase IV and bromelain shows that these enzymes are also members of the papain family.

1) Turk V. (1986) Cysteine Proteinases and Their Inhibitors, Walter de Gruiter, Berlin-New York.

2) Katunuma N. and Kominami E. (1989) Intracellular Proteolysis, Japan. Scient. Press., Tokyo.

3) Bond J.S. and Butler P.E. (1987) Intracellular Proteases, Ann. Rev. Biochem.,56, 333-364.

4) Shaw E. (1990) Cysteinyl Proteinases and Their Selective Inactivation. In : Advances in Enzymology, 63,271-347.

5) Kamphuis I.G., Drenth J., Baker E.N. (1985) J. Mol. Biol. 182, 317-329.

6) Ritonja A., Popović T., Kotnik M., Machleidt W. and Turk V. (1988) FEBS Lett. 228,341-345.

7) Ritonja A, Čolić A., Dolenc I., Ogrinc T., Podobnik M. and Turk V. (1991) FEBS Lett. 283, 329-331.

8) Ritonja A. Rowan A.D. Buttle D.J., Rawlings N.D. and Barrett A.J. (1989) FEBS Lett. 247, 419-429.

9) Ritonja A., Buttle D.J., Rawlings N.D., Turk V. and Barrett A.J. (1989) FEBS Lett. 258, 109-112.

10) Musil Dj., Zučić D., Turk D., Engh R.A., Mayr I., Huber R., Popović T., Turk V., Towatari T., Katunuma N. and Bode W. (1991) EMBO J.(vol. 10, Sept. issue)

Plasma Membrane Metallo-Endopeptidases

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Ectoenzymes This presentation is concerned with a group of peptidases located at cell-surfaces. They are, by definition ectoenzymes, being integral membrane proteins exhibiting a highly asymmetric topology, such that the bulk of the enzyme, including the active site, is at the external surface of the membrane. Their functional roles therefore concern the hydrolysis of peptide substrates in the extra- and inter-cellular spaces. These may include bioactive peptides released by secretory processes in the immediate vicinity or at a distance from the peptidase; while in the gut the same enzymes play a role in protein digestion. Although cell-surface peptidases may be recycled by membrane internalization, they never have contact with peptides in the cytoplasm and hence play no part in intracellular protein turnover. Their physiological roles are: (1) termination of peptide signals, (2) activation of peptides, (3) protection of cells from unphysiological peptide signals and (4) scavenging of peptides for nutritional purposes. Twelve peptidases, all abundant in renal and intestinal brush borders, have been characterized in some detail. Most are exopeptidases: aminopeptidases N, A, P and W; dipeptidyl peptidase IV; peptidyl dipeptidase A; carboxypeptidases M and P; gamma-glutamyl transpeptidase and membrane dipeptidase. Additionally, there are two metallo-endopeptidases, which are the subject of this review.

Endopeptidase-2: an enzyme of restricted distribution and low efficiency in hydrolysing peptides. This enzyme was first recognized in 1981 as a rat renal microvillar endopeptidase, distinguishable from endopeptidase-24.11 by its insensitivity to the specific inhibitor, phosphoramidon. It is an SS-linked tetramer, with two dissimilar subunits of approximately 80kDa. Immunohistochemistry reveals it to be in the brush border of the juxta-medullary nephrons and in the apical membrane of the intestine, extending from the duodenum to the rectum. Weak reactivity was detected in the thyroid and salivary glands, but so far in no other organs. The antibody to the rat enzyme cross reacts with mouse tissues, revealing an identical distribution and demonstrating a close relationship with meprin. Indeed structural and catalytic properties point to a family of peptidases, including rat endopeptidase-2, mouse meprin and human PABA-peptide hydrolase (an intestinal brush border enzyme capable of hydrolysing Bz-Tyr-4-benzoate). However, there are some differences in the detailed catalytic properties: endopeptidase-2 attacks azocasein more slowly and PABA-peptide more rapidly than does meprin. cDNA cloning is in progress for all three peptidases and the derived sequences are awaited with much interest.

Endopeptidase-24.11: an enzyme with a wide distribution and an important role in the inactivation of neuropeptides. This enzyme was discovered in 1969 in rabbit renal brush borders, but it has a very wide, though not ubiquitous distribution in many cell types, including other brush borders, endocrine cells, myo-epithelia, neurons and Schwann cells. For several model membrane systems, such as renal microvilli, choroid plexus and brain striatal synaptic membranes, endopeptidase-24.11 is the enzyme that initiates the attack on a range of biologically active peptides. These include tachykinins (eg substance P), bradykinin, enkephalins, angiotensins and the natriuretic peptides. Its primary specificity is for a hydrophobic amino acid residue in the P_1' position, but subsite interactions and conformational features greatly influence the efficiency of hydrolysis of peptides, there being a 100-fold difference in the kcat/Km ratios for substance P and LHRH. Except for the latter peptide, endopeptidase-24.11 seems to be much more efficient than endopeptidase-2 in hydrolysing neuropeptides, but apart from a slow attack on interleukin-1 beta, it has little ability to hydrolyse large peptides or protein substrates (for which endopeptidase-2 is a more effective enzyme). Atrial and brain natriuretic peptides are likely physiological targets for endopeptidase-24.11. In the nervous system the enzyme has two quite distinct locations: in peripheral nerve it is a marker for Schwann cell membranes, which ensheath nerve fibres. In the brain it has a well defined distribution in the striatal-nigral pathway, where it is a marker for a population of neurons and located on axonal membranes and at synapses, in both pre- and post-synaptic membranes. There is now substantial evidence that endopeptidase-24.11 is a major enzyme in terminating some peptidergic signals.

KSMP - A Membranal Metalloendopeptidase that Splits Off the Tails of Receptor Kinases

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The kinase splitting membranal proteinase (KSMP) is a Zn^{++} metalloendopeptidase which was originally identified by its ability to split off the carboxyterminus tail of the catalytic subunit (C) of cAMP-dependent protein kinase (PKA) (Alhanaty and Shaltiel, BBRC, 89, 323 (1979), Alhanaty et al., PNAS 78, 3492 (1981) ; Curr. Topics in Cell. Regul. 27, 267 (1985)). It is a neutral proteinase (pH optimum 6.8-7.4), has a subunit M.W. of $\sim$ 90 kDa, is inhibited by EDTA, EGTA and chymostatin (at $\geqslant 10^{-3}$M), but not inhibited by phosphoramidon, leupeptin, antipain, elastinal, pepstatin, PMSF, TLCK, TPCK and DTNB. In addition to PKA, KSMP splits off the carboxyterminus tails of the EGF- and insulin receptor kinases. The three protein kinases have in common a cluster of acidic amino acid residues at their tails (positions 327-337, 979-991 and 1268-1286, respectively). These tails are clipped off in a conformation-dependent manner, i. e., no proteolysis occurs if the substrates are pre-denatured (Alhanaty et al., PNAS 78, 3492 (1981); Seger et al., J.B.C 263, 3496 (1988), EMBO J. 8, 435 (1989)). This negatively charged cluster of amino acids constitutes a key biorecognition element for KSMP since: (1) monoclonal antibodies (mAb-103) against a branched polyamino acid containing clusters of D and Y cross react with the C subunit of PKA but not with its KSMP cleavage product (C'), in which the C-terminal tail is clipped off; (2) mAb-C9, a monoclonal antiidiotype of mAb-103, specifically binds to the active site of KSMP and inhibits its activity; (3) Synthetic peptides containing a DDYEEEE stretch are competitive inhibitors of KSMP (K_i=24-46 μM); (4) The peptide FDDYEEEEI inhibits the cleavage of all three substrates and prevents the binding of mAb-C9 to KSMP. In view of the important role played by this cluster of acidic amino acids in the substrate recognition of receptor kinases, KSMP provides a useful tool for studying the relationship between the structure and function of growth factor and hormone receptors, while the monoclonal antibodies and the peptide inhibitors may be used to elucidate the physiological role of KSMP.

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Carboxypeptidase E/H and the Processing of Bioactive Peptides

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Most peptide hormones and neurotransmitters are produced by limited proteolysis of precursors at specific sites. In many cases, an endopeptidase initially cleaves these precursors at pairs of basic residues, or occasionally at single basic residues. Then, a carboxypeptidase removes these basic residues from the C-terminus of the peptides. A single carboxypeptidase appears to be involved with the processing of a wide variety of peptide hormones and neurotransmitters[1]. This enzyme has been designated EC 3.4.17.10, and is alternatively known as carboxypeptidase E (CPE), carboxypeptidase H, and enkephalin convertase. CPE is a member of the metallocarboxypeptidase gene family, with approximately 20% amino acid homology between CPE, CPA, CPB, and mast-cell CPA, and approximately 40-50% homology between CPE, CPN, and CPM.

Although carboxypeptidase E is not the rate-limiting enzyme in the production of most peptides, there are several cases where CPE appears to be rate-limiting. One example is the conversion of rat β-endorphin 1-27 into β-endorphin 1-26, which requires removal of a C-terminal His residue. This step occurs very slowly in tissues which produce β-endorphin, and both forms of the peptide are found in moderate levels. Purified CPE slowly cleaves the C-terminal His from synthetic peptides that resemble the C-terminus of β-endorphin, supporting the proposition that CPE is involved with this step *in vivo*[2]. Since β-endorphin 1-27 is a potent opiate antagonist, whereas β-endorphin 1-26 is neither an agonist or an antagonist, this processing step has important consequences for the biological activity of the peptide.

In order to understand the regulation of CPE activity, we have used both *in vivo* and *in vitro* systems. In a variety of cell culture systems, as well in intact animals, the levels of CPE mRNA and/or CPE activity have been found to be regulated by conditions that alter the production of peptide hormones and neurotransmitters. However, CPE is regulated much more slowly than the peptides, and the magnitude of the changes in CPE levels is much lower than the magnitude of the changes in peptide levels. Also, in several systems, conditions that alter peptide levels had no effect on CPE mRNA and/or activity. These findings indicate that CPE is not coordinately regulated with the peptide substrates, which is consistent with the non-rate-limiting role for this enzyme in the production of most peptide hormones. In the cases where CPE activity is regulated, this could have a large impact on the small number of peptide for which CPE is the rate-limiting step.

The gene for rat CPE has recently been isolated and partially sequenced as a preliminary step in the identification of regulatory elements[3]. The 5' flanking region contains consensus sites for several trans-acting factors, including SP-1, NF-1, Pan-1, AP-2, and others. The intron/exon organization of the CPE gene differs from the organization of the genes for CPA and CPB, with only a single intron conserved between these three genes. This conserved intron is located at the junction between two folding domains proposed for CPA. The homology between CPE and CPA/B is spread out over all of the exons of the CPA/B genes, and most of the exons of the CPE gene. The last exon of the CPE gene has no homology with any of the other carboxypeptidases, consistent with the proposal that this C-terminal region is the membrane-binding anchor of the protein[4].

Although the catalytically important amino acid residues within CPA and B are conserved within CPE, the pH optima of these enzymes are substantially different. CPA and B are maximally active at neutral pH, whereas CPE is maximally active in the acidic pH range (pH 5-6) with extremely low activity at pH 7. To determine whether this represents a fundamental difference in the active site residues of CPE, we examined the effect of pH, temperature, and metal substitutions on the kinetic properties of substrate hydrolysis. The results of these analyses indicate that the acidic pH optimum for CPE is primarily due to a Km effect, rather than a Vmax effect. This is consistent with an active site for CPE which resembles that of CPA/B. In addition, there appear to be two ionizable groups that are responsible for the decrease in substrate binding at pH values > 6. This indicates that CPE activity is highly regulated by pH over the physiological range of 5-7. Thus, pH may be the primary factor in the intracellular regulation of CPE: processing of peptide precursors does not occur in the endoplasmic reticulum or Golgi apparatus, which posses a neutral internal pH, and only starts once the peptides are segregated into the trans-Golgi network, which is slightly acidic in pH.

1. Fricker, L.D. in: Peptide Biosynthesis and Processing (ed. L.D. Fricker) CRC Press, Boca Raton (1991)
2. Smyth, D.G., Maruthainer, K., Darby, N.J., and Fricker, L.D. J Neurochem 53:489 (1989)
3. Jung, Y-K., Kunczt, C., Pearson, R., Dixon, J.E., and Fricker, L.D. Mol Endocrin in press (1991)
4. Fricker, L.D., Das, B., and Angeletti, R.H. J Biol Chem 265:2476 (1990)

Mast Cell Carboxypeptidases and Serine Proteases

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Mast cells are readily distinguished from other hematopoietic cells by their characteristic secretory granules, which contain multiple cell-specific serine proteases and a carboxypeptidase [designated mast cell carboxypeptidase A (MC-CPA)] bound to highly sulfated proteoglycans. Mucosal mast cells present in the gastrointestinal tracts of helminth-infected mice express mouse mast cell protease (MMCP) -1 and MMCP-2, but not MMCP-5 or MMCP-6, and little, if any, MC-CPA (1-8). In contrast, mouse serosal mast cells express MC-CPA, MMCP-3, MMCP-4, MMCP-5, and MMCP-6, but not MMCP-1 or MMCP-2 (1-8). An immature population of mast cells can be obtained by culturing mouse bone marrow cells for 1-3 wk in the presence of interleukin-3 (IL-3) rich WEHI-3 cell conditioned medium (9). These *in vitro*-differentiated bone marrow-derived mast cells ($BMMC_W$) can give rise to both mucosal mast cells and serosal mast cells when injected *in vivo* into mast cell-deficient W/W^V mice (10). Mouse $BMMC_W$ contain abundant levels of MC-CPA, MMCP-5, and MMCP-6 mRNAs, but do not contain MMCP-2 or MMCP-4 mRNAs. Thus, $BMMC_W$ must cease to express MC-CPA and MMCP-5, and begin to express MMCP-1 and MMCP-2 when they differentiate into mucosal mast cells. Alternatively, these BMMC must express MMCP-4 when they differentiate into serosal type mast cells.

Culture of mouse bone marrow cells in medium containing recombinant c-kit ligand (rKL), rIL-3, or IL-3-rich WEHI-3 cell-conditioned medium resulted in cell populations consisting of <30%, >99%, and >99% mast cells, respectively, as defined histochemically. Although mouse rKL was not as selective as mouse rIL-3 in its ability to induce mast cells, only the cells in the rKL-treated cultures expressed the same protease transcripts found in serosal mast cells (Gurish et al., submitted to J. Exp. Med.). To determine if rKL directly or indirectly induced this transcriptional phenotype, safranin$^-$ $BMMC_W$ were maintained in medium containing rKL, rIL-3, or both cytokines. $BMMC_W$ exposed to rIL-3 alone or in the presence of rKL remained safranin$^-$, did not express MMCP -4 mRNA, and contained reduced levels of MMCP-6 mRNA. In contrast, $BMMC_W$ exposed to rKL alone became safranin$^+$, expressed high levels of MMCP-4 mRNA and MMCP-6 mRNA; however, they contained low amounts of histamine and MC-CPA enzymatic activity relative to *in vivo*-differentiated serosal mast cells. rKL is thus a pluripotent cytokine that induces progenitor mast cells to preferentially transcribe those proteases found in serosal mast cells, but it is a relatively ineffective granule maturation factor. Other *in vitro* studies revealed that IL-3, IL-4, and IL-10 were three cytokines that controlled expression of the mucosal mast cell proteases MMCP-1 and MMCP-2. These are the first studies to define the transcriptional phenotype of mast cells derived from bone marrow progenitors by any cytokine, to compare and distinguish the phenotypes elicited by four cytokines capable of acting on bone marrow progenitors and immature mast cells, and to demonstrate an antagonistic effect of IL-3 on mast cell differentiation.

The MC-CPA gene was found to be expressed relatively early along with FcϵRI when progenitor cells differentiate into $BMMC_W$, $BMMC_{IL\text{-}3}$, or $BMMC_{KL}$. Inspection of the 5' flanking region of the human and mouse MC-CPA

genes revealed a potential binding motif for the GATA family of transcription regulatory factors, indicating a potential role for these *trans*-acting factors in protease gene regulation. GATA-1, GATA-2, and GATA-3 were found to be expressed in several mouse and rat mast cell lines that contain MC-CPA and other proteases in their cytoplasmic granules (11). GATA-1 mRNA was not detected in P815 cells, an immature mouse mastocytoma-derived cell line that lacks electron dense granules and has low levels of secretory granule proteases. Because the 5′ flanking regions of the mouse and human MC-CPA genes contained a conserved GATA-binding motif 51-base pairs upstream of their translation-initiation sites, the ability of GATA-binding proteins to regulate the promoter activity of the MC-CPA gene was examined in rat basophilic leukemia cells, mouse P815 cells, and transfected mouse P815 cells that expressed GATA-1. In all three mast cell lines, the promoter activity of the MC-CPA gene depended on the GATA-binding site. GATA-1, GATA-2, and GATA-3 are thus the first DNA-binding proteins identified in mast cells which regulate the promoter activity of a gene that encodes a secretory granule protease.

References

1. Serafin, W.E., E.T. Dayton, P.M. Gravallese, K.F. Austen, and R.L. Stevens. 1987. J. Immunol. 139:3771.

2. Reynolds, D.S., R.L. Stevens, W.S. Lane, M.H. Carr, K.F. Austen, and W.E. Serafin. 1990. Proc. Natl. Acad. Sci. USA 87:3230.

3. Reynolds, D.S., R.L. Stevens, D.S. Gurley, W.S. Lane, K.F. Austen, and W.E. Serafin. 1989. J. Biol. Chem. 264:20094.

4. Serafin, W.E., D.S. Reynolds, S. Rogelj, W.S. Lane, G.A. Conder, S.S. Johnson, K.F. Austen, and R.L. Stevens. 1990. J. Biol. Chem. 265:423.

5. Serafin, W.E., T.P. Sullivan, G.A. Conder, A. Ebrahimi, P. Marcham, S.S. Johnson, K.F. Austen, and D.S. Reynolds. 1991. J. Biol. Chem. 266:1934.

6. McNeil, H.P., K.F. Austen, L.L. Somerville, M.F. Gurish, and R.L. Stevens. J. Biol. Chem., in press

7. Reynolds, D.S., D.S. Gurley, K.F. Austen, and W.E. Serafin. 1991. J. Biol. Chem. 266:3847.

8. Le Trong, H., G.F.J. Newlands, H.R.P. Miller, H. Charbonneau, H. Neurath, and R.G. Woodbury. 1989. Biochemistry 28:391.

9. Razin, E., J.N. Ihle, D. Seldin, J-M. Mencia-Huerta, H.R. Katz, P.A. LeBlanc, A. Hein, J.P. Caulfield, K.F. Austen, and R.L. Stevens. 1984. J. Immunol. 132:1479.

10. Nakano, T., T. Sonoda, C. Hayashi, A. Yamatodani, Y. Kanayama, T. Yamamura, H. Asai, T. Yonezawa, Y. Kitamura, and S.J. Galli. 1985. J. Exp. Med. 162:1025.

11. Zon, L.I, M.F. Gurish, R.L. Stevens, C. Mather, D. Reynolds, K.F. Austen, and S.H. Orkin. J. Biol. Chem., in press.

ACTIVATION OF PANCREATIC PROCARBOXYPEPTIDASES

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Procarboxypeptidases A and B, the inactive precursors of carboxypeptidases A and B, are unique among major pancreatic proenzymes in that they release a very long peptide (94 and 95 amino acid residues long, respectively) upon activation by limited proteolysis. Here we discuss the activation process and the structural features of the two zymogens from porcine origin. The study was started with the aim of gaining insight into some basic issues: the possible effect of the released activation segments on the activation process, the definition of the sequence of molecular events that take place, and the structural reasons of proenzyme inactivity, among others.

The activation processes of both proenzymes share some common characteristics: initial proteolysis starts at the border between carboxypeptidase and activation segment and further proceeds from the C–terminal end of the latter; the generated active carboxypeptidase participates in both cases in the trimming of the released activation peptides; and finally, generation of carboxypeptidase activity has been observed to be dependent on the degree of degradation of the severed activation fragments in the two cases.

There are, on the other hand, some differences between the two processes studied. In the first place, the A form generates two species of CPA whereas a single CPB is generated from PCPB. However, this does not affect activation since no correlation has been found between the presence of either form of CPA and the rate of expression of enzymatic activity. The main difference lies in the rate of activation, which is biphasic in the A form and monophasic and much faster in the B form. Since both zymogens are converted into carboxypeptidases in a comparably short time, the differences observed in activation rate might be a consequence of differences in the type of interaction between the enzyme and the fragment(s) released or, in other words, to differences in the inhibitory capabilities of the activation fragments. Preliminary experiments had shown that the activation segment isolated from PCPA acted as a strong inhibitor of the active enzyme (1), whereas no such inhibitory potential has so far been observed for any activation fragment derived from PCPB.

We have analyzed the process of conversion of porcine procarboxypeptidases A and B into the fully active carboxypeptidases at the molecular level (2,3). Preparative isolation and amino acid and sequence analysis of the activation segment and fragments derived from it have been performed and the sequence of events during activation has been formulated.

Participation of carboxypeptidases in the process of trimming its own activation segment is a remarkable characteristic readily deduced from the analysis of the fragments generated. In the case of PCPB, a further demonstration of the participation of active carboxypeptidase B in this proteolytic action is furnished by the results obtained in activations performed in the presence of carboxypeptidase inhibitors. These experiments, where the proteolytic degradation of the activation segment fragments is slowed down by the presence of inhibitors, are also valuable in making possible the detection of short-lived intermediates which would be otherwise undetectable in their absence (4).

The picture resulting from the activation experiments commented so far would be incomplete without consideration of the three-dimensional structures of the zymogens. The three-dimensional crystal structures of procarboxypeptidases A and B, and the solution structure of the activation domain of the B form have been recently obtained (5, 6). Their observation permits the visualization of the target points for activation and the definition of the structural basis of zymogen inactivity. The observed differences in the rate of activation and in the residual activities against peptide substrates between PCPA and PCPB may also be explained by small differences in the three-dimensional structures of the zymogens, in the type of contacts established between enzyme and activation domain and in the location of target points for proteolysis.

Comparison of the crystal and solution structures of activation domain B shows that the globular structure is preserved after isolation. As expected, those regions showing a higher degree of disorder in the solution structure largerly coincide with surface areas which are in contact with the enzyme in the intact zymogen. This results indicate that the long intermediate fragments detected during the activation process are also likely to maintain their original globular conformation, since the activation domain constitutes a limit in the degradation of the activation segment.

REFERENCES

(1) San Segundo, B., Martínez, M.C., Vilanova, M., Cuchillo, C.M. & Avilés, F.X. (1982) *Biochim. Biophys. Acta* 707, 74-80.
(2) Vendrell, J., Cuchillo, C.M. & Avilés, F.X. (1990) *J. Biol. Chem.* 265, 6949-6953.
(3) Burgos, F.J., Salvà, M., Villegas, V., Soriano, F., Méndez, E. & Avilés, F.X. (1991) *Biochemistry* 30, 4082-4089.
(4) Villegas, V., Salvà, M., Ventura, S., Guasch, A. Vendrell, J & Avilés, F.X. Workshop on *Innovations on proteases and their inhibitors.* Madrid, October 1991
(5) Coll, M., Guasch, A., Avilés, F.X. & Huber, R. (1991) *EMBO J.* 10, 1-9
(6) Vendrell, J., Billeter, M., Wider, G., Avilés, F.X. & Wüthrich, K. (1991) *EMBO J.* 10, 10-15.

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The Crystal Structure of Thrombin as a Starting Point for Designing Inhibitors

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Thrombin plays a central role in thrombosis and hemostasis, but is also implicated in wound healing and in various disease processes. It is therefore a prime target for inhibitor therapy, so that its spatial structure is of great value for designing knowledge-based inhibitors.

We have determined and refined the high-resolution X-ray crystal structure of the covalent complex formed between human α-thrombin and D-PheProArg chlorometylketone (PPACK-thrombin) [Bode et al., 1989; Bode et al., 1991]. Using the refined coordinates of PPACK-thrombin, the X-ray crystal structures of two slightly different human α-thrombin-hirudin complexes [Rydel et al., 1990; Grütter et al., 1990], of two bovine thrombin structures (with and without bound fibrinopeptide) [Martin et al., 1991; Martin et al., 1992], and of human α-thrombin ternary complex containing a covalently bound fibrinopeptide A fragment and a non-covalently-bound peptide derived from the hirudin tail segment [Stubbs et al., 1991] have been solved.

The thrombin B-chain exhibits the characteristic polypeptide fold of trypsin-like proteinases; several extended insertions form large protuberances, however. The most remarkable feature at the thrombin surface is a prominent canyon-like active-site cleft, shaped and narrowed by "insertion loops." The specificity of thrombin for distinct macromolecular substrates and inhibitors is in accord with this narrow and deep cleft. Hirudin, a 65 residues long polypeptide from the leech Hirudo medicinalis, occupies the whole active-site cleft, thus preventing access of substrates to the active-site residues.

The strongly furrowed thrombin surface around the active site is well-suited to bind small synthetic inhibitors. The structure-based de novo design of novel non-peptidic drugs is, nevertheless, still very difficult and might be confusing. As documented for the specific arginine-based antithrombotic

argatroban [Matsuzaki et al., 1989], even docking attempts with known "lead" compounds, found and improved by classical screening procedures, can lead to wrong results.

The structures of thrombin complexes with several arginine and benzamidine-derived inhibitors have now been established crystallographically derived either from analogous trypsin or from thrombin structures [Bode et al., 1990; Turk et al., 1991; Brandstetter et al., 1991]. These structures stress the importance of intermolecular hydrogen bonds with the S3-subsite and of the steric fit of the amino and the carboxy-terminal hydrophobic inhibitor moieties into the characteristic aryl-binding site and the hydrophobic S-2 cavity of thrombin. Particularly surprising is the finding that the distal amidino or guanidyl group of the central basic residue must not necessarily interact in a symmetrical manner with the specificity determining carboxylate of Asp 189 at the bottom of the S1-pocket, but can point towards the hydrophobic "back" of this pocket. These experimental structures are starting points for a systematic modification of the "lead" compounds and the design of more potent and effective inhibitors. First attempts in this direction are promising.

References

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S.R. and Hofsteenge, J. (1989) EMBO J., 8, 3467-3475.

Bode, W., Turk, D. and Karshikov, A. (1991) submitted for publication.

Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Fenton, J. W. and Roitsch, C. (1990) Science 249, 277-280.

Grütter, M.G., Priestle, J.P., Rahnel, J., Grossenbacher, H., Bode, W., Hofsteenge, J. and Stone, S.R. (1990) EMBO J. 91, 2361 -2365.

Martin, P.D., Robertson, W., Turk, D., Huber, R., Bode,W. and Edwards, B.F.P. (1991), submitted for publication.

Martin, P.D., Kunjummen, R., Kumar, V., Bode, W., Huber, R. and Edwards, B.F.P. (1992), manuscript in preparation.

Stubbs, M.T., Oschkinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S.R. and Bode, W. (1991), submitted for publication.

Matzusaki, T., Sasaki, C., Okumura, C. and Umeyama, H. (1989) J. Biochem. (Tokyo) 105, 949-952.

Bode, W., Turk, D. and Stürzebecher, J. (1990) Eur. J. Biochem. 193, 175-182.

Turk, D., Stürzebecher, J. and Bode, W. (1991) FEBS Let. 287, 133-138.

Brandstetter, H., Turk, D., Hoeffken, W., Grosse, D., Huber, R., Stürzebecher, J., Martin, P.D., Edwards, B.F.P. and Bode, W. (1991), submitted for publicaion.

A NEW LATENT (L) CONFORMATION OF SERPINS: EVIDENCE FOR A MOBILE REACTIVE CENTRE

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The serpins are unique amongst the families of serine protease inhibitors in that they have a large and unconstrained reactive centre loop[1]. Recent crystallographic studies[2] indicate that this loop can exist as an exposed helix, and earlier work[3] had shown that proteolysis of the reactive centre resulted in the re-insertion of the cleaved peptide into the large A-sheet that forms the predominant feature of the molecule. Evidence is provided here that the reactive centre loop in the intact serpins is mobile and that inhibitory activity is dependent on a folding of the N-terminal portion of the loop with potential movement in and out of the A-sheet. The finding that this movement can be induced to give a new stable latent (L) conformation supports the concept of a flexible reactive centre that can adapt its conformation to meet differing physiologic requirements and suggests a structural basis for the latent form of plasminogen activator inhibitor and the heparin activation of antithrombin.

The evidence on which these deductions are based is summarised in the figure which shows, in diagram form, the relation of the reactive centre loop P_1-P_{14} to the 5-stranded A sheet. a) Represents the structure of cleaved inhibitory serpins with the loop reinserted in the sheet. b) Is the structure of the non-inhibitory serpin ovalbumin [2,4] in which the loop is locked in a helical form due to mutations in the P_{10}-P_{14} region[5]. c) Represents a proposed model of the partially reinserted loop to give the native active 'canonical' form; overinsertion of this loop, beyond 6 residues, occurs on exposure to 0.9M guanidinium chloride with all the inhibitory serpins, to give a loss of activity and physical properties that match those of latent DPAI-1. d) The technique of Schulze et al[6] can be used to insert various length synthetic loop peptides and form binary complexes with consequent loss of inhibitory activity.

Changes in thermal stability (e and f) are seen to match the movement in and out of the A sheet of the extra strand, thus the native S form undergoes a considerable increase in stability on cleavage at P_1, but this S-R change does not occur when movement of the loop is blocked in the P_{10}-P_{14} stalk area as with ovalbumin shown in cleaved and noncleaved forms or with the P_{10} proline variant of antithrombin whose cleaved form cP_{10} is identical to that of uncleaved native S antithrombin. f) As predicted the induced latent L-form of antithrombin has a thermal stability that matches that of the hexapeptide binary complex BC6.

These findings are supported by other physical measurements and ancillary studies of site specific mutants of various serpins and of antithrombin with and without added heparin. The findings strongly

support the proposed linked mobility of the reactive centre and the A-sheet[7], that evolution has adopted to give modulation of serpin activity and an ability to vary exposure of the reactive centre to proteolytic attack. The topology of the reinsertion process must await further crystallographic structures.

References

1. Huber R & Carrell RW (1989) *Biochemistry* **28**: 8951-8966.

2. Stein PE, Leslie AGW, Finch JT, Turnell WG, McLaughlin PJ & Carrell RW (1990) *Nature* **347**: 99-102.

3. Loebermann H, tukuoka R, Diesenhofer J & Huber R (1984) *J Mol Biol* **177**: 531-556.

4. Wright HT, Quian HX & Huber R (1990) *J Mol Biol* **213**: 518-528.

5. Perry DJ et al (1991) *FEBS Letts* **286**: 2-4.

6. Schulze AJ, Baumann U, Knof S, Jaeger E, Huber R and Laurell C-B (1990) *Eur J Biochem* **194**: 51-56.

7. Stein P & Chothia C (1991) *J Mol Biol* **221** (in press).

CURRENT STATUS ON CYSTATINS

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Within the last ten years it has become evident that newly discovered protein inhibitors of cysteine proteinases, named cystatins, offer a new insight into the processes in which they participate. The cystatins are tight and reversibly binding inhibitors of the most papain-like cysteine proteinases. They form a superfamily of sequentially homologous proteins subdivided into three families, the stefins, the cystatins and the kininogens. The recently discovered cathelin, a protein inhibitor of cathepsin L which we isolated from pig leucocytes indicates that a new family of cysteine proteinase inhibitor may exist.

The stefins (also family 1) are single-chain proteins with Mr of about 11000, which lack disulfide bonds and carbohydrates. Whereas human and rat stefin A were found in high concentrations in various types of epithelial cells, the human and rat stefin B are found to be widely distribuited amongst different cells and tissues. Stefin B forms disulfide linked inactive dimers. In addition to stefins of animal origin, in rice there are structurally related proteins, named oryzacystatins.

The cystatins (also family 2) have Mr of about 13000 and contain two disulphide bonds. These proteins occour at relatively high concentrations in many biological fluids. The best known representatives of this family are chicken cystatin and human cystatin C. Human cystatin C variant (point mutation at position 68) is a major constituent of the amyloid fibrils in patients from Iceland suffering from hereditary cerebral hemorrhage with amyloidosis. With the exception of the rat cystatin C, all other members of this family described so far are not glycosilated.

The kininogens (family 3) are single chain proteins. They are three distinct types of kininogens: H-kininogens with Mr of about 120000, L-kininogens with Mr of about 68000 and T-kininogens(known as "acute phase protein") with Mr of about 68000. They are glycoproteins. They have been found in plasma and other secretions of mammalian species. By sequence analysis was found that teh kininogen molecule is composed of three cystatin-like domains. Second and the third domain are inhibitory active. The kininogens are strong inhibitors of papain and cathepsins L and weaker inhibitors of cathepsin H and particularly of cathepsin B. The crystal structure of chicken egg white cystatin was determined. It was suggested that the highly conserved region Gln53-Gly57 (QVVAG region) folds into a tight -hairpin loop, which together with a second hairpin loop (Pro103-Trp104 residues) and the N-terminus form a hydrophobic wedge-shaped "edge" highly complementary to the active site cleft of papain as shown by docking experiments. This proposed "trunk model" was

recently verified by the crystallized stoichiometric complex of recombinant human stefin B and papain .

Structural analysis of stefins, cystatins and three domains of the kininogens revealed that they share extensive sequence homology, enough to be classified as members of one superfamily. Recently, a scheme for the evolution of mammalian cystatins was proposed based on the numbers of copies of cystatin-like domains and the presence or absence of disulphide bonds.

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1) Barrett A.J., Rawlings N.D., Davies M.E., Machleidt W., Salvesen G. and Turk V. (1986) In: Proteinase Inhibitors (Barrett A.J. and Salvesen G., eds.) Elsevier, Amsterdam, pp 515-569.

2) Fritz H., Schmidt I. and Turk V. (1990) Proteinase Inhibitors and Biological Control.,. Biol. Chem. Hoppe-Seyler, 371 suppl., Walter de Gruyter Berlin.

3) Bode W., Engh R., Musil Dj., Thiele U., Huber R., Karshikov A., Brzin J. Kos J. and Turk V. (1988) EMBO J., 7, 2593-2599.

4) Stubbs M.T., Laber B., Bode W., Huber R., Jerala R., lenarčič B. and Turk V (1990) EMBO J., 9, 1939-1947.

5) Turk V and Bode W. (1991) FEBS Lett., 285, 213-219.

6) Rawlings N.D. and Barrett A.J. (1990) J. Mol. Evol., 30, 60-71

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SIGNAL TRANSDUCTION PATHWAYS FOR WOUND-INDUCIBLE PROTEINASE INHIBITOR GENES IN PLANTS.

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Several signalling molecules have been identified that regulate the expression of genes coding for proteinase inhibitor proteins that are synthesized in plant leaves in response to predator and/or pathogen attacks. Regulatory molecules include oligosaccharide fragments of plant and fungal cell walls (1,2), methyl jasmonate (3), a recently discovered polypeptide of 18 amino acids (4),and plant hormones such as abscisic acid (5) and auxin (6). Methyl jasmonate, placed on wicks near tomato, tobacco or alfalfa plants, is transported through volatile airborne molecules to plants and is highly specific in activating proteinase inhibitor genes in the leaves of these plants (3). Methyl jasmonate is thought to be interacting directly with the intracellular signal transduction pathways close to the gene level. Interplant communication mediated by methyl jasmonate has been demonstrated (3). We have recently isolated an eighteen amino acid polypeptide from tomato leaves that powerfully activates the expression of proteinase inhibitor genes in tomato plants (4). This polypeptide, called systemin, is the most potent inducer of the two proteinase inhibitor genes yet found. Radioactively labelled systemin is transported throughout young tomato plants when place directly on leaf wounds in the manner consistent with a systemic signal (4). The structure and properties of systemin and its cDNA and gene will be presented. Antisense constructs of the cDNA, under the regulation of the CaMV 35S promoter, when stably integrated into the tomato genome and expressed, result in plants that only weakly respond to wounding (7). This indicates that the expression of prosystemin is necessary for systemic signalling. The synthesis of prosystemin and the generation of systemin by wounding will be discussed. (Supported in part by Washington State College of Agriculture and Home Economics Project #1791 and grants from NSF and the McKnight Foundation.)

REFERENCES:

1. Darvill, A.G. and Albersheim, P. 1984. Phytoalexins and their elicitors: A defense against insects and pathogens. Ann. Rev. Plant Physiol. 35, 243-275.

2. Ryan, C.A. 1987. Oligosaccharide signalling in plants. Ann. Rev. Cell. Biol. 3 317.

3. Farmer, E.E. and Ryan, C.A. 1990. Interplant communication: Airborne methyl jasmonate induces the synthesis of proteinase inhibitor genes in plant leaves. Proc. Natl. Acad. Sci. USA 87, 7713-7716.

4. Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. 1991. A polypeptide from tomato leaves activates the expression of proteinase inhibitor genes. Science (in press).

5. Pena-Cortez, H. Sanchez-Serrano, J.J., Mertens, R. and Willmitzer, L. 1989. Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci. USA 86, 9851-9855.

6. Thornburg, R.W. and Li, X. 1990. Auxin levels decline in tobacco foliage following wounding. Plant Physiol. 93, 500-504.

7. Thornburg, R.W., Cleveland, R.E. and Ryan, C.A. 1987. Wound-inducible expression of potato inhibitor II gene in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA 84, 744-748.

CEREAL INHIBITORS OF SERINE PROTEASES ACTIVE AGAINST PHYTOPHAGOUS INSECTS.

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A substantial fraction of the albumins and globulins from cereal endosperms is represented by a complex protein family that includes trypsin inhibitors and monomeric, dimeric and tetrameric inhibitors of heterologous α-amylases (For reviews, see 1 and 2). This group of inhibitors which may play a protective role against insect predators, is encoded by a multi-gene family which is dispersed over four out of the seven homoeology groups of chromosomes in wheat and barley, as has been determined by electrophoretic analysis of the proteins and by Southern hybridization analysis with cDNA probes, using the appropriate aneuploid lines. An up-dated compilation of characterized subunits and chromosomal location of their genes appears in Table 1.

Table 1 Genomic distribution of genes encoding α-amylase/trypsin inhibitors

Inhibitory activity (aggregation)	Protein (syn.)	Clones	Gene locus (syn.)	Chromosome no. genome (arm)
α-amylase (monomeric)	BMAI-1 (Horv1)		Imha-H1	?
	WMAI-1 (0.28)	pUP-28	Imha-D1	6D(S)
	WMAI-2		Imha-B1	6B(S)
α-amylase (dimeric)	BDAI-1	pUP-44	Idha-H1	?
	WDAI-1 (0.53)		Idha-B1-1	3B(S)
	WDAI-3		Idha-B1-2	3B(S)
	WDAI-2 (0.19)		Idha-B1	3D(S)
α-amylase (tetrameric)				
1st subunit	BTAI-CMa		Itha-H1 (Cma1)	1(7H)
	WTAI-CM2		Itha-B1	7B(S)
	WTAI-CM1	pCT-2	Itha-D1	7D(S)
	RAI		Itha-R1	?R
2nd subunit	BTAI-CMb		Itha-H2 (Cmb1)	4(4H)
	WTAI-CM16	pCT-3	Itha-B2	4B(S)
	WTAI-CM17		Itha-D2	4D(S)
3rd subunit	BTAI-CMd	pUP-38	Itha-H3 (Cmd1)	4(4H)
(2 copies)	WTAI-CM3B	pCT-1	Itha-B3	4B(S)
	WTAI-CM3D		Itha-D3	?D
Trypsin	BTI-CMe	λcCMe λgCMe	Itr-H1 (Cme1)	3(3H)
	WTI?			?A
	?	pCT-4		?A, B, D?
	RTI		Itr-R1	3R
Trypsin	BTI-CMc		Itr-H2 (Cmc1)	1(7H)
Unknown	?	pUP-23	?	?
	?	pCT-88	?	?A, B, D?
Unknown	?	pUP-13	?	?

Although some experiments concerning wheat monomeric α-amylase inhibitor WMAI-1 may be discussed (4), w'll focus our attention in recent findings concerning barley trypsin inhibitor CMe.

A cDNA encoding trypsin inhibitor CMe from barley endosperm has been cloned and characterized. The longest open reading frame codes for a typical signal peptide of 24 residues followed by a sequence which is identical to the known amino acid sequence of the previously reported inhibitor protein except for three more aminoacids at the C-terminal (VVL). Southern-blot analysis of wheat-barley addition lines has shown that chromosome 3H of barley carries the gene(s) for CMe. This protein is present at less than 2%-3% of the wild-type amount in the mature endosperm of the mutant Risø 1508 with respect to Bomi barley, from which it has been derived, and the corresponding steady state levels of the CMe mRNA are of the same order (≈1%). One or two copies of the CMe gene (synonym gene locus ItrH1) per haploid genome have been estimated both in the wild type and in the mutant and DNA restriction patterns are identical in both stocks, so neither a change in copy number nor a major rearrangement of the structural gene account for the markedly decreased expression. The mutation at the lys 3a locus in Risø 1508 has been previously mapped in chromosome 5H. A single dose of the wild-type allele at this locus (Lys 3a) restores the expression of gene CMe in chromosome 3H to normal levels, indicating that the expression of gene CMe is regulated in trans by the gene at the lys 3a locus (3).

The genomic clone for CMe has a duplication in a region of ≈4 kbp, the first copy being identical to the cDNA. The gene has no introns. In the promoter of CMe, besides the typical TATA box and the -300 endosperm box, two long imperfect repeats of ≈600 bp are to the found. At -2000 bp of the translation initiation, there is a retroproson-like sequence of ≈300 bp with sequence similarities to the WIS-2 element of wheat and the COPIA element of Drosophila. Transient expression studies in protoplasts derived from developing barley endosperms and coleoptiles have been carried out using chimeric constructs with different parts of the CMe promotor fused to the reported GUS gene.

Transgenic tobacco plants carrying a chimeric gene carrying the 35S CaMV promoter and the structural sequence from the CMe cDNA have been obtained, and insect tests against Agrotis ipsilon and Spodoptera littoralis have been performed. Increased mortality in the range of 30-40% over controls have been observed with larvae of both species.

REFERENCES

(1) García-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987). In: Miflin, B.J. (ed) Oxford Surveys of Plant Molecular and Cell Biology. Vol. 4, pp. 275-334. Oxford University Press.

(2) García-Olmedo, Salcedo, G., Sanchez-Monge, R., Hernandez-Lucas, C., Carmona, M.J., Lopez-Fando, J.J., Fernandez, J.A., Gomez, L., Royo, J., García-Maroto, F. and Carbonero, P. (1991). In: "Barley: Genetics, Molecular Biology and Biotechnology" (ed. P.R. Shewry). C.A.B. Intern (in press).

(3) Rodriguez-Palenzuela, P., Royo, J., Gomez, L., Sanchez-Monge, R., Salcedo, G., Molina-Cano, J.L., García-Olmedo, F. and Carbonero, P. (1989). Mol. Gen. Genet. 219: 474-479.

(4) García-Maroto, F., Carbonero, P. and García-Olmedo, F. (1991) Plant Mol. Biol. 17: 1005-1011.

Wound-induced expression of proteinase inhibitors in plants.

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The proteinase inhibitor II (pin2) gene family is constitutively expressed in flowers of potato and tomato, and in potato tubers. In addition, its mRNA accumulates in the plant foliage as a result of transcriptional activation of the gene family following mechanical wounding. This accumulation is not restricted to the damaged leaves as pin2 mRNA also accumulates in distal, non-damaged tissue. The plant hormone abscisic acid (ABA) is involved in the pin2 induction upon wounding. ABA treatment results in pin2 mRNA accumulation in leaves in the absence of any apparent damage. Consistent with this, a rise in endogenous ABA concentration is observed upon wounding which peaks before maximal pin2 mRNA accumulation is reached. Moreover, plant mutants deficient in ABA synthesis fail to accumulate pin2 after wounding, and no change in their low ABA level is detected. Pin2 activation in mutant plants is triggered by ABA treatment. Constitutive pin2 expression is, however, not affected by the ABA-deficiency, and thus potato droopy mutant plants have wild-type levels of pin2 mRNA in tubers and flowers.

The plant growth regulator jasmonic acid (JA) has been suggested to be involved in wound-induced responses in different plant species. It induces pin2 expression in the foliage in the absence of any mechanical damage, and it is also able to do so in an ABA-deficient background. These results can be explained by two alternative hypotheses: either there is a JA step downstream to the ABA requirement in the wound signal transduction chain leading to pin2 transcriptional activation or JA and ABA are located in different pathways, eventually converging in pin2 gene activation.

Plants react to wounding by accumulating newly synthesized products whose function is mainly related to wound healing and prevention of subsequent pathogen attack. Ubiquitous ABA involvement in plant responses to mechanical damage has been firmly established with the isolation from a potato ABA-induced leaf cDNA bank of several wound-induced clones. Among them, the cathepsin D inhibitor displays the same expression pattern as pin2 being, in addition to its wound-induced gene activation in the plant foliage, also constitutively present in tubers and flowers. In contrast, the gene encoding threonine dehydrase and another clone sharing high similarity to the bovine lens leucine aminopeptidase are expressed in flowers and wounded leaved but their mRNAs are absent (threonine dehydrase) or barely detectable (leucine aminopeptidase) in tubers. An opposite pattern regarding organ-specificity is exhibited by a gene with sequence similarities to rice oryzacystatin. In this case, constitutive expression is detectable in tubers but not in flowers of potato plants.

RNA corresponding to the described clones accumulate upon wounding in the damaged leaves but their abundance in non-damaged systemically-induced ones differs from one another. In addition to its responsiveness to ABA, JA treatment leads to accumulation of mRNA from all the above mentioned genes. These results suggest that ABA and JA are linked in the wound signal transduction pathway and, moreover, that organ specificity of these clones have to involve additional signals.

Other studies have shown the presence in potato tubers of proteins with inhibitory activity towards proteinases. Most of them are also induced in the plant foliage upon wounding, as is for instance the case of the proteinase inhibitor I (directed against serine proteinases with trypsin-like activity), the 22 kd tuber protein (also a serine proteinase inhibitor), and the metallocarboxypeptidase inhibitor (active against metalloexopeptidases). This accumulation of proteinase inhibitors observed after wounding may serve a defensive function to the plant. A decrease in the nutritional value of the tissue is induced by pests feeding on the plant which is systemically extended to other tissues that would be subsequently ingested by these pests. In this way, an array of inhibitors with different specificities towards proteinases would counteract the different enzymes present in the digestive tract.

A single pin2 promoter is able to drive constitutive expression of a reporter gene in tubers and flowers, and wound-induced expression in leaves, of transgenic potato plants. Deletion analyses of this promoter have determined a region governing expression in tubers and

wounded leaves, whilst sequences required for flower expression are apparently located elsewhere. Whether expression in tubers and wounded leaves is controlled by the same, or different, cis-regulatory motifs is however not known. This pin2 promoter has been scanned for interactions with nuclear proteins which may be relevant in regulation of gene expression. Whilst several DNA-binding activities have been identified in nuclear extracts from both pin2 expressing and non-expressing plant tissues, none bind to the important cis-regulatory elements defined by deletion analyses, or show differences in binding activity which could be related to the transcriptional status of the pin2 gene family.

Expression and hormonal regulation of proteolytic activities in germination of cereal seeds.

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We have characterized the proteolytic activities (proteases in cooperation with carboxypeptidases) involved in the different stages of germination of maize (Zea mays) grains. A sequential expression of different groups of proteases (aspartic, cysteine, serine and metallo-proteases) with pH optima in the acidic range has been found by using specific protease inhibitors. Pepstatin-sensitive proteolytic activity (aspartic-protease activity) is dominant in resting grains. Germination is accompanied by the appearance of a proteolytic activity which can be enhanced by low molecular weight thiol compounds and inhibited by thiol-protease inhibitors, which is indicative of the involvement of cysteine protease(s). This burst of cysteine protease activity is coincident with the disappearance of the main storage protein fractions. We conclude from this that cysteine protease(s), with an acid pH optimum, are good candidate(s) for the proteolytic attack of stored protein reserves in maize. After this stage, where cysteine-protease activity is dominant, a period with larger total proteolytic activity starts, coincidentally with the expression of the different types of other proteolytic activities (serine, aspartic and metallo proteases), in addition to the cysteine protease activity above mentioned. When the development of the carboxypeptidase activity during germination was analyzed, the highest activities were found during the earlier and later stages. This result is indicative of a cooperative interaction between carboxypeptidase and endoproteolytic systems in order to obtain a more effective mobilization of storage proteins in germinating maize grains. The phytohormones, gibberellic acid (GA_3) and abscisic acid (ABA) which can stimulate or inhibit, respectively, the total proteolytic activity in extracts from germinating grains, exert a differential effect on the different proteolytic activities here detected.

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BOVINE PANCREATIC TRYPSIN INHIBITOR (BPTI) AS A MODEL FOR STUDIES OF PROTEIN EVOLUTION AND PROTEIN FOLDING

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A comparison of the gene sequenceS of BPTI and that of the closely-related protein, bovine spleen inhibitor, indicated that the nucleotides coding for the inhibitor sites of these proteins had diverged more rapidly than had the remainder of the genes, including the introns.[1] Similar observations were made by others with different protease inhibitor families.[2,3] This prompted a comparsion of the protein sequences of serine proteases to determine if their active site regions demonstrated a comparable hypervariability. Significant hypervariability was found with some proteases, especially the kallikreins, suggesting that they have also evolved recently under positive selective pressures for functional differences between them.[4] The proteases and their inhibitors seem to be special in exhibiting such positive selection, which is very difficult to detect in other protein families.

BPTI has been studied primarily as a model subject for studies of protein structure, flexibility, and folding. Its three Cys residues have been used as probes of the gross conformational properties of the unfolded, reduced polypeptide chain by their tendency to form disulphide bonds between various pairs of Cys residues and by the stabilities of those disulphide bonds. This approach has been used to determine the protein folding pathway of BPTI, which is the most detailed and best-characterized folding pathway available.[5] Further understanding of the BPTI folding mechanism requires more detailed structural information about the partially-folded intermediates. Previous studies have used the trapped intermediates,[6] but protein engineering methods make it possible to construct more appropriate analogues of any intermediate in homogeneous form[7]. Cys residues not involved in disulphide bonds are replaced by Ser, which are considered to mimic best the partially ionized Cys residues of the folding protein. The conformational properties of these intermediates are being characterized by 2-D NMR techniques. The results obtained thus far and their significance for protein folding will be presented.

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1. T.E. Creighton & I.G. Charles, Sequences of the genes and polypeptide precursors for two bovine protease inhibitors, *J. Mol. Biol.* **194**, 11-22 (1987).
2. M. Laskowski Jr. et al., Ovomucoid third domains from 100 avian species: Isolation, sequences and hypervariability of enzyme-inhibitor contact residues, *Biochemistry* **26**, 202-221 (1987).
3. R.E. Hill & N.D. Hastie, Accelerated evolution in the reactive centre regions of serine protease inhibitors, *Nature* **326**, 96-99 (1987).
4. T.E. Creighton & N.J. Darby, Functional evolutionary divergence of proteolytic enzymes and their inhibitors, *Trends Biochem. Sci.* **14**, 319-324 (1989).
5. T.E. Creighton, Protein folding, *Biochem. J.* **270**, 1-16 (1990).
6. D.J. States, T.E. Creighton, C.M. Dobson & M. Karplus, Conformations of intermediates in the folding of the pancreatic trypsin inhibitor, *J. Mol. Biol.* **195**, 731-739 (1987).
7. N.J. Darby, C.P.M. van Mierlo & T.E. Creighton, The 5-55 single-disulphide intermediate in folding of bovine pancreatic trypsin inhibitor, *FEBS Letters* **279**, 61-64 (1991).

NMR Structures of Protein Proteinase Inhibitors in Solution.

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Protein proteinase inhibitors have had an important role in the development of the NMR method for protein structure determination in solution. For example, because of its ready availability, high stability in solution and favorable NMR-spectral properties, the basic pancreatic trypsin inhibitor (BPTI) has been used time and again to develop and optimize new NMR experiments for studies of proteins (e.g., 1-3). BPTI was also the first globular protein for which complete sequence-specific NMR assignments and a NMR determination of the secondary structure were obtained (4-6). Subsequently the bull seminal protease inhibitor IIA (BUSI IIA) was the first protein for which the determination of the three-dimensional structure by NMR in solution was completed (7-9). Our interest in this class of proteins has kept up over the years. For example, the structure of

hirudin was solved (10), work in collaboration with the group of Prof. F.X. Avilés resulted in the NMR structure of the activation domain from porcine procarboxipepdidase B (11,12), and we just completed the determination of highly refined structures of BPTI and several homologs of BPTI. In connection with the continued work on BPTI it became possible to extend the NMR observations to numerous individual molecules of hydration water (13). This lecture will be mostly devoted to the recent refinement of the BPTI structure and the observations made on protein hydration in aqueous solution.

REFERENCES

1. K. Wüthrich and G. Wagner, FEBS Lett. **50**, 265-268 (1975). "NMR Investigations of the Dynamics of the Aromatic Amino Acid Residues in the Basic Pancreatic Trypsin Inhibitor."

2. K. Nagayama, K. Wüthrich, P. Bachmann and R.R. Ernst, Biochem. Biophys. Res. Comm. **78**, 99-105 (1977). "Two-Dimensional J-Resolved ^{1}H NMR Spectroscopy of Biological Macromolecules."

3. Anil Kumar, G. Wagner, R.R. Ernst and K. Wüthrich, J. Amer. Chem. Soc. **103**, 3654-3658 (1981). "Buildup Rates of the Nuclear Overhauser Effect Measured by Two- Dimensional Proton Magnetic Resonance Spectroscopy: Implications for Studies of Protein Conformation."

4. A. Dubs, G. Wagner and K. Wüthrich, Biochim. Biophys. Acta **577**, 177-194 (1979). "Individual Assignments of Amide Proton Resonances in the Proton NMR Spectrum of the Basic Pancreatic Trypsin Inhibitor."

5. G. Wagner, Anil Kumar and K. Wüthrich, Eur. J. Biochem. **114**, 375-384 (1981). "Systematic Application of Two-Dimensional ^{1}H Nuclear-Magnetic-Resonance Techniques for Studies of Proteins. 2. Combined Use of Correlated Spectroscopy and Nuclear Overhauser Spectroscopy for Sequential Assignments of Backbone Resonances and Elucidation of Polypeptide Secondary Structures."

6. G. Wagner and K. Wüthrich, J. Mol. Biol. **155**, 347-366 (1982). "Sequential Resonance Assignments in Protein ^{1}H Nuclear Magnetic Resonance Spectra. Basic Pancreatic Trypsin Inhibitor."

7. P. Strop, G. Wider and K. Wüthrich, J. Mol. Biol. **166**, 641-667 (1983). "Assignment of the ^{1}H Nuclear Magnetic Resonance Spectrum of the Proteinase Inhibitor IIA from Bull Seminal Plasma by Two-Dimensional Nuclear Magnetic Resonance at 500 MHz."

8. M.P. Williamson, D. Marion and K. Wüthrich, J. Mol. Biol. **173**, 341-359 (1984). "Secondary Structure in the Solution Conformation of the Proteinase Inhibitor IIA from Bull Seminal Plasma by Nuclear Magnetic Resonance."

9. M.P. Williamson, T.F. Havel and K. Wüthrich, J. Mol. Biol. **182**, 295-315 (1985). "Solution Conformation of Proteinase Inhibitor IIA from Bull Seminal Plasma by ^{1}H Nuclear Magnetic Resonance and Distance Geometry."

10. H. Haruyama and K. Wüthrich, Biochemistry **28**, 4301-4312 (1989). "Conformation of Recombinant Desulfatohirudin in Aqueous Solution Determined by Nuclear Magnetic Resonance. "

11. J. Vendrell, G. Wider, F.X. Avilés and K. Wüthrich, Biochemistry **29**, 7515-7522 (1990). "Sequence-specific ^{1}H NMR Assignments and Determination of the Secondary Structure for the Activation Domain Isolated from Pancreatic Procarboxypeptidase B."

12. J. Vendrell, M. Billeter, G. Wider, F.X. Avilés and K. Wüthrich, EMBO J. **10**, 11-15 (1991). "The NMR Structure of the Activation Domain Isolated from Porcine Procarboxypeptidase B.

13. G. Otting and K. Wüthrich, J. Amer. Chem. Soc. **111**, 1871-1875 (1989). "Studies of Protein Hydration in Aqueous Solution by Direct NMR Observation of Individual Protein-Bound Water Molecules."

Structural studies of proteases and their protein inhibitors.

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Serine and cysteine proteases play a central role in digestion, protein degradation and bioregulation. Their activity may be controlled by activation from inactive proenzyme forms and by inhibition by natural inhibitors. Detailed structural studies have provided a basis for understanding the principles of activation, activity and inhibition of proteases. In serine proteases the functional significance of molecular flexibility was first documented (1).

The natural serine protease inhibitor BPTI served as a model to develop biophysical and biochemical techniques (eg protein NMR, hydrogen-deuterium exchange, experimental folding studies) and theoretical methods (eg molecular dynamics and *ab initio* folding studies). In the last 20 years many large families of 'small' natural serine protease inhibitors have been characterized in structure and function. They differ in polypeptide chain folds but have a similar canonical substrate-like conformation of their protease binding loops (1-6, 8-14). They have a common mode of function and are reversibly cleaved by proteases, an exception being hirudin (20), a natural inhibitor of thrombin, which blocks the enzyme binding site in a non-orthodox way by use of its globular N-terminal portion while its extended C-terminal tail fills the anion binding exo-site.

In mammalian plasma a different class of large serine protease inhibitors (serpins) occus which are regulators of serine proteases such as leucocyte elastase and thrombin. They function rather differently to the small inhibitors and, as structure analyses of α_1-protease inhibitor (7) and ovalbumin (17) have shown, undergo a transition to an inactive form. This transition involves an interesting extension of a pre-existing β-sheet (21).

Much less is known of natural inhibitors of cysteine proteases but two members of the cystatin super-family, cystatin (15, 16) and stefin in complex with papain (19) have now been structurally defined. In contrast to the serine protease inhibitors, they do not act in a substrate-like manner but occlude the enzyme binding site.

All of these inhibitors provide frameworks for the

development of specific drugs. Such studies are being vigorously pursued with leucocyte elastase (11,12) and thrombin (18,20) for replacement therapy of emphysema and thrombosis.

Pancreatic procarboxypeptidases have structurally and functionally (concerning their mechanism of inhibition) much in common with protease-protease inhibitor complexes (24).

Annexins inhibit blood clothing by a very different mode, probably through competition for membrane binding sites. Their unusual structures integrate features of integral membrane proteins and soluble proteins and lead inhibitor research into a new area (22,23).

References

1. Huber R., Bode W., In:*Accounts of Chemical Research* 1978; **11**:114-122.
2. Walter J.,Steigemann W.,Singh T.P., Bartunik H., Bode W. and Huber R., *Acta Cryst.* 1982; **B38**: 1462-72.
3. Papamokos E., Weber E., Bode W., Huber, R., Empie M.W., Kato I. and Laskowski M. Jr., *J.Mol.Biol.* 1982; **158**: 515-537.
4. Bolognesi M., Gatti G., Menegatti E., Guarneri M., Marquart M., Papamokos E. and Huber R., *J.Mol.Biol.* 1982; **162**: 839-868.
5. Chen Z., Bode, W., *J.Mol.Biol.* 1983; **164**: 282-311.
6. Marquart M., Walter, J., Deisenhofer J., Bode, W. and Huber R., *Acta Cryst* 1983; **B39**: 480-490.
7. Löbermann H., Tokuoka R., Deisenhofer, J. and Huber R., *J.Mol.Biol.* 1984; **177**: 531-556.
8. Bode W., Walter J., Huber R., Wenzel H.R. and Tschesche H., *Eur.J.Biochem.* 1984; **144**: 185-190.
9. Wlodawer A., Walter J., Huber R., and Sjölin L., *J.Mol.Biol.* 1984;**180**: 301-320.
10. Wang D., Bode W. and Huber R., *J.Mol.Biol.* 1985; **185**: 595-624.
11. Bode W., Papamokos E., Musil D., Seemueller U. and Fritz H., *The EMBO Journal* 1986; **5**: 813-818.
12. Bode W., Wei A., Huber R., Meyer E., Travis J. and Neumann S., *The EMBO Journal* 1986; **5**: 2453-2458.
13. Bode W., Papamokos E. and Musil D. *Eur.J.Biochem.* 1987; **166**: 673-692.
14. Grütter M.G., Fendrich G., Huber R. and Bode, W., *The EMBO Journal* 1988; **7**: 345-351..
15. Bode W., Engh, R., Musil D., Thiele U., Huber R., Karshikov A., Brzin J., Kos J. and Turk, V.,*The EMBO Journal* 1988;**7**: 2593-2599.
16. Bode, W., Greyling H.J., Huber R., Otlewski, J. and Wilusz T., *FEBS Letters* 1989; **242**: 285-292.
17. Wright H.T., Oian, H.X. and Huber, R., *J.Mol.Biol.* 1990;**213**: 513-528.

18. Bode W., Mayr I., Baumann U., Huber R., Stone St.R. and Hofsteenge J., *EMBO J.* 1989; **11**: 3467-3475.
19. Stubbs M., Laber B., Bode W., Huber R., Jerala R., Lenarcic B. and Turk V., *The EMBO Journal* 1990; **9**:1939-1947.
20. Rydel T., Ravichandran K.G., Tulinsky A., Bode W., Huber R., Fenton J.W. and Roitsch C., *Science* 1990; **249**: 277-280.
21. Schulze A.J., Baumann U., Knof S., Jaeger E., Huber R. and Laurell C.B., *Eur.J.Biochem.* 1990; **194**: 51-56.
22. Huber R., Römisch J. and Paques E., *The EMBO Journal* 1990; **12**: 3867-3874.
23. Huber R., Schneider M. Mayr I., Röhmisch J. and Paques E., *FEBS Lett.* 1990; **275**: 15-21.
24. Coll, M., Guasch, A., Aviles, F.X. and Huber, R., *The EMBO Journal* 1991; **10**: 1-9.

POSTERS

THE INTERACTION BETWEEN HUMAN CATHEPSIN G AND BPTI.

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Cathepsin G, from human neutrophil leukocytes, is probably responsible for the degradation of proteins in several pathological conditions and the production of angiotensin II, a potent vasoconstrictor (1). The substrate binding site of Cathepsin G is closely related to other serine proteases, like human leukocytic elastase (2). Several protein inhibitors showed affinity for cathepsin G (3), even if the basic pancreatic trypsin inhibitor BPTI was not known as active toward the enzyme. In the present study, we found that the interaction between Cathepsin G and BPTI is weak but not negligible (Fig.1), and strongly substrate-dependent (Fig.2), but it could be seen as a good model for protein-protein interaction (4).

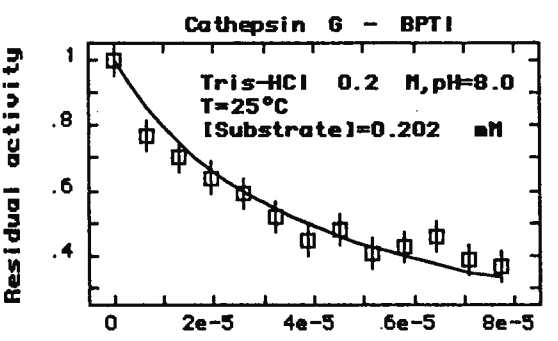

Fig.1

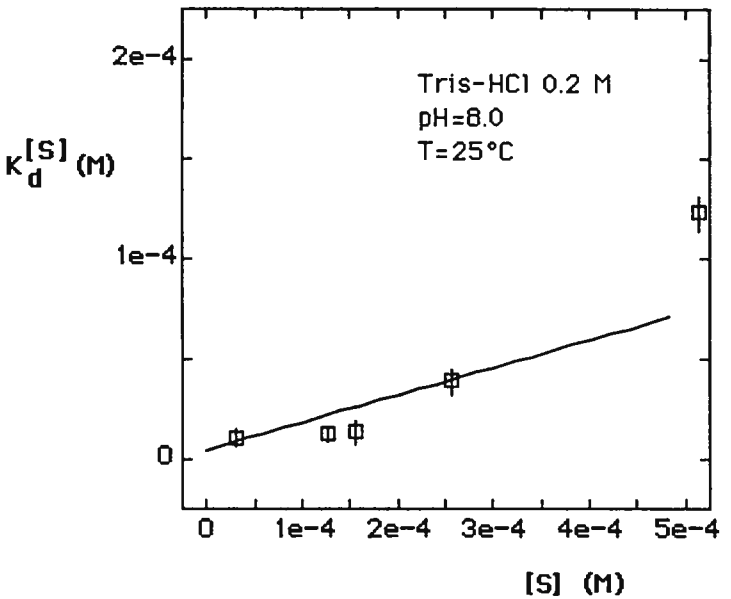

Fig.2

The determination of the apparent equilibrium dissociation constants (at substrate concentration equal to zero), and its pH-dependence were also determined.

The raw data were analyzed following the scheme proposed by Bieth (5), and the results will be discussed according to a substrate-inhibitor competition model.

REFERENCES

1) J.C.Powers, "Advances in Inflammation Research", 11, 145-157 (1986)
2) K.Nakajima, J.C.Powers, B.M.Asheand, M.Zimmerman, J.Biol. Chem., 254, 4027-4032 (1979)
3) A.J.Barrett, Meth.in Enz., 80, 561-565 (1981)
4) E.Fioretti, M.Angeletti, D.Passeri, and F.Ascoli, J.Prot.Chem., 8, 51-60 (1989)
5) J.Bieth, Bayer-Symposium V "Proteinase Inhibitors", 463-469 (1974)

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INHIBITION OF CHYMOTRYPSIN BY C1-INHIBITOR: CORRELATION WITH REACTIVE CENTRE SEQUENCES.

C1-inhibitor is a serine protease inhibitor active against C1s, C1r, factor XIa, factor XIIa, kallikrein, nerve growth factor and plasmin. These proteinases all cleave on the carboxy-terminal side of arginine residues. In their interaction with C1-inh, all form stable 1:1 complexes (proteinase:inhibitor) and under certain conditions release a small peptide from C1-inh. This arises following cleavage between the P1 arginine and P'1 threonine residues.

We have investigated the inhibitory activity of various C1-inh molecules against chymotrypsin, a protease with specificity for bulky hydrophobic residues. C1-inh(hu) inhibited chymotrypsin activity against Suc-Ala-Ala-Pro-Phe-pNA (Kass 7.3 x 10^3). Sequence analysis of the released peptide showed that cleavage occurred between the P2 alanine and P1 arginine (ie the P2 alanine had become the functional P1 residue). C1-inh(Ri), a dysfunctional protein with a P1 arginine to histidine substitution, had a five fold increase in its inhibitory activity against chymotrypsin (Kass 3.4 x10^4), and sequence analysis of the released peptide showed that the cleavage had occurred between the new P1 histidine and the P'1 threonine. This demonstrated that the inhibitory activity is not restricted to one site within the reactive centre. Rabbit C1-inh also forms a complex with chymotrypsin and human C1s, and its reactive centre sequence has been determined. This data and the results from similar structural and functional analysis of C1-inh from other species, currently in progress, will be interpreted with reference to their reactive centre sequences.

In addition to the above, we are characterising two dysfunctional C1-inhibitor proteins with mutations within the 'hinge' region. C1-inhibitor(Mo) has a substitution of a threonine to alanine at the P9 residue and C1-inhibitor(We) has a substitution of a valine to glutamic acid at the P13 residue. Both these proteins have been expressed in COS cells and structural-functional analysis of these is in progress.

USE OF SOLUBLE AND IMMOBILIZED SUBTILISIN

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Subtilisin is a versatile catalyst for hydrolysis and synthesis of acyl esters (1, 2). As an alternative in our work on hydrolysis and synthesis of acyl-sugars, subtilisin -either soluble on insolubilized- has also been employed. The differences in selectivity of acylation and deacylation of sucrose by subtilisin Carlsberg, native or insolubilized on diatomaceous earth (Celite), will be reported.

1. A. Zaks & A.M. Klibanov, JACS 108, 2767-2768 (1986).
2. S. Riva et al., JACS 110, 584-589 (1988).

SOYBEAN PROTEASE INHIBITORS IN GERMPLASM AND IN PROCESSED FOODS

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Monitoring the inactivation of protease inhibitors

Active protease inhibitors can limit the digestibility and bioavailability of soy protein, and, at least in the rat, consumption of dietary protease inhibitors can result in development of pancreatic adenomas. In order to assess the nutritional quality and healthfulness of food containing protease inhibitors, it is necessary to measure these specific inhibitors accurately. In soy products, one complication is the occurrence of multiple soybean protease inhibitors, including the Kunitz trypsin inhibitor (KTI) and the double-headed Bowman-Birk trypsin and chymotrypsin inhibitor (BBI). Both KTI and BBI exist as several isoforms, which are derived from different genes or are produced by proteolysis, and other inhibitors are also present. It is therefore impossible to establish the exact protease inhibitor composition of a sample through enzymatic assay, especially in samples with low residual inhibitor activity, such as toasted soy flours or soy protein isolates. We have found that immunoassays using monoclonal antibodies offer the specificity and sensitivity necessary to analyze complex, processed food samples (1-5). The antibodies and ELISA methods enable measurement of active KTI and BBI specifically, in the presence of denatured inhibitors. In addition, monoclonal antibodies can distinguish among the isoforms of KTI and between KTI which is free and molecules which are bound to trypsin (6). The highly specific antibodies distinguish BBI from closely related Bowman-Birk type inhibitors in lima bean. In addition to ELISA, the antibodies can be used for immunoblotting or affinity chromatography. Using the monoclonal antibody-based ELISA's, we found that the microenvironment in soy flour appears to catalyze the heat inactivation of BBI to a greater extent than KTI. Thus, although BBI is much more stable than KTI in the pure state, it is KTI which is responsible for the heat-stable trypsin inhibitory activity of soy flour.

Active protease inhibitors, which may limit the nutritional quality of foods, also have potentially beneficial effects. They have been shown to inhibit carcinogenesis, in both *in vitro* and *in vivo* systems, and to stimulate human T cells. More data on the health effects of protease inhibitors are needed to guide further development of food processing strategies for optimizing the content of protease inhibitors in soy foods. High resolution techniques, such as the monoclonal antibody-based ELISA's for measuring KTI and BBI, could help optimize production of the most nutritious and healthful soy protein product. In addition, immunochemical methods may also help determine the biological fate of ingested protease inhibitors (7).

Development of new soybean cultivars based on immunochemical screening of soybean germplasm.

Although the monoclonal antibodies proved useful to detect protease inhibitors in processed foods, we thought that they may have applicablity at an earlier stage--the development of new cultivars. As a first step in this direction, we analyzed an experimental line of soybeans, L81-4590, a KTI-null isoline of Williams 82, developed by Professor T. Hymowitz, University of Illinois. The isoline, grown isolated in a greenhouse, produced near zero levels of KTI as determined by ELISA (about 10,000-fold lower than Williams 82). However, KTI could be detected in field-grown samples, probably as a result of cross-pollination by KTI-expressing cultivars (8). If the KTI-null isolines are to be used commercially, the ELISA method could be an important quality control test. Preliminary screening of the USDA Soybean Germplasm Collection indicated that there is considerable variation in the content of protease inhibitors, indicative of a genetic potential to produce soybeans with enhanced nutritional value and requiring minimal processing (e.g., a KTI-BBI-lectin triple null), and, possibly, cultivars with higher anti-carcinogenic activities (perhaps a BBI-rich line).

Browning reactions and antigenicity of food proteins.

Since non-enzymatic browning reactions can alter protein structure in a number of ways, we studied the effects of the reactions on the ability of protein to form antigen-antibody complexes (9). Using KTI as a model protein, we evaluated processing-induced changes in antigenicity with two monoclonal antibodies. The results suggested that relatively mild conditions of heating food proteins with carbohydrates can reduce the antigenicity of the protein, prior to the occurrence of extensive browning and that judicious selection of epitope specificity can yield ELISA's sensitive to early stages of non-enzymatic browning.

Technology transfer

Because of the potential commercial value of these methods, the Agricultural Research Service has patented some of the antibody methods discussed above (10, 11). We hope that this will encourage commercial development of products based on this technology.

References

1. Brandon, D. L., Haque, S., and Friedman, M. "Interaction of monoclonal antibodies with soybean trypsin inhibitors." J. Agric. Food Chem. 35:195-200. 1987.
2. Brandon, D. L., Bates, A. H., and Friedman, M. "Enzyme-linked immunoassay of soybean Kunitz trypsin inhibitor using monoclonal antibodies." J. Food Sci. 53. 97-101. 1988.
3. Brandon, D. L., Bates, A. H., and Friedman, M. "Monoclonal antibody-base enzyme immunoassay of the Bowman-Birk protease inhibitor of soybeans." J. Agric Food Chem. 37. 1192-1196. 1989.
4. Friedman, M., Gumbmann, M. R., Brandon, D. L., and Bates, A. H. "Inactivation and analysis of soybean inhibitors of digestive

enzymes. in Kinsella, J. E., and Soucie, W. G. (eds.) Food Proteins. American Oil Chemists' Society, Champaign, IL. 296-328. 1989.

5. Brandon, D. L., Bates, A. H., and Friedman, M. "ELISA Analysis of Soybean Trypsin Inhibitors in Processed Foods." in Friedman, M. (ed.) Nutritional and Toxicological Impact of Food Processing. Plenum Press, NY. 1991. 321-337.
6. Brandon, D. L., and Bates, A. H. "Definition of functional and antibody-binding sites on Kunitz soybean trypsin inhibitor isoforms using monoclonal antibodies." J. Agric. Food Chem. 36. 1336-1341. 1988.
7. Billings, P. C., Brandon, D. L., and Habres, J. M. "Internalization of the Bowman-Birk Protease Inhibitor by Intestinal Epithelial Cells." Eur. J. Cancer 27:903-908. 1991.
8. Friedman, M., Brandon, D. L., Bates, A. H., and Hymowitz, T. "Comparison of a commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: Composition, nutritional value, and effects of heating." J. Agric. Food Chem. 39. 327-335. 1991.
9. Oste, R. E., Brandon, D. L., Bates, A. H., and Friedman, M. "Effect of Maillard browning reactions of the Kunitz soybean trypsin inhibitor on its interaction with monoclonal antibodies." J. Agric. Food Chem. 38. 258-261. 1990.
10. Brandon, D. L., Bates, A. H., and Friedman, M. "Monoclonal antibodies to soybean Kunitz trypsin inhibitor and immunoassay methods. U. S. Patent No. 3,959,410. 1990.
11. Brandon, D. L., Bates, A. H., and Friedman, M. "High affinity monoclonal antibodies to Bowman-Birk inhibitor and immunoassay methods. U. S. Patent No. 5,053,327. 1991.

REGULATION OF GERMINATION BY ABA IN *Cicer arietinum*: CHARACTERIZATION OF PROTEINS INVOLVED

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Abscisic Acid (ABA) plays an important role in maintaining the embrionary state and preventing germination. ABA promotes the synthesis of specific proteins including reserve proteins, lectins and agglutinins.

Exogenously added ABA promotes the synthesis of a polypeptide of MW=32,000 in embrionic axes of chickpea, that could be a regulator of transcription (Rodriguez et al., 1985); also, exogenously added ABA induces the synthesis of five polypeptides in embrionic axes of this legume species (Colorado et al., 1991).

Our present project aims to identify the activities of proteinase inhibitors during the germination of chickpea and to investigate their possible regulation by ABA, Calcium and other factors regulating germination.

REFERENCES

Colorado et al., 1991. Calcium dependence of the effects of Abscisic Acid on RNA synthesis during germination of *Cicer arietinum* seeds. Physiol. Plant. in press.

Rodriguez et al., 1985. Altered development of polysomal RNA activity in chickpea (*Cicer arietinum*) embryonic axis. Effects of Abscisic Acid and temperature. Planta 164, 517-523.

MECHANISM-BASED INHIBITORS OF PROLINE-SPECIFIC PEPTIDASES

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A large number of regulatory peptides include proline residues at special locations in their sequences. They are important for the structure of the peptides and they are exposed cleavage points for proline-specific peptidases. Prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV) belong to these enzyme group. To perform detailed studies of regulatory peptide processing, specific and selective inhibitors are required. Thus, we developed N-peptidyl-O-acyl hydroxylamines as specific irreversible inhibitors for PEP and DP IV. The compounds represent a new concept in mechanism-inhibition. Both acyl residues may be used to specifically design inhibitors of special application tasks.

Structure and Activity of a cyclic phosphinic hexapeptide peptide, a potent inhibitor of bacterial collagenase

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A cyclic phosphinate peptide has been synthesized as transition-state-analogue and evaluated as inhibitor of a zinc bacterial collagenase.

The basis of the activity of this peptide is attributed to the similarity of the tetrahedral phosphorus oxyanion moiety to the tetrahedral intermediate in peptide substrate arising from the addition of a zinc-water molecule to the carbonyl group of the cleaved peptide bond.

Preliminary conformational analysis of this cyclic hexapeptide utilizing the ^{1}H-NMR spectroscopy and NOE restrained molecular dynamics will reported.

The biological activity and conformational preference of this peptide allow us to propose a tentative model of the inhibitor-collagenase interactions.

Functional characterization of a new tryptase from bovine mast cells: search for its processing role.

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A seryl protease (tryptase) was purified from bovine mast cells present in connective tissue of bovine liver capsule. The enzyme was solubilized with 2 M NaCl and the apparent molecular weight determined by gel filtration in 1 M NaCl was 360 ± 40 kDa. By SDS-PAGE followed by fluorography, two bands radiolabelled with [^{3}H]-DFP with molecular masses around 40 kDa were detected. Bovine tryptase activity is inhibited by some non-proteic seryl protease inhibitors; among the high molecular weight inhibitors, only BPTI, which is also localized in bovine mast cells, specifically interacts with the enzyme with a $K_I \approx 10^{-9}$ M.

Bovine tryptase is very reactive toward synthetic coumarin-containing substrates with pair of basic aminoacids at the site of cleavage. Preliminary experiments with prohormone substrates containing dibasic or monobasic sites, indicated that the enzyme cleaves at the carboxyl site of Lys-Arg, Arg or Lys residues with a different efficiency depending on the sequences flanking the hydrolysis site. These experiments indicate that the enzyme could process proproteins and could be eventually involved in the maturation of BPTI precursor.

Design of mechanism-based inactivators for the prototypal zinc protease, carboxypeptidase A. Soumitra S. Ghosh[1], Shahriar Mobashery[2] and E.T.Kaiser[3]. The Salk Institute Biotechnology/Industrials Associates, Inc.[1], San Diego, Wayne State University[2] , Detroit and Rockefeller University[3], New York, U.S.A.

We report our studies on the mechanism-based inactivation of carboxypeptidase A (CPA) using the inhibitors, (R)-2-benzyl-5-cyano-4-oxo-pentanoic acid (**I**), N-cyanoacetyl-L-phenylalanine (**II**) and N-(3-chloropropionyl)-L-phenylalanine, (**III**). The rationale for the design of **I** was based on the known propensity of CPA to carry out proton abstraction from ketone substrate analogues [Nashed, N.T. and Kaiser, E.T. (1986) J. Am. Chem. Soc., 98, 1940-1947]. It is suggested that that enzymic deprotonation on the C-5 methylene moiety of **I** followed by isomerization generates an α-ketoketenimine intermediate, which partitions between turnover and irreversible enzyme inactivation. The enzyme inactivation exhibited pseudo-first order kinetics and was fully prevented in the presence of the competitive inhibitor, benzylsuccinic acid. The inactivation rate constant, k_{inact}, was evaluated to be 0.083 ± 0.003 min^{-1}, and from the partition ratio of 28 ± 3, k_{cat} was calculated as 1.78 ± 0.06 min^{-1}. The reversible inhibitor constant (K_i) was measured at 1.8 ± 0.5 μM, indicative of a high affinity of CPA for **I**. However, K_m for the inactivation process was determined at 4.93 ± 0.43 mM. Kinetic analysis as well as labelling studies with the radioactive form of the inactivator revealed that the stoichiometry for CPA modification by **I** approaches 1:1 ratio.

The concept was extended to include the peptidic molecules, **II** and **III**, from which CPA-catalysed proton abstraction presumably leads to the transient formation of a ketenimine and an α, β-unsaturated amide, respectively. Subsequently, it is proposed that these intermediates trap an active site nucleophile, resulting in irreversible covalent modification of the protein. In competition with the inactivation process, the enzyme hydrolyses the amide bonds of these molecules. Partition ratios of 1180 ± 40 and 1680 ± 60 were determined for **II** and **III**, respectively. N-Acrolyl-L-phenylalanine, the putative intermediate from **III**, was independently studied to test the validity of ther mechanistic scheme and was observed to be an active site-directed inactivator of CPA.

Kinetics of Autolysis of μ- and m-Calpain in the Presence and Absence of Phosphatidylinositol and Erythrocyte Ghosts

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Autolysis of μ-calpain is an intermolecular process at all Ca^{2+} concentrations between 25 and 2000 μM. Autolysis of m-calpain is an intramolecular process at Ca^{2+} concentrations of 1000 to 5000 μM but is an intermolecular molecular process at Ca^{2+} concentrations of 100 to 350 μM. Although phosphatidylinositol lowers the Ca^{2+} concentration required for autolysis, it does not change the mechanism of autolysis, i.e., autolysis is still intermolecular in the presence of phosphatidylinositol. Association with erythrocytes membranes (inside-out vesicles) has no effect on the Ca^{2+} concentration required for autolysis. Consequently, association with a cell membrane does not seem to stimulate autolysis as proposed by the membrane-activation theory. Moreover, autolysis of calpain would be hindered, not stimulated, by association with a cell membrane because an intermolecular mechanism would require that the membrane-associated molecule collide with a second calpain molecule in the cytoplasm.

Consequently, it seems unlikely that the calpains are "activated" by association with cell membranes, and it is probable that some other mechanism is involved in activation of calpain activity in living cells. This other mechanism may involve Ca^{2+} binding to specific sites on the calpain molecule, with each site responsible for a particular activity.

NEUTRAL ENDOPEPTIDASE IS HIGHLY EXPRESSED ON CELLS OF AN OSTEOBLASTIC PHENOTYPE

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Neutral endopeptidase EC 3.4.24.11 or enkephalinase (NEP) is a zinc ectoenzyme present on the surface of many cell types and is widely distributed in mammalian tissues including the kidney, brain tissue and in the immune system. The amino acid sequence of human NEP is identical with that of the common acute lymphoblastic leukemia antigen (CALLA, CD10) and is highly conserved in different species. NEP is implicated in the metabolism and regulation of a variety of peptides including the enkephalins, Substance P and atrial natriuretic factor.

We have purified NEP from bovine kidney membranes by Triton X-100 extraction and various chromatographic procedures. Utilizing our selective two-stage enzymatic assay of high sensitivity towards NEP (FEBS Lett. 255:237, 1989), we assessed NEP levels in various mouse bone marrow derived cells (BBRC 172:620, 1990). Various levels of NEP were expressed by mouse marrow stromal cell lines, with the highest levels found in cells (MBA-15) of an osteoblastic phenotype. Other cell lines of an osteoblastic phenotype also express high levels of NEP. Treatment of certain clones of MBA-15 with 1α,25-dihydroxyvitamin D_3 increased the level of NEP expression fourfold, while having no effect on alkaline phosphatase levels. These results raise the possibility that NEP may play a role in bone remodelling and may serve as a novel differentiation marker of osteoblastic cells.

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CHANGES OF MULTICATALYTIC PROTEINASE ACTIVITY IN TISSUES OF INSULIN-DEFICIENT RATS

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The multicatalytic proteinase (MCP), also designated prosome or proteasome, is a high-molecular-mass, multisubunit protein with a characteristic, cylinder-shaped structure. The enzyme is of ubiquitous occurrence in eukaryotes, suggesting a fundamental role of the molecule in cellular metabolism (1). A number of different functions have been proposed, such as post-transcriptional modulation of translational activity (2) but the physiological function(s) of the enzyme remains to be elucidated. As experiments in the insulin-deficient state of fasted, young rats have shown that there is a progressive fall in skeletal muscle protein synthesis during the first days of fasting, whereas skeletal muscle protein breakdown remains essentially unchanged during this period (3), we used this experimental approach to investigate whether any, and if so, what changes in activity and/or content of the MCP can be measured in tissues of fasted as well as fasted and re-fed rats. The results of these experiments, conducted on gstrocnemius muscle, thymus and testicular tissue show a differential response of the MCP in these three organs and favour the idea of a relationship of the MCP and steps associated with cellular protein synthesis rather than protein degradation.

(1) Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G.,Hegerl, R. and Baumeister, W. (1989) FEBS Lett. 251, 125-131
(2) Kuehn, L., Dahlmann, B. and Kopp, F. (1990) FEBS Lett. 261, 274-278
(3) Emery, P.W., Cotelíessa, L., Holness, M., Egan, C. and Rennie, M.J. (1986) Biosc. Rep. 6, 143-153

CYSTEINE PROTEINASE INHIBITOR FROM SEA ANEMONE STRUCTURALLY DIFFERS FROM CYSTATINS

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Cysteine proteinase inhibitors are distributed in mammalian tissue and body fluids as well as in plants and insects. The most studied are cystatins, tight and reversible binding inhibitors of papain-like cysteine proteinases, which form a superfamily of sequentially homologous proteins (1). The X-ray crystal structure of chicken cystatin and papain-stefin B complex showed that the highly conserved QVVAG region (present in most members of the cystatin superfamily, i.e. stefins, cystatins and kininogens) is located in the first β -hairpin loop. Beside this loop, the amino terminal "trunk" and second hairpin loop are involved into the mechanism of interaction of cystatins with their target enzymes (2,3).

The amino acid sequence of the recently discovered cathelin, a protein inhibitor of cathepsin L (4) and potato cysteine proteinase inhibitor (5), assigns that new families of cysteine proteinases may exist. To support this belief we isolated and characterized a strong inhibitor of cysteine proteinases from sea anemone. Data from the partial amino acid sequence of the inhibitor clearly show that this inhibitor structurally does not belong to the cystatin superfamily. The 5,6 kDa inhibitor obtained from pineaplle stem acetone powder was also sequenced. Its amino acid sequence is not comparable with cystatins. Both inhibitors were biochemically characterized.

Further structural studies including christallography will explain the nature of the interaction of these inhibitors with cysteine proteinases. The conserved QVVAG region is not present thus indicating that a different mechanism of interaction might be expected.

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1) Turk V., Bode W. (1991) FEBS Lett. 285, 213-219.

2) Bode W., Engh R., Thiele U., Huber R., Karshikov S.A., Brzin J., Kos J., Turk V. (1988) EMBO J. 7, 2593-2599.

3) Stubbs M.T., Laber B., Bode W., Huber R., Jerala R., Lenarčič B., Turk V. (1990) EMBO J. 9, 1741-1750.

4) Ritonja A., Kopitar M., Jerala R., Turk V. (1989) FEBS Lett. 255, 211-214.

5) Brzin J., Popović T., Drobnič-Košorok M., Jerala R., Turk V. (1989) In: Intracellular Proteolysis Mechanisms and Regulations (Katunuma N., Kominami E., eds) pp 398-400, Japan Sci.Soc. Press, Tokyo.

Kinetics of inhibition of cysteine proteinases by their protein inhibitors: Established structure-function relationship and open questions

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A major portion of the lysosomal proteinases responsible for intracellular protein degradation are cysteine proteinases. Their activity is controlled by protein inhibitors, mainly cystatins, stefins and kininogens.

The X-ray crystal structure chicken cystatin has been solved and a model for its interaction with papain has been proposed [1,2]. This model has been confirmed and extended by X-ray analysis of a [Ser^3]stefin B-papain complex [3]. Very recently, X-ray crystallography of human cathepsin B has revealed important differences to the known structures of the plant cysteine proteinases papain and actinidin [4].

In order to assess the functional significance of the proposed structural models, we have studied the kinetics of inhibition of papain, actinidin, human cathepsin B and H by a variety of natural and recombinant cystatins and stefins. Equilibrium dissociation constants (K_i) and rate constants of complex formation (k_{on}, k_{off}) were determined under carefully controlled conditions [5,6].

The results confirmed that, unlike small serine proteinase inhibitors, cystatins and stefins do not interact with their cognate enzymes in a simple substrate-like manner. Although the functional significance of the proposed contact regions was supported by kinetic data, single or double amino acid exchanges within these regions and truncations had very different effects on binding to different cysteine proteinases. The observed slow binding of stefins to cathepsin B involving an isomerization step in case of the cathepsin B-stefin B complex may reflect the effect of an "occluding loop" in the structure of cathepsin B [4].

It seems that the functional properties of protein inhibitors of cysteine proteinases cannot easily be predicted by intuitive computer modelling. The rather complex interactions of the three discontinuous binding sites of the inhibitors with the cognate enzymes would have to be considered in a quantitative manner to explain the observed kinetic differences.

References

1. Bode, W. et al. (1988) EMBO J. **7**, 2593-2599
2. Machleidt, W. et al. (1988) FEBS Lett. **243**, 234-238
3. Stubbs, M.T. et al. (1990) EMBO J. **9**, 1939-1947
4. Musil, D. et al. (1991) EMBO J., in press
5. Thiele, U. et al. (1990) Biol. Chem. Hoppe Seyler **371**, Suppl., 125-136
6. Machleidt, W. et al. (1991) Biomed. Biochim. Acta **50**, in press.

The Conformation of the Reactive Site Loop of α_1PI Probed by Limited Proteolysis

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Members of the serpin superfamily differ from other serine proteinase inhibitor superfamilies in several characteristics that suggest they may operate by a mechanism different from the standard mechanism of inhibition. Proteolytic cleavage within the reactive site loop (RSL), a region that is thought to form intermolecular contacts with proteinases, enhances the conformational stability of many serpins so that they now fail to unfold in 8 M urea. This stabilization is likely due to increased favorable interactions within the dominant structural feature of serpins, the A-β-sheet (Loebermann et al., J. Mol. Biol. 177,-531-556, 1984). The conformation of this sheet in native and proteinase-complexed α_1PI was probed by examining the stability of the protein after cleavage at different sites along the RSL. Synthetic peptides corresponding to portions of the α_1PI reactive site loop incorporated to different extents into native, proteolytically modified and proteinase-complexed serpins. The effects of insertion of the various peptides upon the conformational stability and inhibitory activity of these different forms were investigated. These data are discussed in terms of the proteinase inhibitory mechanism of serpins and the probable conformation of the RSL.

PROTEOLYSIS IN THE YEAST ENDOPLASMIC RETICULUM ?

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In mammalian cells vacuolar proteins that fail to fold or assemble correctly are usually retained in the endoplasmic reticulum (ER) and then degraded by a mechanism distinct from lysosomal degradation. The present work is aimed at determinig whether a similar protein degradation system exists in the yeast ER .

Many of the yeast vacuolar proteases are synthesized as inactive precursor proteins (pro-forms) that are translocated into the ER. Afterwards, the proteins transit through the ER and Golgi apparatus, and before or upon arrival to the vacuole, the propeptide segment is proteolytically removed to generate the mature active enzymes.

By pulse-labeling and immunoprecipitation of lysates followed by SDS-PAGE, we have observed that certain mutations in the structural genes for the yeast vacuolar proteases proteinase A (PrA) and carboxypeptidase Y (CPY), PEP4 and PRC1 respectively, cause these proteins to accumulate in the pro-form. This is the case for Lys_{341}-CPY and His_{275}-PrA obtained by directed mutagenesis of the respective wild type genes. Other inactive PrA mutants with the same phenotype (pro-form accumulation) have been obtained by in vitro mutagenesis of the PEP4 gene using hydroxylamide. Some of the mutants analysed so far in pulse-labeling experiments exhibit a loss of immunoprecipitable material during 30 minutes chase, which could be due to proteolytical break-down. To determine whether these mutant proteins are degraded in the ER we are analysing their stability in a sec18 mutant, since this mutation blocks the transport from the ER to the Golgi apparatus at the restrictive temperature.

INHIBITION OF RECOMBINANT HIV-1 AND HIV-2 PROTEASES USING NON-PEPTIDE COMPOUNDS WITH SHAPE COMPLEMENTARITY TO THE ACTIVE SITE, Rafael Salto, Dianne L. DeCamp, Paul S. Furth, Paul Ortiz de Montellano, Irwin Kuntz and Charles S. Craik, Departments of Pharmaceutical Chemistry and Biochemistry/Biophysics, UCSF, San Francisco CA 94143-0446

Recombinant HIV-1 and HIV-2 proteases have been expressed and purified to homogeneity from *E. Coli.* The enzymes have been used to test the inhibitory effects of compounds with shape complementarity to the active site of the HIV-1 protease. The compounds were discovered using the three-dimensional structure of the enzyme and the DOCK algorithm of Kuntz *et al.* (1982, J. Mol. Biol. 161, 269). One of the compounds identified in the initial computer search was bromperidol. Haloperidol, a closely related compound and approved antipsychotic drug with know pharmaceutical properties including oral activity and wide tissue distribution, was chosen as a lead compound for further development. Haloperidol inhibits the HIV-1 and HIV-2 proteases in a competitive fashion with a Ki of approximately 100 μM (DesJarlais *et al.* (1990) PNAS 87, 6644-6648). It is highly selective, having little inhibitory effect on pepsin activity and no effect on renin at concentrations as high as 5 mM. Approximately 80 derivatives of haloperidol were prepared and the ability of the compounds to act as competitive inhibitors was investigated on both the HIV-1 and HIV-2 protease. Of the derivatives that were better inhibitors of the HIV-1 protease one exhibited an IC50 of approximately 7 μM. Several of the inhibitors show a different selectivity in their ability to inhibit the HIV-1 and HIV-2 proteases. A derivative with an IC50 of 15 μM was co-crystallyzed with the HIV-1 protease. The differences in the IC50's of the derivatives for the HIV-1 protease *versus* the HIV-2 protease suggest that the inhibitors bind to the enzymes in a similar mode and that the differences in binding result from structural variations at the active site between the two enzymes. The three-dimensional structure of the HIV-1 protease-inhibitor complex will provide insight regarding the binding affinities of the various haloperidol derivatives. New inhibitors are being designed based on this structure and will be discussed.

Nonpolar and Electrostatic Interactions in the Thrombin-Hirudin Complex

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Hirudin is a polypeptide of 65 amino acids that is a potent and specific inhibitor of thrombin. The crystal structures of the thrombin-hirudin complex indicate that both electrostatic and nonpolar interactions play important roles in the formation of the complex. We have investigated the quantative importance of nonpolar interactions with the N- and C-terminal regions of hirudin by using site-directed mutagenesis. The results obtained at a number of different positions can be rationalized in terms of the crystal structure. In one position (Phe56), the results cannot be interpreted in terms of the crystal structure and it is suggested that an unfavourable conformational change is necessary for the binding of this residue. Cooperative interactions in the binding of adjacent residues have also been investigated and in all cases examined no cooperativity was observed.

Detection and Characterization of Intermediates in the activation Process of Procarboxypeptidase B from Porcine Pancreas

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Porcine procarboxypeptidase B (PCPB) is activated when trypsin produces a single cleavage at the boundary between the activation region (residues 1-95) and the carboxypeptidase B (CPB) region. Subsequently, a definite sequence of cleavages occurs at the C-terminal of the released activation segment giving rise to several intermediary fragments (Fx) and, finally, to a proteolitically resistant globular peptide (F3, residues 1-81). The high speed at which this process takes place hindered the isolation of all of these fragments, some of which were predicted on the basis of the sequence of the protein and on the overall effects [1].

By addition of specific inhibitors of carboxypeptidases, which allowed to partially freeze the above process, we have confirmed that the released CPB participates in the shortening of the activation segment and its derivatives (Fx), all of which were isolated. Both organic and peptidic inhibitors have been used to detect derivatives that act like a substrate for CPB and their products (F1a, F1b, F1g, F1d, F2a, F2b, F3, F4 and F5).

A comparative analysis of the activation processes of porcine monomeric procarboxypeptidases A and B (PCPA and PCPB) is made on the basis of the above studies, and on the light of the recently solved crystal tertiary structures of both proenzymes [2] and of the NMR structure of the globular activation domain from the former [3]. According to these, the protein segment connecting the globular activation domain and the active enzyme is folded in a a-helix (longer in the A than in the B form) which tightly binds to the enzyme. After the first trypsin cut at the N-terminal end of the enzyme region, the generated carboxypeptidase quickly trims the C-terminal of the activation segment, probably in an intermolecular mechanism. In the case of PCPB, the trimming promotes the dissociation of the remaining activation fragment -probably by the unfolding of the helical connecting segment- and the appearance of full carboxypeptidase activity. In contrast, in the case of PCPA, the removal of the C-terminal residue of the primary activation fragment only decreases by ~ 50% its affinity to the carboxypeptidase moiety, and the subsequent and slow action of trypsin is required to complete the activation.

[1]- Burgos F.J., Salva M., Villegas V., Soriano F., Mendez E. & Avilés F. X. (1991) Biochemistry 30, 4282- 4089.

[2]- Coll M., Guasch A. Avilés F.X. & Huber R. (1991) EMBO J. 10, 1-9.

[3]-Vendrell J., Billeter M., Wider G., Avilés F.X. & Wüthrich K. (1991) EMBO J. 10, 11-15.

CONCLUDING REMARKS

F. X. Avilés

CONCLUDING REMARKS

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One of the most evident conclusions that can be drawn from the meeting is that proteases are still in the leading front of the research in molecular biology. Proteases not only played a key role in the stablishment of the foundations of enzyme conformation an mechanisms of action, but are providing in nowadays a solid ground for the development of new methodological approaches -such as protein enginneering and drug design- and for the obtention of an integrated view of biochemical regulation through protein processing and turnover. They are used as elaborate models for structure- function relationship studies, and many new proteolytic enzymes and inhibitors are isolated, cloned and found to be involved in a pletora of biochemical processes and in their regulation.

The opening talk of Prof. Neurath stressed this wide span of the research on proteases and the fertility of its contribution to molecular biology : from the initial crystallizations of pancreatic proteases and inhibitors at the end of the first third of our century to today's understanding of the role of proteolysis in problems of great social impact, such as carcinogenesis, viral infection, connective tissue degradation and anaphylactic reactions. He and other speakers also pointed out the importance and activity of the research on intracellular and tissular proteases (lysosomal, granulocyte and membrane bound proteases , and proteases of specialized tissues) in higher eukaryotes.

Another of the recurrent topics in the meeting were the mechanisms for the regulation of protease action. Clearly, the use of milder and faster methods for protease isolation and the wealth of structural information derived from DNA cloning, allowed to recently detect and characterize an increasing number of zymogens. Many precursor processing proteases and several proteolytic cascades have also been characterized. At this respect, the own and reviewed results presented by several speakers on the coagulation cascade, on the detailed structure of several of its components, and on the design of antithrombotic and hemolytic drugs, were impressive. However, important questions related to zymogen activation still await clarification, particularly for those isolated in the last years or in minute quantities, such as many non-digestive zymogens.

The work on the alternative method for protease regulation, the interaction with inhibitors, was shown to be one of the most active in this field : from the characterization, DNA cloning and engineering of several new inhibitors -with an outstanding presence of those of cysteine proteinases- to the already traditional use of protease inhibitors as small models for protein structure-redesign and protein dynamics studies, and to the biomedical applications. Well selected and attractive exemples on the last issue were presented, particularly on the use of the measurements of proteases and inhibitors levels as a precise diagnostic and prognostic tool in polytraumatic patients. Also, on the administration of inhibitors (such as those of PMN elastase and thrombin) as therapeutic drugs in pulmonar emphysema and septicema, among other clinical cases.

The intracellular regulatory role of proteases and inhibitors was further stressed in different talks about their potential involvement in signal transduction (calpain-calpastatin, KSMP), on the characterization of membrane-located peptidases (ectopeptidases) and on the properties of several precursor and peptide -processing proteases (endopeptidase 24.11, carboxypeptidase E and MC...). Also, an overview was presented on their involvement in the controlled turnover of proteins (mainly cysteine proteinases and inhibitors).

A significant fraction of the metting was devoted to plant proteinases and inhibitors, a very active although still nascent field when compared to the research carried out in other organisms. Emphasis was made on the studies on inhibitors of proteases in plants (particularly solanacea, tobacco, and cereals) and on the wound or plague induction of them. The potential biotechnological application of the derived information for the control of pests was impressive and illustrated how basic research in this field is interlocked with applied research.

Metalloproteases had an important representation, either for endopeptidases (endopeptidase 2, endopeptidase 24.11, KSMP...) and for exopeptidases (amino- peptidases N, A, P, W, carboxypeptidases P and M, carboxypeptidases A, B, E, MC...). Three-dimensional information is only available in a few cases. Investigations on the neuropeptide/ hormone processing carboxypeptidase E and on mast-cell carboxypeptidase are enlightening the structural basis that allowed these enzymes to diverge from the pancreatic parents and adquire structural or functional properties adapted to its peptide processing function, such as the shift in optimum pH in the former case and the binding to proteoglycans in the latter. Also, comparison of potential pro-pieces in these carboxypeptidases favoured an interesting discussion about how they are regulated, and how its activation differs from the activation processes described for pancreatic procarboxypeptidases A and B, also treated in a subsequent talk.

It was also apparent that proteases (and precursos), inhibitors, and their complexes, are among the proteins for which most detailed three-dimensional and dynamic information has been obtained. Some outstanding examples were shown on the use of protease inhibitors (BPTI in the first position, how not!) for the structural and dynamical characterization of proteins in solution, and for the development of NMR as a method for protein determination in this state. Also, on the use of the same molecules for protein folding studies. Impressive were also the examples on the structure-function characterization by X-ray crystallography of isolated molecules or complexes shown for members of each of the main protease families and complementary inhibitors : serine, aspartic, cysteine and metallo -proteases.

As previously commented, the enormous biochemical background collected for these molecules over the years favours its selection for modern protein engineering studies and for drug design. Thus, the generation of engineered forms of trypsin with a narrower specificity than the wild forms or with the insertion of a metal switch that allows to regulate its activity in the presence of absence of specific transition metals, were bright examples presented. Also, the design of peptide or organic based inhibitors of renin, HIV proteinase and thrombin, among others, of great biomedical potentiality. The closing conference of Prof. Huber, elegantly summarized the present knowledge on the principles that govern activation, activity and inhibition of proteases, a difficult task given the massive information already available and the tendency of nature to diversify.

In spite of the variety of topics, it must be admitted that some important issues in the field were not treated or underrepresented in the meeting or treated only in the poster sessions : proteases in prokaryotes and yeast, proteasomes, ubiquitin and ATP- dependent proteases, connective tissue and matrix degrading proteases...etc , and many other related with the pletora of organelles, specialized cells, tissues and organisms in which proteases and inhibitors play a role in natural or pathological states. This justifies why meetings of different structure, goals and size have to be organized in a field with such a tremendous vitality.

LIST OF INVITED SPEAKERS

Workshop on
INNOVATIONS ON PROTEASES AND THEIR INHIBITORS: FUNDAMENTAL AND APPLIED ASPECTS

List of Invited Speakers

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INNOVATIONS ON PROTEASES AND THEIR INHIBITORS:
FUNDAMENTAL AND APPLIED ASPECTS

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