Instituto Juan March
de Estudios e Investigaciones

41 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Three-Dimensional Structure of Biological Macromolecules

Organized by

T. L. Blundell, M. Martínez-Ripoll, M. Rico and J. M. Mato

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The lectures summarized in this publication were presented by their authors at a workshop held on the 8th through the 10th of May, 1995, at the Instituto Juan March.

Depósito legal: M. 21.083/1995
I. S. B. N.: 84-7919-086-8
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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INTRODUCTION

T. L. Blundell, M. Martínez-Ripoll, J. M. Mato and M. Rico

Instituto Juan March

Workshop on "Three-dimensional Structure of Biological Macromolecules"

Date: 7-10 May 1995, Fundación Juan March, Madrid

With the development of biotechnological techniques and macromolecular engineering, there is an increasing need to elucidate the three-dimensional structure of proteins nucleic acids and multi-macromolecular complexes both in crystals as well as in solution. Rational proposals for the obtention of, e.g., novel proteins with improved or new biological activities can only be advanced from a detailed knowledge of their three-dimensional structure at atomic level. Conversely, protein engineering methods can be used to modify proteins structurally with the objective of getting a deep understanding of their biochemical function and thus delineating precise structure-function relationships, which has been a main objective in biochemistry from very long. Interactions between biological macromolecules with themselves or with a wide range of effectors is currently a very active field of work, where the knowledge of the precise structure of the interacting interfaces in the corresponding complexes is crucial. Interactions between protein-nucleic acids, antigen-antibody or drug-receptor are just examples of the importance of this field, which is not confined to the academic area, but it also has a high industrial and economic relevance.

X-ray Crystallography is a well proven, long-established tool for determining the structures of biological macromolecules in the crystal state. In the last decade, NMR spectroscopy has emerged as an alternative technique to obtain molecular structures also at atomic level. The aim of this workshop on "Three-dimensional Structure of Biological Macromolecules" was to provide detailed and rational information on the theoretical and practical aspects of these two techniques in relation with solving the three-dimensional structure of biomacromolecules. The workshop was directed to a wide range of scientists, from crystallographers and NMR experts, who could learn about the latest advances and methodological developments in the two techniques, to biochemists and molecular biologists who were initiated in the practical aspects of these techniques as well as in understanding and correlating function and structure.

No technique has so profoundly influenced the field of structural biochemistry as X-ray diffraction analysis when applied to crystals of macromolecules. It is among the most prolific technique available for providing significant new data with, in principle, no limit in molecular size, and therefore including, in addition to proteins, viruses, ribo-and-deoxyribonucleic acids and nucleoprotein complexes. X-ray Crystallography attracts workers from many different disciplines such as Chemistry, Biochemistry, Biophysics, Zoology, Physics and Mathematics, and

the different insights and skills contributed by these workers are vitally important in this multidisciplinary subject. As the material to be studied must be in the crystalline state, the first limiting step to achieve an X-ray diffraction model is to get crystals suitable for the analysis, and although the crystallization process is still far from being a classical analytical procedure and contains a substantial component of trial and error, there are many approaches to crystallize biological macromolecules, all of which aim at bringing the solution to a supersaturation state. Although protein Crystallography has advanced rapidly in the last decades and in some cases protein structure can be determined in a straightforward manner, new methods and ideas in certain areas must still be developed.

For the determination of three-dimensional structures at atomic level of biological macromolecules in solution, the technique of choice is multidimensional NMR spectroscopy. As compared to X-ray Crystallography, NMR methods present the advantage of providing the structure in solution, a medium more similar to the conditions prevailing in vivo. Also, highly valuable kinetic and dynamic information can be obtained about important biochemical processes, like, e.g., the detection and structural characterization of intermediates in the protein folding reaction. The size of proteins susceptible of being analyzed by NMR method is, however, limited at present to the range of 20-25 kDa and requires, in the upper limit, proteins labelled with stable isotopes like ^{15}N and/or ^{13}C. The technique is still evolving and an extension of its applications is expected.

Tom L. Blundell
Martín Martínez-Ripoll
José M. Mato
Manuel Rico

FIRST SESSION:

Chairperson: Wolfgang Kabsch

PROTEIN STRUCTURE-BASED DRUG DESIGN

Tom L Blundell

ICRF Unit of Structural Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London, WC1E 7HF and Biotechnology and Biological Sciences Research Council, Central Office, Polaris House, North Star Avenue, Swindon, SN2 1UH.

Knowledge of the three-dimensional structures of proteins defined by high resolution X-ray analysis provides a basis for design of novel molecules. The design process takes place as part of an engineering bicycle. The protein is characterised as a ligand complex, the three-dimensional structure of which is defined by X-ray analysis; this provides a basis for hypotheses concerning modification of either the protein or the ligand. In the protein engineering cycle, the protein is modified by site-directed mutagenesis, and the mutant recombinant protein is reexamined chemically and structurally. In the drug or vaccine design cycles, the ligand is often modified chemically in order to provide an improved complementarity with the protein. The drug-design cycle will be illustrated with the design of anti-hypertensives based on the crystal structures of human and mouse renin-inhibitor complexes and the design of AIDS antivirals based on the crystal structures of HIV proteinases-inhibitor complexes. I shall also use the structure of serum amyloid P-component to discuss approaches to combat amyloidosis, the study of porphobilinogen deaminase to understand acute intermittent porphyria, and the structures of SH3 and PH domains as targets for intervention in signal transduction, of interest in cancer. My lecture will describe new approaches to protein modelling and prediction which are important where no X-ray structure is available as a basis for design.

The Role of NMR Spectroscopy in Structural Biology.

Kurt Wüthrich, Institut für Molekularbiologie und Biophysik,
Eidgenössische Technische Hochschule Hönggerberg, CH-8093 Zürich, Switzerland.

With the use of DNA recombination and chemical synthesis an unlimited array of different polypeptide sequences can be generated, but the relationship of the resulting compounds with corresponding biological functions can only be rationalized through knowledge of the three-dimensional structure. Therefore, in biological and biomedical research the area of structural biology, in particular atomic resolution studies of the three-dimensional structure of biological macromolecules and their intermolecular interactions, has never before had as central a role and enjoyed as much popularity as today. Although X-ray diffraction with single crystals has for two decades, until 1984, been unique in its potential for efficient determination of three-dimensional molecular structures, it presently shares this role with nuclear magnetic resonance spectroscopy in solution.[1–3] In this presentation the role of NMR in structural biology will be reviewed, in particular with respect to its ability to complement structural data that can be obtained with diffraction experiments in single crystals.

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3. Wüthrich, K. (1993) in *DNA and Chromosomes, Cold Spring Harbor Symposia on Quantitative Biology,* **58,** 149–157. Hydration of biological macromolecules in solution: surface structure and molecular recognition.

The crystal structure of coenzyme B_{12} dependent methylmalonyl-CoA mutase

F. Mancia, A. Nakagawa, N.H. Keep, P. Leadlay and P.R. Evans

MRC Laboratory of Molecular Biology, Cambridge, UK

Methylmalonyl-CoA mutase is a member of a class of enzymes that bind the cobalt-containing 5´-deoxyadenosyl-cobalamin cofactor (better known as coenzyme B_{12}) and catalyse unusual 1,2 intramolecular rearrangements in which a hydrogen atom is exchanged with a group on an adjacent carbon (Halpern *et al.*, 1985). For methylmalonyl-CoA mutase, which catalyses the interconversion between (2R)-methylmalonyl-CoA and succinyl-CoA, it is the COSCoA group that migrates. These reactions are thought to involve radical intermediates, with the cofactor acting as a source of free radicals, but the experimental evidence for the underlying mechanism is unclear (Keep *et al.*, 1993). There is nevertheless evidence for the formation of an initial radical: this is the 5´-deoxyadenosyl radical that arises from the homolysis of the Co-C bond of coenzyme B_{12}. This is the only metal-carbon bond known in nature. At present there are ten known members of this class of enzymes, and structural information is just beginning to emerge, with this structure and the recently published structure of a B_{12}-binding fragment of the methylcobalmin-dependent methionine synthase (Drennan, *et al.*, 1994).

Methylmalonyl-CoA mutase is one of only two adenosylcobalamin-dependent enzymes present in both animals and microrganisms. In the bacterium *Propionibacterium shermanii* it is a key enzyme in the fermentation to propionate, whilst in mammalian liver it is responsible for the conversion of odd-chain fatty acids and branched-chain amino acids to succinyl-CoA for further degradation. The *P.shermanii* enzyme is an α,β heterodimer of 150kD total molecular weight with one active site per dimer. We have solved the structure of the ternary complex between the active recombinant protein expressed in *E.coli* (McKie *et al.*, 1990), coenzyme B12, and the partial substrate desulpho-CoA. This has been achieved using data from crystals in two closely related crystal forms, $P2_1$ (Marsh *et al.*, 1988) and $P2_12_12_1$, each of which contains two dimers in the asymmetric unit (300kD total molecular weight in the asymmetric unit). A model has been built into a 3Å resolution map, produced by averaging the two copies of the molecule in each crystal form (four copies in all), with experimental phases from MAD data on a Pt derivative for the orthorhombic form, and 3 isomorphous derivatives of the monoclinic form. Refinement of this model to 1.97Å resolution is currently in progress.

Each subunit has essentially a two domain architecture. In the catalytic α chain, the B_{12} is sandwiched between a C-terminal flavodoxin α/β domain and an N-terminal α/β TIM barrel. A conserved histidine from the flavodoxin-like domain provides axial coordination to the cobalt atom in a very similar way to that seen in methionine synthase. The cobalamin lies on top of the barrel. The coenzymeA molecule is bound along the axis of the barrel, pointing into a cavity on the 5'-deoxyadenosyl or catalytic side of the B_{12}. The inactive β subunit shows a similar topology, though the flavodoxin-like domain does not lie on top of the barrel but is completely displaced away from it.

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McKie, N., Keep, N.H., Patchett, M. and Leadlay, P. (1990), *Biochem. J.*, **269**, 293-298.

NEW NMR TECHNIQUES APPLIED TO THE STUDY OF A CONFORMATIONAL SWITCH Hitoshi Kuboniwa, Nico Tjandra, Stephan Grzesiek, Andy Wang, John Marquardt, and Ad Bax Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, Maryland 20892, USA

The affinity of calmodulin for its target enzymes is modulated by calcium. Many of the newest three- and four-dimensional NMR techniques have been used to obtain an as accurate as possible picture of the calcium-free state of the protein, thereby permitting a detailed comparison with the X-ray structures previously reported for Ca^{2+}-ligated calmodulin. The secondary structure of the Ca^{2+}-free and -ligated states is nearly identical, and the flexibility previously observed by NMR in the "central helix" of the Ca^{2+}-ligated form is also found in the Ca^{2+}-free state. However, the residual rotational anisotropy of the individual domains at a quantitative level appears incompatible with the Herzberg model for the structure of the Ca^{2+}-free state. This conclusion was derived from ^{15}N relaxation studies alone, prior to calculation of its structure. Structure calculations were based on an unusually large number of ^{1}H-^{1}H, ^{13}C-^{1}H, ^{15}N-^{1}H and ^{13}C-^{15}N and ^{13}C-^{13}C J couplings, and on a very large number of ^{1}H-^{1}H NOEs obtained, in part, by a novel "reverse labeling" strategy. ^{13}C-^{13}C and ^{13}C-^{15}N J couplings confirm that in the Ca^{2+}-free state, residues in the hydrophobic cores of the two small domains are subject to significantly more disorder compared to the same residues in the Ca^{2+}-ligated state of the protein, when bound to target peptide.

SECOND SESSION:

Chairperson: Kurt Wüthrich

X-ray Structure Analysis of Mitochondrial Creatine Kinase

Karin Fritz-Wolf[1], Thomas Schnyder[2], Theo Wallimann[2], Wolfgang Kabsch[1]

[1] Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstraße 29, 69028 Heidelberg, Germany

[2] Institute for Cell Biology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Creatine kinase (CK, EC 2.7.3.2) catalyzes the reversible phosphoryl transfer from phosphocreatine to ADP. The enzyme is present in tissues of high and fluctuating energy requirements and exists in tissue-specific (M, muscle type; B, brain type) as well as in compartment-specific (cytosolic and mitochondrial) isoforms. The cytosolic forms are homo- or heterodimeric (MM-, BB-, or MB-CK), whereas the mitochondrial creatine kinases (Mi-CK) form octamers. Mi-CKs are found exclusively in the mitochondrial intermembrane compartment being attached to the inner membrane. This enables the enzyme to utilize the intramitochondrially produced ATP for the synthesis of phosphocreatine, which is exported into the cytosol where it is used for the regeneration of ATP by the cytosolic isoforms. This interplay between the cytosolic and mitochondrial CKs, with the easily diffusable (phospho)creatine serving as an energy shuttle system between the cellular compartments, is referred to as the "phosphocreatine circuit" [reviewed in Wallimann et al.(1992)Biochem.J.281,21-40.].

Octameric Mi-CK isolated from chicken cardiac muscle has been crystallized in two tetragonal crystal forms (type III: a = b = 12.6nm , c = 14.5nm, space group $P4_2$; type IV : a = b = 14.4nm , c = 22.0nm, space group $P422$) which diffract to 0.3nm resolution. The phase problem has been solved at low resolution (0.6nm) by the method of isomorphous replacement for type III crystals soaked in buffer containing mercurial compounds. The analysis of the difference Patterson map between native and derivative data sets revealed eight labeling sites per asymmetric unit (two per monomer). The orientation of the octamer in the unit cell was determined from the native diffraction intensity data by the rotation function, which quantifies the degree of overlap between the original and the rotated Patterson function. In addition, the analysis of the rotation function revealed a 422 point group symmetry for the Mi-CK octamer. The asymmetric unit contains one tetramer since one of the 2-fold axes of the octamer coincides with a crystallographic 2-fold axis. The

fourfold symmetry axis of the octamer is found in the ab-plane of the crystal lattice at an angle of 77.3° from the a-axis. With the knowledge of the non- crystallographic symmetry the eight binding sites obtained from the difference Patterson map could be explained as two different labelling sites per Mi-CK monomer. An electron density map was calculated at 0.6nm resolution and improved by averaging the density of the non-crystallographically related monomers in the asymmetric unit. The low-resolution map of the molecule is compatible to all the electron optical work done so far on Mi-CK octamers. Data up to 0.3nm resolution were collected in our laboratory as well as at the Synchrotron facilities in Hamburg from native as well as a number of mutant Mi-CK crystals. Based on the low-resolution map, phases of the higher resolution data were obtained by the phase extension method which exploits the redundancy in the asymmetric unit. The computed electron density map at 0.3nm resolution was of sufficient quality to allow a complete assignment of the 380 amino acid residues of each Mi-CK monomer. Refinement of the atomic structure is still going on; at present (Feb. 95) it has been refined to an R-factor of 26.7% thereby imposing strict non-crystallographic symmetry. Details of the structure will be described in the lecture.

NMR STRUCTURES OF POTENTIAL DRUG TARGETS

Stephen W. Fesik, Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, IL 60064.

Due to the development of heteronuclear multi-dimensional NMR methods, the availability of isotopically labeled proteins, and computational tools for analyzing NMR data, three-dimensional structures of biomacromolecules and molecular complexes can be rapidly determined. The structural information gained from the NMR experiments could aid in the design of molecules that bind and block the function of proteins that are important for critical cell processes. These proteins may be involved in signal transduction, binding to DNA, or carrying out key enzymatic reactions. In this presentation, the solution structures of proteins and protein/ligand complexes will be described. Each of these proteins are potential drug targets and of pharmaceutical interest. NMR structures will be described for the following: a stromelysin/inhibitor complex, Shc SH2/phosphopeptide complex, N-terminal PH domain of pleckstrin (1,2), a C-terminal fragment of *E. coli* topoisomerase I, the DNA-binding domain of the human papilloma virus E2 protein, and the *ets* domain of Fli-1 (3,4).

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STRUCTURAL STUDIES OF THE SIGNAL TRANSDUCTION PROTEIN CHEY.

Miquel Coll, Lluís Bellsolell, Maria Solà, Antonio Párraga, Cristina Vega.
Centro de Investigación y Desarrollo-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.
Philippe Cronet, Eva Lopez, Luis Serrano.
European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, Germany.

The protein CheY is the response regulator in the signal transduction system for bacterial chemotaxis. A chemotactic response occurs when an attractant molecule is released from the transmembrane receptor. The signal is transmitted to the cytoplasmic part of the receptor where the attached effector protein CheA, a histidine kinase, autophosphorylates. In the presence of Mg^{2+} the phosphoryl group of phospho-CheA is transferred to CheY. The phosphorylated Mg^{2+}-CheY is the active form of the regulator and docks in the flagellar switch proteins FliM and FliG inverting the direction of the flagellum rotation. A large conformational change is believed to occur upon phosphorylation but the phosphorylated form of Mg^{2+}-CheY remains unknown, its crystallization being hindered by the short half life of the phospho-Mg^{2+}-CheY species. We have determined the three-dimensional crystal structure of the metal-bound CheY observing important rearrangements in the active site and at the functional surface of the protein when compared to the apo-CheY structure (Bellsolell *et al.*, 1994). In addition seven different mutants of CheY, affecting residues of the active site and other functional areas, have been prepared and their three-dimensional crystal structures have been solved. Based on them a detailed picture of the functional and mobile parts of the protein is emerging. We are starting to understand how changes in the active site area get amplified and propagate to the docking surface of CheY.

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PUBLIC LECTURE

Genomes, protein superfamilies and drug discovery

Tom L Blundell

Biotechnology and Biological Sciences Research Council, Polaris House, North Star Avenue, Swindon, Wiltshire, SN2 1UH

and

ICRF Unit of Structural Molecular Biology, Department of Crystallography, Birkbeck College, Malet Street, London, WC1E 7HX

Information in biology flows from nucleic acids to proteins, which carry out most of the functions in living organisms. Protein functions are usually modified in disease states and, even where they are not, inhibition of activity can be useful in therapy.

Over the past ten years drug discovery has increasingly been pursued through the selection of relevant protein targets, often enzymes or receptors, and the identification of useful leads by using assays to screen data bases of synthetic chemicals or natural products from microbial fermentations or wild plants. Knowledge of the target protein can then assist in the optimisation of the candidate drug.

In the past three years complete sequences of individual chromosomes of simple eukaryotes have indicated that the functions of many gene products cannot be identified from sequence alone. However, recognition of distant relationships between structurally and functionally similar molecules can provide clues as to functions of newly sequenced proteins. This can be of value in identifying members of protein superfamilies - receptors, growth factors, proteinases, signal transduction enzymes - that are useful targets in drug discovery.

THIRD SESSION:

Chairperson: Tom L. Blundell

PROTEIN FOLDING STUDIED BY NMR AND OTHER PHYSICAL TECHNIQUES

Christopher M. Dobson

Oxford Centre for Molecular Sciences
University of Oxford, New Chemistry Laboratory
South Parks Road
Oxford OX1 3QT
United Kingdom

We are using a combination of physical techniques to provide a structural description of the events that occur during the folding of c-type lysozymes and α-lactalbumins. Of particular interest with regard to the mechanism of folding is evidence for the formation of a collapsed 'molten globule' state very rapidly after initiation of folding, for heterogeneity and rearrangement events within this state prior to subsequent steps in folding, and for varying degrees of cooperativity in the development of persistent structure within domains and sub-domains of the native-like fold. On the basis of the experimental results we identify some of the determinants of the folding process and speculate on the importance of these in driving protein molecules to their native states.

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Structural Studies on Larger Viruses

Jonathan Grimes[1], Ajit Basak[1], Patrice Gouet,[1] Geof Sutton[1,2], Polly Roy[1,2], Nick Burroughs[3], Peter Mertens[3], B.V.V. Prasad[4] and David Stuart[1].

1. Laboratory of Molecular Biophysics, Rex Richards Building, South Parks Road, Oxford, OX1 3QU, UK., **2**. NERC Institute of Virology, Mansfield Road, Oxford, UK., **3**. Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Surrey, UK., **4**. Baylor College, Houston, Texas, USA.

There is now a wealth of information on the structure of the capsids of the smaller isometric viruses, which has shed light on various aspects of the biology of these systems. However as we go up in complexity we rapidly arrive at viruses which retain underlying icosahedral symmetry but have many more copies of more proteins and about which we know very much less. The idea of quasi-equivalence, whereby the stabilizing contacts are assumed to be preserved throughout the surface lattice of a complex capsid, is powerful in making sense of viruses at the level of crude electron micrographs but usually falls down when we look in detail at what's going on. Since this is true for relatively simple viruses, where path dependent conformational switching seems to be the real underlying mechanism governing correct assembly, we might assume that the situation will be even messier for larger systems.

We are studying the structures of some orbiviruses. These are animal viruses, which belong to the same family as the better known rotaviruses and reoviruses, which cause significant human disease (Holmes, 1994). Bluetongue virus is the classic orbivirus; it has a proteinaceous capsid from which an outer layer can be stripped away to reveal a 700 Å core particle (Prasad *et al.*, 1988). The core is robust and penetrates the host cell intact. Once inside the cell the core soaks up nutrients from the host which are used to produce viral RNA which is extruded from the core into the cytoplasm of the host cell. These little factories have a complex architecture. The outer shell which is lost on cell penetration consists of two proteins with uncertain stoichiometry, VP2 and VP5. The core itself has a small number of proteins with enzymatic activity (the number of copies of each of these is uncertain but is thought to be of order of 12) and much larger numbers of VP3 and VP7. These latter two components can self assemble (when co-expressed in an heterologous expression system) into particles which, apart from their emptiness, are indistinguishable from virus derived cores.

VP7 is present at the level of 780 copies per core, arranged on a T=13 lattice (Prasad *et al.*, 1988). The 38kDa protein forms trimers in solution and these are the building blocks for the bristly outer surface of the core. We have now determined, by X-ray crystallography, the structures of this molecule in 3 different crystal forms (Basak *et al.*, 1992, Grimes *et al.*,1995, Grimes *et al.*, unpublished). In addition we have determined the structure of a trimeric fragment of VP7 from a related virus, African horse sickness (Basak *et al.*, in preparation). The structure of VP7 is rather different to that of other viral coat proteins observed to date. It possesses an upper trimerization domain that resembles the head of influenza virus haemagglutanin and a larger lower domain packed full of helices which may be capable of some relative articulation about greasy contacts to facilitate interactions with the underlying layer of protein.

The inner protein layer is made up of 120 copies of VP3 (Mertens and Burroughs, unpublished). This protein is more mysterious in structure; we have been unable to obtain crystals of the isolated 100kDa protein. To move forward to a more complete understanding of the structure and assembly of the cores we have combined the information from the X-ray structures with that from electron cryo-microscopy. Reconstructions have been calculated at almost 20 Å resolution (Prasad, unpublished) and simple fitting procedures used to optimally place the X-ray structure in the EM

map. This process is straightforward and unambiguous. Although the EM reconstruction is at far from atomic resolution we are able to import much more detailed information from the X-ray analysis. In this way we can map with some precision the points of interaction between adjacent molecules of VP7 and also between VP7 and VP3. Perhaps of more immediate interest is the observation that the VP7 molecules do no behave as rigid bodies but flex somewhat in forming the surface lattice of the core. Now that we are able to dissect the VP7 contributions cleanly away from the EM reconstruction the underlying structure of the 120 molecules of VP3 is revealed. It seems that these molecules form a T=1 lattice with a 'dimer' forming the icosahedral protomeric unit. The great complexity of the T=13 arrangement of VP7 is simplified by the use of building blocks. The first step is to assemble, in solution, trimers. Next there are a preferred set of contacts between dimers of trimers which are used wherever possible. Finally a less frequently observed set of contacts allow the trimers to plug any remaining gaps in the capsid. In this respect the virus is returning to something rather close to the original concept of quasi-equivalence.

Very recently an X-ray structure of VP7 has revealed that very large conformational changes can occur in this molecule, in response to the rather gentle crystal packing forces (Grimes *et al.*, unpublished). These motions are much larger than those required for core assembly, which raises interesting possibilities for the biological functions of VP7.

Finally we are advancing with a crystallographic analysis of the whole core (Burroughs *et al.*, unpublished). This is a substantial undertaking and we do not expect to have solved the structure in time for the present meeting!

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NMR studies on the folding of α/β parallel proteins.

Manuel Rico
Instituto de Estructura de la Materia, CSIC, Madrid (Spain).

An important question concerning protein folding is whether proteins with similar tertiary structure and no sequence homology follow similar folding processes. In trying to answer that question, a number of studies have been performed in our lab, in collaboration with the group of Dr. Serrano at EMBL. These studies have been focussed on the folding properties of three structurally related proteins belonging to the α/β parallel family of proteins, Che Y, flavodoxin and P21-ras. First, we have analyzed, as a model of early steps in their folding pathways, the helical propensities of peptide fragments encompassing all the α-helices of the three proteins. Second, a larger fragment (the C-terminal segment 79-129) of the smallest member of the family, the chemotactic protein Che Y, has been examined and characterized structurally with an aim of modelling possible folding intermediates. Third, we have assigned the ^{1}H and ^{15}N spectra of Che Y and determined its three-dimensional structure in solution by NMR methods combined with restrained molecular dynamics calculations. The structure adopted by Che Y in solution is extremely similar to the structure in the crystalline state and consists of five β strands arranged in a parallel sheet with the topology β_2-β_1-β_3-β_4-β_5 and the five amphiphilic helices clustering into two groups: α_1+α_5 lying on one side and α_2-α_3-α_4 on the opposite side of the central β-sheet. Wild-type Che Y presents a unimolecular, but complex, kinetic folding mechanism that is dominated by a slow phase compatible with a *trans-cis* isomerization (Lys 109-Pro 110). Also, an hyperexposed hydrophobic residue, Phe 14, contributes negatively to the native-state stability. Thus a double mutant, F14N and P110G, has been used in studies of H/D chemical exchange of amide backbone protons with solvent deuterons. Some 25-30 amide protons have been found to exchange very slowly, thus meaning that they are protected from exchange either by hydrogen bonding, solvent unaccesibility or both. As expected, they are mainly located in helices α_1 and α_5 and in the central strands β_1-β_3-β_4 just in the hydrophobic region where contact between these elements of secondary structure takes place. These slowly-exchanging amide protons will constitute the crucial elements to probe the structure of a refolding intermediate, what will be addressed by a combination of quench-flow and 2D-NMR methods. All the above information will be used in a convergent way in order to describe the folding pathway of α/β parallel proteins.

Protein Crystallography in Madrid: One Year's Work

Romero, A., Sanz-Aparicio, J. and Martínez-Ripoll, M.

Departamento de Cristalografía
Instituto de Química-Física Rocasolano, CSIC
Serrano 119, E-28006 Madrid (Spain)

Our contribution is aimed to emphasize the establishment of Protein Crystallography in Madrid as the result of a multi-effort work sponsored by the Spanish Research Council (CSIC), and to offer our collaboration to the research teams with interest in the knowledge of three-dimensional structure of macromolecules of biological meaning.

As the basic needs of the laboratory have been fulfilled recently, we will present the results achieved in the period of one year only, including the study of several proteins implied in electron transfer processes (**flavodoxins**), enzymatic inhibition (**aspartic proteinases inhibitor complexes**), fibroblasts growth factors (**FGF**) and hydrolysis of glycosylic bonds (**β-glucosidase**).

We will report on the tertiary structure of *Desulfovibrio desulfuricans* ATCC 27774 flavodoxin that has a molecular mass of 15 KDa, isolated and purified by Caldeira *et al.* at the CTQB (Portugal). This enzyme represents an interesting case of showing the most positive E2 redox potential among the remaining flavodoxins with elucidated structure. Since the modulation of the redox potentials is known to be derived from the binding strength of the FMN group to the apoprotein, the found structure-properties correlations will be discussed.

As a part of our collaboration with the laboratory of Prof. Blundell at the Birkbeck College (London), the structures of three complexes of the aspartic proteinase endothiapepsin, co-crystallized with synthetic chymosin inhibitors, will be presented. They have been refined up to crystallographic R factors between 0.16 and 0.18, at resolutions of 2 Å. The general conformation of the inhibitors as well as the location and nature of the specific subsites will be described in terms of the X-ray models.

The third project, which is a product of the collaboration with Prof. Giménez (Centro de Investigaciones Biológicas, CSIC), is related to one of the two closely related proteins known as fibroblast growth factors (FGF). The so called aFGF-154 (acidic form of the FGF) has been crystallized. The crystals show monoclinic C2 symmetry with a cell of a=116.1, b=48.6, c=98.3 Å, β=126.7°, diffract up to 2.4 Å resolution and the structure has been refined to an R value of 0.24.

We will finally report on the crystallization and preliminary X-ray diffraction analysis of a type I β-glucosidase encoded by the bgIA gene of *Bacillus polymyxa*, isolated and purified at the Instituto de Agroquímica y Tecnología de Alimentos, CSIC, by Polaina *et al.* This enzyme, crystallized using both Na-K-phosphate and Na-citrate precipitants, builds nice crystals which diffract up to 3 Å and shows orthorhombic $C222_1$ symmetry in a cell of dimensions a=155.4, b=209.4 and c=209.7 Å. Analysis of both size exclusion HPLC, measured density and crystallographic data, indicated that this crystal form contains an octamer in the asymmetric unit.

Solution structure of the ets domain of Fli-1 when bound to DNA

Edward T. Olejniczak[1], Heng Liang[1], Xiahong Mao[2], David G. Nettesheim[1], Liping Yu[1], Robert P. Meadows[1], Craig B. Thompson[2] and Stephen W. Fesik[1]

1. Pharmaceutical Discovery Division Abbott Laboratories, Abbott Park, Il 60064, USA 2. Howard Hughes Medical Institute and Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago IL. 60637 USA.

The ets family of transcription factors regulate a variety of important biological processes. In mammals they control the development of the haematopoietic and immune system and are involved in the regulation of T-cell activation and tissue remodeling. Members of this family have also been found to act as oncogenes as a result of chromosomal translocation or viral transformation.

Here we report on the three dimensional structure of the DNA binding domain of human Fli-1 when bound to DNA. The structure was determined using multidimensional NMR on a $^{13}C/^{15}N$ labeled protein when complexed to an unlabelled 16 bp DNA fragment containing the concensus binding site for Fli-1. Structures were obtained using NOE constraints and a distance-geometry simulated annealing optimization protocol. Further insight into the structure were obtained from mutational studies of the protein. Comparisons are made between the structure of the DNA binding domain of Fli-1 and the structures of other DNA binding motifs.

Disruption of the target G-C base-pair by the HhaI methyltransferase

Saulius Klimašauskas[a,c], and Richard J. Roberts[b]

[a] *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA*
[b] *New England Biolabs, Beverly, MA 01915, USA.*
[c] *Present address: Institute of Biotechnology, Vilnius 2028, Lithuania.*

The crystal structure has been determined at 2.8 Å resolution for a chemically-trapped covalent reaction intermediate between the HhaI DNA cytosine-5-methyl-transferase, S-adenosyl-L-homocysteine and a duplex 13-mer DNA oligonucleotide containing methylated 5-fluorocytosine at its target (Klimašauskas *et al., Cell* 1994, 76: 357). The DNA is located in a cleft between the two domains of the protein and has the characteristic conformation of B-form DNA, except for a disrupted G-C base pair that contains the target cytosine. The cytosine residue has swung completely out of the DNA helix and is positioned in the active site.

The structure highlights the contacts of M.HhaI with the orphan guanine residue, but little is known about the contacts made with target cystosine during the initial binding interaction. We have investigated the effects of replacing the target cytosine by other, mismatched bases, including adenine, guanine, thymine and uracil. We find that M.HhaI binds more tightly to such mismatched substrates and can even transfer a methyl group to uracil if a G:U mismatch is present. Other mismatched substrates in which the partner guanine is changed exhibit similar behaviour. This suggests that the binding specificity of M.HhaI is G-GC, where '-' represents the absence of a base in the major groove. We propose that the DNA methyltransferases have evolved from mismatch binding proteins and that base flipping was, and still is, a key element in DNA-enzyme interaction.

CRYSTAL STRUCTURE OF THE CELLULOSE-BINDING DOMAIN OF THE CELLULOSOME FROM CLOSTRIDIUM THERMOCELLUM

José Tormo[1], Raphael Lamed[2], Ely Morag[3], Edward A. Bayer[3], Thomas A Steitz[1]

[1]Department of Molecular Biophysics and Biochemistry, and Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06520, USA.
[2]Department of Molecular Microbiology and Biotechnology, Tel Aviv Universtity, Ramat Aviv, Israel.
[3]Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel.

Cellulose is the most abundant renewable resource on Earth, accounting for about half of the organic material in the biosphere. In additions to its ecological and technological importance, the high degree of crystallinity of the substrate makes cellulose degradation a problem of fundamental interest.

Cellulases of bacteria and fungi are composed of a group of functional domains, which promote the efficient degradation of cellulose. The hydrolytic function is carried out by the catalytic domains, and often a separate cellulose-binding domain (CBD) serves to target the catalytic domain to its substrate. In many cases, cellulases are organized into discrete multisubunit structures thad had been called cellulosomes, first described for *Clostridium thermocellum*. Most of the cellulosomal subunits are cellulases, but a new class of central scaffolding polypeptide integrates the various subunits into the cohesive complex. Like the cellulolytic subunits, the "scaffoldin" is also partitioned into a multiplicity of functional domains. One of these is a high-affinity CBD, consisting of around 160 amino acid residues.

We have solved the crystal structure of the major CBD from the scaffoldin subunit, of the cellulosome from *Clostridium thermocellum* by the standard method of multiple isomorphous replacement and refined the model to a crystallographic R factor of 20% at 1.7 Å resolution. The molecule adopts a jelly-roll ß-sandwhich fold, and is structurally analogous to the plant and animal lectins. It contains a groove in the same region where these proteins bind sugars. The walls of the groove are rich in amino acids that are known to interact with sugars, especially aromatic residues. The three-dimensional structure also shows a region remarkedly flat and hydrophilic which probably binds to crystalline cellulose.

FOURTH SESSION:

Chairperson: Thomas L. James

Solution Structure of the Single Stranded DNA Binding Protein encoded by the *Pseudomonas* Bacteriophage Pf3.

C.W. Hilbers, R.H.A. Folmer and R.N.H. Konings, NSR Center for Molecular Structure, Design and Synthesis, Laboratory of Biophysical Chemistry, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands.

M. Nilges, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

Single stranded DNA binding proteins (ssDBPs) perform a variety of key functions in prokaryotic and eukaryotic cells. They play indispensable roles in DNA replication, repair and recombination and can function as specific regulators of gene expression, e.g. as transcription activators.
In the lecture the structure determination of the homodimeric single stranded DNA binding protein encoded by the filamentous *Pseudomonas* bacteriophage Pf3 will be discussed. Hetero nuclear multidimensional NMR techniques have been applied to the mutant (Phe 36—>His) that was succesfully designed to reduce the protein's tendency to aggregate. The protein monomer is composed of a five stranded antiparallel ß-sheet from which two ß-hairpins and a large loop protrude. The structure of the Pf3 protein will be compared to that of the gene V protein of bacteriophage Ff, a protein with a similar function and DNA binding properties, yet with a quite different amino acid sequence. The folding of the Pf3 protein turns out to be very similar to that of the major cold shock protein of *Escherichia coli*, a single-stranded DNA binding protein with an entirely different sequence, biological function and DNA binding characteristics. The remarkably similar folding in these proteins suggests the occurrence of a motif which may be common to a large number of single stranded nucleic acid binding proteins.

form, and that it is identical to the native form in its specific mitogenic activity and in its requirements of exogenous heparin for inducing mitogenesis (6). Finally, we looked for simpler compounds that emulate heparin in fibroblast growth factor mitogenesis assays. After a relatively long screening process, we found that inositol hexasulfate, like heparin, elicited a full mitogenic activity and also strongly protected the protein against the effects of high concentrations of denaturing agents like urea (6).

The three-dimensional structure of the free and the inositol hexasulfate bound acidic fibroblast growth factor was established on the basis of 871 unambiguously assigned NOE upper distance constraints, uniformly distributed along the entire primary structure. The three-dimensional structures of the bound and the free protein effectively follow the same folding pattern (β-trefoil). However, an examination of the set of the ten structures that best fit the NOE constrains in each case, shows that some of the regions of the free protein are poorly defined structurally. These differences are strikingly depicted in the Ramachandran plot of the ten structures obtained for the free and bound protein. Comparison of the Ramachandran plot for the two forms of the protein show a significant increase in high-defined β-sheet in the bound protein. The regions with poorer defined structures appear to be located near the N-terminal region of the protein. This is clearly depicted in a histogram of the inverse of the contribution of each residue to the global r.m.s.d. of the best ten structures in each case. It has been shown that this region of the protein interacts with the cell membrane receptor specific for fibroblast growth factors, and that synthetic peptides encompassing this region compete with the whole protein in binding to its cell membrane receptor, and also partially mimic the mitogenic activity of the whole protein (7). In summary, the structures we have computed on the basis of ^{1}H-NMR data suggest that binding to heparin considerably increases the three dimensional structural definition of one of the regions of the protein that is recognized by its specific cell membrane receptor. This should cause a significant enhancement in the affinity of acidic fibroblast growth factor for its receptor following heparin binding.

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GUILLERMO GIMÉNEZ
Centro de Investigaciones Biológicas
C.S.I.C. - c/ Velázquez 144
28006 MADRID (Spain)

STRUCTURAL BASIS OF THE ACIDIC FIBROBLAST GROWTH ACTIVATION BY INOSITOL HEXASULFATE

Acidic and basic fibroblast growth factors were first isolated from brain and bovine pituitary on the basis of their mitogenic activity towards some established fibroblast cell lines. It was later shown that the mitogenic spectrum of these proteins is remarkably broad: they induce cell division in all mesoderm and neuroectoderm derived cells. It has also become progressively evident that these proteins have many other physiological activities. Thus, fibroblast growth factors are important modulators of the endocrine effects of TRF on the anterior pituitary, and of FSH on the secretion of oestrogen and progesterone by the granulosa ovarian cells; they are implicated in food-intake regulation at the level of the lateral hypothalamic area, they regulate the secretion of tissue plasminogen activator by endothelial cells, and they are powerful vasodilators. So far, no clear-cut functional differences between acidic and basic fibroblast growth factors have been reported (1).

Acidic and basic fibroblast growth factors bind strongly to heparin and to the sulphated glycosaminoglycan moiety of heparan sulphate, a common glycoprotein of the extracellular matrix. Actually, acidic and basic fibroblast growth factors are also known as heparin binding growth factors (1). It has been shown by infrared spectroscopy that the binding of heparin induces conformational changes in fibroblast growth factors (2).

Fibroblast growth factors trigger cell division by binding to specific high affinity receptors with tyrosine kinase activity, at the cell surface (3). This interaction requires heparin (4). Consequently, the actual mitogen is the heparin-bound fibroblast growth factor. In the case of the basic fibroblast growth factor, the heparan sulfate of the cell surface is sufficient for receptor binding. However, exogenous heparin is always required to induce mitogenesis with acidic fibroblast growth factor.

Molecular clues to the heparin requirement in fibroblast growth factor-induced mitogenesis may be provided by high resolution structural studies of the heparin-bound growth factor. Since the heparin preparations are highly heterogeneous such studies have to be carried out with heparin mimics having well defined chemical structures. A first attempt has been the determination by X-ray crystallography, of the structure of acidic fibroblast growth factor bound to sucrose octasulfate. This compound binds tightly to fibroblast growth factors and, like heparin, strongly stabilizes them (5). However, these studies did not show any differences between the free and sucrose octasulfate bound acidic fibroblast growth factor. It was subsequently shown that fibroblast growth factor is inactive in the presence of sucrose octasulfate. Furthermore, the form of the acidic fibroblast growth factor used in the crystallographic studies, which had been obtained by site directed mutagenesis, did not require the presence of exogenous heparin in the mitogenesis assay. We also found that high salt concentrations, such as those used to crystallize acidic fibroblast growth factor, stabilize the protein in a manner similar to heparin.

In order to investigate the structural consequences of heparin binding, we first made the choice of using NMR. We also decided to work with a polypeptide as identical as possible to the native one. The native acidic fibroblast growth factor is a 154 amino acid protein. This is larger than the biggest protein whose three-dimensional structure has been solved by ^{1}H-NMR. Our studies also had to be carried out at neutral pH because serious problems with protein stability in other conditions. We have, nevertheless, isolated shortened forms of the protein that do not show any appreciable functional differences from the native form. Thus, we have shown that a 132 amino acid fibroblast growth factor is as stable as the full length

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DNA-binding proteins and their interaction with DNA

R. Kaptein.
Bijvoet Center for Biomolecular Research, Utrecht University,
Padualaan 8, 3584 CH Utrecht,
The Netherlands

NMR has contributed considerably to our understanding of DNA sequence recognition by proteins. Several solution structures of DNA binding domains of gene regulatory proteins have been solved, an early example being the lac repressor headpiece in 1985. In particular, for several sub-families of zinc-finger proteins the structural information came first from NMR studies and was later confirmed by X-ray crystallography. We have been studying various classes of DNA-binding proteins such as prokaryotic repressors (lac and lexA repressors), and eukaryotic transcription factors including the nuclear hormone receptors (glucocorticoid and retinoic acid receptors) and POU-domains proteins.
The complex of lac repressor headpiece with an 11 bp lac operator half-site has been studied in great detail. Both structural and dynamic aspects of the protein-DNA complex will be discussed. When a full structural study of protein-DNA complex is not possible one can obtain useful information using docking procedures, when the structure of the protein is known. We have developed the program MONTY for this purpose, which uses a Monte Carlo sampling procedure. A simplified force field is used that represents H-bond and van der Waals interactions. In addition, biochemical data such as from footprinting, contact point analysis and mutagenesis can also be taken into account. The program will be illustrated with the lexA repressor, which interacts with a variety of SOS operators in E. coli.

FIFTH SESSION:

Chairperson: Cornelis W. Hilbers

Conformational Flexibility in Nucleic Acids

Thomas L. James, Carlos González, He Liu, Uli Schmitz, Marco Tonelli, and Nicolai Ulyanov, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446.

Sequence-dependent structural variations in nucleic acids are important for recognition by proteins, drugs and mutagens. There are indications that the three-dimensional arrangement of hydrogen-bonding sites, bases, and helix geometry may play an important role in protein recognition, with specific promoter sequences exhibiting 10^3-10^4 times stronger affinity for the transcription factors than random sequences. The short range interactions which influence measurable NMR parameters are quite sensitive to the local molecular environment. Crystal structures of protein/DNA complexes indicate that bound DNA can be modified significantly from canonical B-DNA, but it is not known whether the particular DNA sequence had some tendency for these structural deformations in the absence of protein. This lack of knowledge is chiefly due to the lack of high-resolution structures for DNA sequences which are specifically recognized by proteins. Within the past couple years, the quality of DNA structure determination via NMR has improved sufficiently to provide an accurate, high-precision structure of nearly any DNA double helix of length less than 15 base pairs if sufficient care and effort are expended. Although the number of well-determined structures is not high, we are able to draw a few tentative generalizations regarding the sequence-dependent structural features of DNA duplexes in solution. While nucleic acid duplexes do assume a "structure" in solution, internal inconsistencies in some of the NOE and scalar coupling data do suggest that there is some conformational flexibility. In particular, NMR data for a DNA-RNA hybrid suggest significantly more structural flexibility in the DNA strand than the RNA, yet the DNA does retain sequence-dependent structural features (*1,2*). For conformationally flexible molecules in solution, structural information from NMR data (both NOE-based and scalar coupling-based) is time-averaged. This complicates the situation, but there are ways to address this complication. We have examined two methods of doing so. One entails the use of molecular dynamics with time-averaged restraints (MD-tar) (*2,3*), which was originally proposed by Torda et al (*4*). Another approach, termed PARSE, we have recently developed generates an ensemble of conformers and then yields an assessment of the probability of each conformer (*5*). For both the MD-tar and PARSE procedures, the experimental NOE and scalar coupling data ensemble are better fit by the conformational ensemble than by the single best rigid structure obtained by conventional rMD.

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STRUCTURE AND FUNCTION OF *E. COLI MET* REPRESSOR: AN ELECTRIC GENETIC SWITCH

Jonathan P. Porter, Kathryn Phillips# and Simon E.V. Phillips

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK, #present address: MRC Virology Unit, Church Street, Glasgow G11 5JR

The *E. coli met* repressor is a small (24 kDa) dimeric protein that controls gene expression in the methionine biosynthetic pathway. It binds to natural operator regions consisting of 2-5 tandem repeats of 8 base pair sequences homologous to the "met box" consensus dAGACGTCT, forming cooperative oligomeric arrays of repressors, one per met box, arranged in superhelices around the DNA. Its three-dimensional structure has been determined in a number of different crystal forms, both alone and in complexes with its corepressor S-adenosylmethionine (SAM) and a synthetic DNA fragment corresponding to a consensus operator. The crystal structure of the repressor-operator complex shows two protein dimers bound to one 18 base pair DNA fragment containing two consensus met boxes, with protein-protein contacts mediating the cooperativity. Pairs of protein antiparallel β-strands lie in the DNA major groove, with side chains contacting the bases, to read the DNA sequence.

The structure of the repressor itself is relatively unperturbed by the binding of the corepressor (SAM), and of operator DNA. The SAM molecules carry a positive charge, and bind to sites on the repressor remote from the DNA-binding site. Despite the lack of induced structural change, however, SAM binding raises the affinity for the operator by a factor of up to 1000. The neutral corepressor analogue S-adenosylhomocysteine (SAHC) also binds to the repressor, but does not raise operator affinity. However, positively charged analogues, such as sinefungin (adenosylornithine - AO) and a synthetic analogue where the SAM sulphur is replaced by nitrogen (NAM) do raise affinity. Calculations of electrostatic potential for the repressor and its complexes suggest that the activation of DNA binding may be explained by long-range electrostatic interaction between the charges on the corepressor and the DNA phosphate groups[1]. The electrostatic calculations assumed the structure of the holorepressor to be the same for the SAM and corepressor analogue complexes. To test this assumption, high resolution crystal structures have now been determined for complexes of repressor with SAHC, AO and NAM. In all cases the overall repressor structure is indeed unchanged, but analogues bind in slightly different conformations, not all of which are fully ordered. The results are, however, consistent with the model of *met* repressor as an electrostatically triggered genetic switch.

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NMR Studies of RNA Recognition by Basic-Domain HIV Regulatory Proteins and by RNP Proteins

Gabriele Varani*, Fareed Aboul-ela, Frederic Allain, Charles Gubser, Peter Howe, Jon Karn and David Neuhaus

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

RNA-protein complexes are involved in many aspects of regulation of gene expression, including protein synthesis, mRNA processing and regulation of mRNA transcription and translation. In contrast to the wealth of biochemical functions, the understanding of the structural basis of RNA recognition by RNA-binding proteins is limited to a handful of crystallographic studies. We are using NMR spectroscopy to determine at high resolution the solution structures of several RNA-protein complexes of up to 23 kDa in molecular weight. These studies have been made possible by advances in synthetic and spectroscopic techniques that allow the determination of RNA structure using the same protocols and approximations that have been succesfully used for protein NMR studies. The RNA and protein components of each complex have been separately isotopically labelled, and multidimensional triple resonance experiments have allowed unambiguous spectral assignments, the identification of intermolecular contacts and the establishment of a very extensive constraint list for structure determination.

The structures under investigation involve two classes of RNA-binding proteins. The RNP domain is found in hundreds of proteins, many from the RNA processing machineries, and recognize single stranded RNA regions via amino acids located on the surface of an antiparallel β-sheet. The basic-domain class of RNA-binding proteins, to which the essential HIV regulatory proteins Tat and Rev belong, recognize instead double helical RNAs at structural distortions induced by non Watson-Crick base pairs or bulges, probably via extended loops within a flexible protein. The structural results highlight the stereochemical complementarity between RNA and protein structures as a primary determinant of the affinity and specificity of RNA-protein recognition. This structural complementarity is often the result of the ligand-induced folding of a flexible RNA structure, and NMR provides a unique insight into these dynamic processes. In addition to widening the understanding of RNA-protein recognition, the high-resolution structure and the dynamic information on the Tat substrate TAR RNA defines an ideal target for the identification of novel antiviral compounds designed to inhibit this essential retroviral function.

Structure and Mechanism in S-adenosylmethionine Synthesis.

George D. Markham, Institute for Cancer Research; Fox Chase Cancer Center, Philadelphia PA, USA.

S-adenosylmethionine (AdoMet) is the primary biological alkylating agent and plays an essential role in metabolism. AdoMet acts as a methyl group donor in many reactions and is a precursor for synthesis of the polyamines spermine and spermidine, among other functions. AdoMet biosynthesis is catalyzed by the enzyme S-adenosylmethionine synthetase (ATP:methionine S-adenosyltransferase) which accelerates the conversion of ATP and methionine to AdoMet, pyrophosphate and orthophosphate (1). The enzyme requires two divalent metal ions such as Mg^{2+} for activity, and is further activated by certain monovalent cations such as K^{+} (2). AdoMet synthetase is highly conserved in evolution, with 70% sequence homology between isoforms from bacteria and mammals. The crystal structure of AdoMet synthetase from *Escherichia coli* has recently been solved by F. Takusagawa and co-workers at the University of Kansas (3). The structure shows that the tetrameric enzyme consists of a pair of dimers with the active site in the interface between two monomers. Site directed mutagenesis and detailed characterization of the resulting mutant proteins is being carried out to determine the roles of certain amino acids in catalysis. Selective mutagenesis is especially informative for AdoMet synthetase since the overall reaction occurs in two steps which can be individually studied: conversion of ATP and methionine to enzyme-bound AdoMet and tripolyphosphate, and the subsequent hydrolysis of tripolyphosphate to pryrophosphate and orthophosphate prior to product release. Tripolyphosphate hydrolysis apparently provides the thermodynamic driving force for AdoMet formation.

The role of the potassium ion activator has been explored by mutagenesis of the cation binding site which contains the carboxylate of glutamate-42 as the only protein side chain ligand to the metal ion. A mutation of glutamate-42 to glutamine (the E42Q mutant) abolishes the 100 fold activation of the rate of AdoMet formation that potassium provides to the wild type enzyme. The mutation has little effect of the rate of tripolyphosphate hydrolysis. The E42Q mutant enzyme has kinetic properties similar to those of the wild type enzyme in the absence of potassium. Crystallographic studies show that the uranyl cation binds at the same site as potassium. Kinetic measurements show that uranyl is a tight binding competitive inhibitor with affinity 10^{4} fold greater than potasssium.

Residues important in interaction with the polyphosphate chain have been identified from the crystal structure and by sequence homologies to inorganic pyrophosphatase (4). The sequence DxGxTxxK(I or L or V)I is found only in proteins known to bind pyrophosphate; these residues are located in the active site of both AdoMet synthetase and inorganic pyrophosphatase. An arginine precedes the lysine in this motif in all reported AdoMet synthetase sequences and is located near the polyphosphate binding site. Alteration of the arginine to leucine results in a dramatic decrease in the rate of the overall reaction, but presteady state kinetic studies show that it has less impact on the rate of either enzyme-bound AdoMet formation or the hydrolysis of added tripolyphosphate.

The results indicate that mutations can selectively alter one function of the bifunctional active site of AdoMet synthetase. The importance of using methods in addition to detailed steady state kinetic studies in elucidation of the consequences of mutations is underscored.

Acknowledgments: This work was carried out at Fox Chase by M. McQueney, R. Reczkowski and J. Taylor, and at the University of Kansas by F. Takusagawa and S Kamitori. The research was supported by grants from the National Institutes of Health.

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STRUCTURE OF LIVER S-ADENOSYL-L-METHIONINE SYNTHETASE

José M. Mato, Jesús Mingorance, Luis Alvarez, María A. Pajares
Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain

S-adenosylmethionine (SAM) sythetase uses methionine and ATP to synthesize SAM, the main methyl donor for transmethylation reactions. Liver SAM synthetase plays three essential functions:

a) It provides SAM, necessary for the many and varied methylation reactions that occur in cells as well as for polyamine synthesis.

b) It is the first step in the conversion of the amino acid methionine into cysteine, which is limiting in the synthesis of the vital antioxidant glutathione (GSH).

c) SAM synthesis is essential for folate cycling, thus allowing DNA and RNA biosynthesis to take place in cells.

In humans, impaired liver SAM-synthetase activity is associated with hypermethioninemia and liver disfunction. There are two different genes for SAM-synthetase in mammals, one expresed specifically in the liver and a second gene expressed in nonhepatic tissues. The rat liver enzyme exists in two different forms, a high- and a low-M_r SAM-synthetase, with molecular masses of 200 and 100 kDa respectively. By using a bacterial expression system we have shown that the high-M_r form of the enzyme is a tetramer and the low-M_r form a dimer of the same 43.7 kDa subunit. Both forms are not in equilibrium. The tetramer is about 15-fold more active than the dimer at physiological concentrations of methionine, and shows lower values for K_{met}^{MET} and K_m^{ATP}. The tetramer can be converted into an active dimer, the less active form, by incubation in the presence of 1.8 M LiBr and into an inactive dimer by modification with N-ethylmaleimide. In addition, both tetramer and dimer can be converted into inactive monomers upon oxidation in the presence of GSSG.

By changing the ten cysteine residues of rat liver SAM-synthetase to serine through site-directed mutagenesis we have observed: a) that Cys_{69} is essential for the formation of the tetramer; and b) that $Cys_{35,57,61}$ are important for the formation of the dimer.

The active site of rat liver SAM-synthetase was investigated with 8-azido ATP. A peptide (267-286) was identified which is well conserved in all SAM-synthetases whose sequence are known (e.g. E. coli, yeast, mouse, human). Moreover, this peptide contains the sequence GxxxxGK which matches a common motif present in several families of proteins that use ATP, known as the P-loop, and which is proposed to interact with the phosphoryl groups of ATP.

POSTERS

Probing Structure of Protein-RNA Complexes by Heteronuclear NMR: Interaction of HIV-1 TAR RNA with Tat

Fareed Aboul-ela, Jonathan Karn and Gabriele Varani
MRC Laboratory of Molecular Biology
Hills Road, Cambridge CB2 2QH, England

The binding of the transcriptional activator protein (*tat*) to the *tat* response element RNA (TAR) is necessary for the proliferation of the HIV retrovirus. The TAR RNA contains a 3 pyrimidine (UCU) bulge. Mutagenesis and chemical modification experiments have demonstrated that the U residue at the 5' end of the bulge and base pairs immediately above and below the bulge site are critical for tat binding. A 37 residue peptide (ADP-1) has been identified which contains the minimal elements necessary to mimic the RNA binding specificity of the full *tat* protein, though with somewhat reduced affinity [1].

We are using 2-d and 3-d heteronuclear NMR to probe the structure of the complex of TAR RNA with ADP-1. Free TAR RNA appears to consist of two stacked A-form helices with a significant amount of flexibility in the UCU bulge region. An allosteric transition is observed in TAR RNA conformation in the presence of ADP1 or even in the presence of a single argininamide. As judged by chemical shifts and NOE patterns, the structure of the RNA in the presence of the argininamide is very similar to that of the RNA in complex with the peptide. However, in contrast to argininamide, ADP-1 shows additional intermolecular NOEs to TAR, suggesting that there are multiple points of contact between the peptide and the RNA. *tat* -derived peptides lacking key residues from the "core" domain, do not bind TAR with the same specificity as the full *tat,* and do not produce the same intermolecular NOE contacts as observed with ADP-1.

We have used over 800 NOE restraints and over 100 torsion angle restraints to determine the structure of the RNA in the presence of the ligands. This structural information, together with NOE contacts observed between ADP-1 and TAR RNA, has been used to model the interaction between ADP-1 and TAR.

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Engineering a GTP binding site on CheY

L. Bellsolell[1], P. Cronet[2], J. Prieto[2], L. Serrano[2], M. Majolero[1] and M. Coll[1]

[1]Dept. Biologia Molecular i Cel.lular, C.I.D.(C.S.I.C.), 08034 Barcelona, (Spain).
[2]EMBL, 6900 Heidelberg (Germany).

With the aim of determining the structural determinants of GTP/GDP binding, we intend to engineer this function into the chemotactic protein CheY, that has almost the same fold as p21 but does not bind mononucleotides. The active sites of both proteins are at the C-terminal edge of the β-sheet, but between different loops. The original CheY active site will be eliminated.

A series of mutants with accumulated substitutions have been prepared. The former of them carry the substitutions necessary to convert the α1−β1 loop of CheY into a phosphate binding loop (P-loop).

The crystallographic structure of the first mutant (CheM1: Met17Gly) has been solved to 2.3Å and that of the second one (CheM2: F14G, S15G, M17G), with Mg^{2+} bound in the original active site, to 1.9Å. The 3-D structures of both mutants are very similar to that of apo-CheY; CheM2 has Mg^{2+} bound in the active site in the same manner as in the native structure Mg^{2+}/CheY(1), but it does not show the conformational change in the α4 helix observed in the native metal-bound structure. In both mutants the conformation of the α1−β1 loop is similar to that of the native structures.

In the fourth mutant, the complete Kinase 1 and 2 motifs have been introduced in the loops α1−β1 and α2−β2 respectively. CheM4 crystallizes in space group $P6_522$, and its 3-D structure has been solved to 2.3Å (2). Compared to CheY, p21 lacks one turn at the N-terminus of α1 helix; this mutant lacks two turns, and the α1−β1 loop interacts with its counterpart from a symmetry-related molecule, related by a two-fold axis. This conformation is not that of the desired P-loop, but this structure indicates that the conformation of the P-loop is not only determined by its sequence, but also by the interaction with neighbouring residues. More mutants have been prepared with additional mutations to stabilize the correct fold of the loop.

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11 AA CYTOPLASMIC LOOP OF BETA SUBUNIT OF HIGH AFFINITY IMMUNOGLOBULIN E RECEPTOR: 2D-NMR STUDY IN SOLUTION AND IN THE PRESENCE OF DOPC VESICLES

Rodolfo R. Biekofsky, Mire Zloh, Malcom Danton, Istvan Toth and William A. Gibbons

University-Industry Centre for Pharmaceutical Research and Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29-39 Brunswick Sq., London WC1N 1AX, U.K.

The receptor with high affinity for immunoglobulin E ($Fc_\epsilon RI$) on mast cells and basophils plays an important role in mediating many of the pathophysiological phenomena associated with allergy. $Fc_\epsilon RI$ is a tetrameric complex, $\alpha\beta\gamma_2$, of non-covalently attached subunits: one IgE-binding α subunit with the binding site in the extracelluar part of the chain, one β subunit and a dimer of disulphide linked γ subunits. Since the huge size of the receptor subunits do not allow a direct structure determination, a long-term project to characterize these proteins by cutting them into smaller domains or structural units has been started. The anisotropic environment of membrane receptors leads to a natural division of these in terms of environment, thus each membrane receptor can be classified into extracelluar, transmembrane and intra-cytoplasmic domains, where the membrane serves to ensure that the various domains have no contact. Beta subunit of $Fc_\epsilon RI$ in rodents is a 243 amino acid chain with four membrane-spanning segments. A 11 AA loop connects beta transmembrane helices 2 and 3. This domain is believed to adopt an amphipathic α-helical structure. In the present work a synthetic 11 AA peptide with the sequence of this loop is being studied by 2D-NMR spectroscopy in solution and in the presence of dioleicphosphatidylcholine (DOPC) vesicles. The comparison of the linear unconstrained peptide in solution with the peptide in the presence of lipidic environment should provide an interesting insight into the structure of the beta subunit, as well as on the induction of structure in peptides due to lipid membranes.

Comparative studies on native catalase HPII and its mutants n201h and h128n.

Jerónimo Bravo, José Maria Ferrán, Chistian Betzel, Thomas Scheneider, Jack Switala , Peter C. Loewen and Ignacio Fita.

Abstract

Catalase (EC 1.11.1.6) has been described as a protective enzyme to neutralize the toxic oxygen-radical by-products, but in fact the biological function of catalase remains unclear. *Escherichia coli* produces two catalases, HPI and HPII which are quite distinct in physical structure and catalytic properties, both from each other and from other catalases. HPII is a monofunctional tetrameric enzyme of 753 aminoacids and one cis-heme d per subunit.

The structures of native, n201h and h128n mutants have recently been determined in our group. The space group is monoclinic $P2_1$, and a whole tetramer is placed in the asymmetric unit. A native and n201h data set have been collected at room temperature up to 2.2 Å. A second native and the h128n data set were collected up to 1.9 Å resolution using criocrystallography techniques.

A possible relationship between the mutated essential residues and the catalatic activity will be presented, as well as a detailed comparison between the structures collected at room temperature and under the nitrogen stream.

RAMÓN CAMPOS-OLIVAS
Instituto de Estructura de la Materia
C.S.I.C. - c/Serrano 119
28006 MADRID (Spain)

^{1}H and ^{15}N assignment of the Ribonuclease α-Sarcine.

The solution structure of the citotoxic ribonuclease α-sarcine from the mold *Aspergillus giganteus* is being studied by heteronuclear multidimensional NMR spectroscopy. The citotoxicity of α-sarcine lays on its ability to cross cell membranes to inactivate the ribosome by hydrolyzing a strictly conserved sequence in the largest rRNA. Therefore, it interacts with membrane lipids and hydrolyzes RNA with high specificity. It shares a limited sequence identity with other bacterial and mammalian enzymes although its larger size (150 aa), its specificity, and its ability to interact with biological membranes are unique. Attempts to crystallize α-sarcin have so far failed. Structure determination and comparative analysis of this protein in structural, dynamic and evolutionary aspects could provide important advances in our understanding of this relevant group of enzymes. It is our working hypothesis that the larger size and higher mobility of α-sarcine would account for its interaction with lipids, not shown by other, shorter ribonucleases.

We recently succeeded in expressing and purifying uniformly ^{15}N-labelled α-sarcine. High quality homo and heteronuclear bidimensional spectra have been recorded and we are currently in the process of assigning the ^{1}H and ^{15}N resonances. Given the size of this protein and its aa composition (13 Pro, 13 Gly, ...), together with the high degeneracy of aromatic resonances observed, the assignment of α-sarcine with conventional bidimensional methods is a formidable task. Therefore, three-dimensional techniques as well as alternative, specific approaches to simplify the problem are now being applied. A few stretches of residues have already been assigned and we are hopeful that we can present most of the assignment and the secondary structure of α-sarcine in this communication.

RAFAEL GIRALDO SUÁREZ
Dept. de Microbiología Molecular - Lab. 452
Centro de Investigaciones Biológicas
C.S.I.C. - c/Serrano 144
28006 MADRID (Spain)

Structure and function of the yeast telomere binding protein RAP1

Introduction

Telomeres are the natural ends of the eukaryotic chromosomes and constitute singular nucleoprotein complexes, not yet fully characterized, with multiple functions in chromosome dynamics (1): protect chromosomes against nucleolytic degradation and end fusions, assure their replication to the end, repress the expression of genes located nearby and have a role in chromosome association, e.g. during meiosis (2). The protein components of telomeres are DNA packing proteins (3) and telomerase, the specialized ribonucleoprotein responsible for telomere replication (4). The interest on telomeres has been recently boosted by the finding that telomere shortening is commonly associated with cellular ageing and tumoral transformation (5), converting telomere-associated proteins into potential targets for structure-based drug design. The DNA component of telomeres has focused no less attention since telomeric sequences, very conserved throughout the evolution, are built on highly repetitive clusters of guanines segregated to one strand (and, therefore, cytosines to its complementary) (6). Telomeric DNA shows the widest structural polymorphism found so far for any kind of natural sequence (7), including double-stranded regions, single-stranded ends (8) and, more interestingly, the potential to form a variety of novel structures, the DNA quadruplexes, based on guanine-guanine or cytosine-cytosine base pairing (9).

RAP1 is a multifunctional DNA binding protein from the yeast *Saccharomyces cerevisiae*. It was first characterized as a transcriptional activator of several essential genes, as well as to be required for the silencing of the mating type *loci* (10). The isolation of conditional mutants in the protein that exhibited alterations in telomere length and stability (11), as well as immunofluorescence studies (12), established that a major role for RAP1 is to be part of the telomeric chromatin (13). As a consecuence, there is a considerable interest in RAP1 and its roles in yeast telomere structure and function.

Results and Discussion

We have cloned and expressed in *Escherichia coli* of both RAP1 and its minimal DNA binding domain (RAP1-DBD) (14) and then purify both proteins to large scale, with the aim of starting crystallization attempts. In parallel, in collaboration with the group of Dr. S.M. Gasser (ISREC, Lausanne, Switzerland), we performed biochemical studies that characterized the interaction of RAP1 with double-stranded telomeric sequences (15), which showed DNA distortion ($KMnO_4$ reactivity) upon binding. DNA bending induced by RAP1 was observed by means of electron and scanning tunneling microscopy, in collaboration with the groups of Drs. J. Sogo and H. Gross (ETH, Zürich, Switzerland) (16). Finally, we found that RAP1 promotes the

formation of parallel DNA quadruplexes by the telomeric G-rich strand after a series of biochemical (17) as well as spectroscopic (Circular Dichroism) (18) sudies. This result has clear implications for the possible role of these DNA structures in chromosomal association (9).

RAP1-DBD presents a very interesting structural problem, since it is one of the largest described so far (335 residues), has no obvious homology to any family of characterized DBDs and it is able to bind to different DNA structures (15, 17-18). We obtained crystals of a complex between RAP1-DBD and a 19 bp telomeric oligonucleotide (unpublished results). The crystals diffract to 2.7 Å with the laboratory CuKα X-ray source and to 2.4 Å with synchrotron radiation (Daresbury, UK, station 9.6). Several datasets were collected using techniques of cryo-crystallography at liquid nitrogen temperatures. They belong to the Trigonal space group $P3_1$ (cell dimensions a=b= 92.3 Å, c= 80.64 Å; $\alpha=\beta= 90^o$, $\gamma= 120^o$) and there are two complexes (41 kDa each) per asymmetric unit. Data processing and reduction has been performed using programs from the CCP4 package. Since soaking experiments with several heavy atoms compounds were unsuccessful, MIR phases were obtained by derivatizing the DNA with iodo-uracil or iodo-cytosine, as well as by substitution of phosphates in the DNA backbone by phosphotioate groups (then reacted with mercurial compounds). The location of the heavy atom sites in the difference Patterson maps was helped by building a model for the DNA packing in the cell (as a pseudocontinuous DNA fiber), using the reflections from the diffused-scattering DNA arcs by means of rotation and translation searches as implemented by X-PLOR. After maximum likelihood phase refinement (MLPHARE), only highly isomorphous derivatives yielded interpretable peaks in difference Fourier maps and a reasonable phasing power (≤ 1.0) to 3.2-3.5 Å. Using the information available, we have recently plot our first experimental electron density maps, in which density can be assigned to the phosphate groups in the DNA. Now the improvement of those maps is under progress (averaging through the non-crystallographic two-fold axis and phase combination with the DNA model) as the previous step in building a model for the complex into the electron density.

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Structure and Dynamics of a DNA•RNA Hybrid Duplex with a Chiral Phosphorothioate Moiety. NMR and Molecular Dynamics with Conventional and Time-averaged Restraints.

Carlos González and Thomas L. James

The three-dimensional structure of two thiophosphate-modified DNA•RNA hybrid duplexes d(GCTATAApsTGG)•r(CCAUUAUAGC), one with R-thiophosphate chirality and one with S-thiophosphate chirality, have been determined by restrained molecular dynamics simulations (rMD). The conformational flexibility of these hybrids have been investigated by employing time-averaged constraints during the molecular dynamics simulations (MD-tar). A set of structural restraints, comprising 322 precise interproton distance constraints obtained by a complete relaxation matrix analysis of the 2D NOE intensities as well as J coupling constants obtained from quantitative simulations of DQF-COSY cross-peaks in deoxyriboses, was obtained. Multiple conformations of the deoxyribose moieties were evident from the scalar coupling constant analysis. Accurate distance constraints, obtained from complete relaxation matrix analysis, yielded a time-averaged solution structure via conventional restrained molecular dynamics which is not compatible with the experimental J coupling constants (root-mean-square deviation in J value ~ 2 Hz). However, vicinal coupling constant information can be reproduced when time-averaged constraints are used during the molecular dynamics calculations instead of the conventional restraints (Jrms ~ 0.6 Hz). MD-tar simulations also improve the NMR R-factors. This improvement is more evident in the DNA than in the RNA strand, where no indication of conformational flexibility had been obtained. Analysis of the MD-tar trajectories confirms that deoxyriboses undergo pucker transitions between the S and N domain, with the major conformer in the S domain. The ribose moieties in the RNA strand, however, remain in the N domain during the entire simulation. Conformations of deoxyriboses in the intermediate E domain are obtained when the average structure is calculated with conventional NMR-restraints. Since these conformations can not account for the experimental J coupling information, and they only appear in a very low population in the MD-tar ensemble, we conclude that intermediate E sugar puckers are artifacts produced by the attempt to fit all the structural constraints simultaneously when in reality more than one conformer is present. Most structural features of the duplex remain the same in the average structure and in the MD-tar ensemble, e.g., the minor groove width, exhibiting an intermediate value compared with those of canonical A- and B-like structures. Calculations with the two duplexes of differing chirality in the modified phosphate have been carried out. Only minor effects in the backbone close to the thiophosphate have been detected.

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NMR SOLUTION STRUCTURE OF THE C-TERMINAL FRAGMENT 205-316 OF THERMOLYSIN

F. Conejero#, C. González*, M.A. Jiménez* & M. Rico*.

#New Chemistry Laboratory. Oxford. U.K.
*Instituto Estructura de la Materia. C.S.I.C. Madrid. Spain

Fragments 205-316, 228-316 and 255-316 of thermolysin, all of them belonging to the C-terminal domain of thermolysin, are able to adopt a very stable globular structure according to CD and inmunochemical data (1,2). The three-dimensional structure of the shortest fragment 255-316 in aqueous solution was determined very recently by ^{1}H NMR (3). It is a dimer in which each subunit presents a globular structure, compossed of three helices, largely coincident with that of the corresponding region in the crystallographic structure of the intact thermolysin (4).

In the present communication the three-dimensional structure of the longer fragment 205-316 in aqueous solution is investigated by ^{1}H NMR. The assignment of the NMR signals was performed by using the standard sequential assignment method. The δ-values measured for all residues within the N-terminal segment 205-257 are quite close to those expected for random coil state and no NOE indicative of an ordered structure could be found. In contrast, the δ-values measured for residues 258-316 are almost identical to that previously reported for these same residues in the shorter fragment 255-316. Furthermore, the set of NOEs found for segment 258-316 of fragment 205-316 of thermolysin is completely similar to that found for the shorter fragment 255-316. The intersubunit NOEs found in fragment 255-316 of thermolysin are also observed in fragment 205-316. The three-dimensional structure was calculated by using distance geometry and restrained dynamic molecular methods. The calculated three-dimensional structure in conjunction to the NMR data discussed above permit to conclude that fragment 205-316 of thermolysin adopts a dimer structure in which each subunit has the 60 C-terminal residues present a well-defined native-like structure whereas the 52 N-terminal residues in a random coil state. Dimer interface is similar to that found in the shortest fragment 255-316.

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Structure of the binding site for inositol phosphates in a PH domain.

M. Hyvonen, M.J. Macias, M. Nilges, H. Oschkinat, M.Saraste and M. Wilmanns.

Phosphatidylinositol bisphosphate is a cellular phospholipid which has been found to bind specifically to pleckstrin homology (PH) domains that are commonly present in signalling proteins. We have studied the complexes of the β-spectrin PH domain and soluble inositol phosphates using both NMR spectroscopy and X-ray crystallography. The specific binding site is located in the centre of a positively charged surface patch of the domain, and basic amino acid are critical for binding. The interactions between PH domains and phosphoinositides could be involved in reversible ahchorage of proteins to membranes, or the domain could participate in a response to a second messenger such as inositol trisphosphate. These interactions may organize cross-roads in cellular signalling.

MOLECULAR MODELLING STUDIES OF LOOP STRUCTURES IN PROTEINS
The Ser100-Gly104 Loop of Lysozyme

Rachid C. Maroun and Graham Bentley, Immunologie Structurale, Institut Pasteur, 25-28, rue du Dr. Roux, 75724 Paris CEDEX 15

A very important problem in biology deals with structural and molecular recognition. Protein loops are frequently present in interactions involving proteins. Loops in proteins consist of varied amino acid sequence and chain length, thus involving a large number of conformational degrees of freedom. Since in most cases the conformation of a loop is not available from observation, the prediction of loop conformations has many potential applications. Yet the usefulness of such approaches is often limited by the accuracy of the results.

We've decided then to evaluate an existing loop structure prediction procedure in order to establish suitable protocols leading to useful results. We have chosen lysozyme as our model protein, given the availability of several crystal structures (many of which have been determined in this laboratory) in varied crystalline forms and coming from different avian species. In addition, the structures determined by X-ray diffraction of these lysozyme antigens show an interesting feature, i.e., in the case of japanese quail lysozyme (JEL), and also in the case of the complex between the anti-lysozyme antibody FvD1.3 and hen egg white lysozyme (HEL), the loop constituted by residues 100-104 (of sequence SDVHG) presents a conformation significantly different from the corresponding segment in the uncomplexed lysozymes from hen egg white, pheasant, and guinea fowl, in which residue 102 is a glycine, and 103 an asparagine. In other words, instead of folding towards the lysozyme catalytic site as in single HEL, the JEL loop is turned away from the site, giving rise to differences of up to 7.5 Å in the Cα positions with respect to other lysozyme structures.

This difference in structure can be ascribed in principle to the substitution at position 102 of the glycine in HEL for the valine in JEL. Indeed, the fact that the (ϕ, ψ) angles of residue 102 in HEL are unfavourable for all amino acids except glycine offers a simple explanation for this difference. Yet, this reasoning alone does not help explain the case of the FvD1.3-HEL complex.

In order to understand this phenomenon, and as a complement to the techniques of X-ray crystallography, we're employing the techniques of computational chemistry as implemented in the Loop Modelling facility of the commercial software QUANTA4.0 (MSI, Cambridge, UK) in order to characterise the factors that determine the structure and stability of the 100-104 lysozyme loop. These factors include the effects of i) aminoacid sequence, ii) crystal packing and near neighbor residue, and iii) solvent.

Our findings will be reported, and propositions will be made to confront it to an experimental program of site-directed mutagenesis of the corresponding loop residues.

^{1}H-NMR Assignment and Solution Structures of Human Acidic Fibroblast Growth Factor and its Complex with Inositol Hexasulfate. Conformational Changes induced upon Ligand Binding. Structure-Function Relationships.

Antonio Pineda-Lucena[1], M. Ángeles Jiménez[2], José L. Nieto[2], Jorge Santoro[2], Manuel Rico[2] and Guillermo Giménez-Gallego[1]
[1]*Centro de Investigaciones Biológicas (C.S.I.C.) Velázquez 144, 28006 Madrid, Spain*
[2]*Instituto de Estructura de la Materia (C.S.I.C.) Serrano 119, 28006 Madrid, Spain*

A truncated form of human acidic fibroblast growth factor of 132 amino acid residues is shown to be as active and stable as the 139 residue molecule initially described, and commonly used in physiological studies. It is shown that inositol hexasulfate is a good substitute for heparin in both activating and protecting acidic fibroblast growth factor. The complex between the shortened form of the protein and inositol hexasulfate was used to determine the structure of activated acidic fibroblast growth factor. The ^{1}H-NMR spectra of free and complexed proteins were totally assigned and their three-dimensional structures computed. The global fold shown by the acidic fibroblast growth factor is the "β-trefoil" motif, that is composed of six β strands forming a barrel structure and other six forming three β-hairpins, which are in a triangular array that caps the barrel. The inositol hexasulfate binds to the protein through the positively charged groups of Lys126, Lys127, Arg133 and Lys142 side-chains. The computed three-dimensional structures suggest that inositol hexasulfate binding limits the conformational flexibility of the protein which may account for the physicochemical and biological differences found between both free and complexed proteins.

STRUCTURAL CHARACTERIZATION OF PARTIALLY FOLDED STATES ON A NASCENT POLYPEPTIDE CHAIN

J.L. Neira, L.S. Itzhaki, G. de Prat Gay, J. Ruiz-Sanz & A.R. Fersht

We have prepared by protein engineering methods and chemical cleavage a family of fragments of the 64 residue protein chymotrypsin inhibitor 2 (CI2), corresponding to the elongation from the N-terminus. Here, we show the results obtained with the larger fragments, by using CD and current homonuclear and heteronuclear NMR techniques.

The peptide CI2(1-50) shows a poor chemical shift dispersion in the amide region.This suggest the absence of stable secondary and tertiary interactions in the fragment.

The unique α-helix of CI2 is formed before the rest of the structural elements, as it is judged from the studies of the peptide CI2(1-53) and CI2(1-60), but it is stabilized by tertiary interactions as a chemical shift study and other studies with shorter fragments have shown.

Deletion of the last residue, CI2(1-63), results in a fragment with native-like structure, as indicated by CD and NMR, but it is less stable to denaturation by temperature. The detailed characterization by NMR has shown that there are no substantial differences in the loop region, compared with the intact protein.

All these results are discussed in terms of the folding behaviour of nascent polypeptides chain and the cooperativity effects.

STRUCTURAL POLYMORPHISM OF d(GA·TC)$_n$ SEQUENCES AND THEIR RECOGNITION BY PROTEINS

M. Ortiz Lombardía, I. García Bassets, D. Huertas, M. Vorlíčková, J. Kypr and F. Azorín.

Departmento de Biología Molecular y Celular
Centro de Investigación y Desarrollo (CSIC)
Jordi Girona 18-26
08034 Barcelona (Spain)

Alternating d(GA·TC)$_n$ sequences are quite abundant in eukaryotic genomic DNA. They can account for about 0.4% of a mammalian genome(1). Interestingly, they are usually found in, or near, transcriptional regulatory regions or in recombinatory hot-spots. SV40 viruses carrying a d(GA·TC)$_{22}$ sequence show an increased genomic instability(2).

These sequences show a remarkable degree of structural polymorphism, including several non-B DNA conformations. d(GA)$_n$ sequences are proposed to form parallel stranded duplexes as well as multistranded complexes(3) but they can, also, form antiparallel duplexes(4). In the presence of appropriate concentration of Zn^{2+} cations and at neutral pH, oligo d(GA)$_n$ forms a duplex with characteristic CD spectrum and electrophoretic mobility. This duplex seems to be antiparallel. The role played by the metal cation is not yet clear, but chemical modification with DMS provide support to the hypothesis that it can co-ordinate with the N7 position of guanines.

On the other hand, short oligo d(TC)$_n$ sequences form antiparallel duplexes at acidic pH, stabilised by the formation of C^+·C pairs(3).

Along with the usual B-DNA duplex, mixtures of both oligo d(GA)$_n$ and oligo d(TC)$_n$ yield other multistranded structures such as intermolecular triplexes both pyr·pur·pyr (at acidic pH) and pyr·pur·pur (at neutral pH, in the presence of Zn^{2+}). These may somehow be considered similar to the conformations found in plasmids H-DNA and H*-DNA, respectively.

We address the question as whether such complex set of structures and conformations could be capable of exercising some biologically relevant control. With this aim, we have partially purified a set of nuclear proteins (ranging from 65kDa to 110kDa) from mouse cells, which show a strong affinity for single-stranded d(TC)$_n$, d(T)$_n$ and, to a lesser extent, for d(C)$_n$ and d(A)$_n$ sequences. No one of them recognises, significantly, d(GA)$_n$ sequences.

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Crystallization of the PAPS reductase from *E.coli.* Preliminary X-ray analysis.

Guillermo Montoya*, Jean Dirk Schwenn# & Irmgard Sinning*

*European Molecular Biology Laboratory, Biological Structures and Biocomputing Programme. #Ruhr University of Bochum Biochemistry of Plants, Faculty of Biology.

Inorganic sulphate is reduced to sulphide by a sequence of enzymatic steps involving ATP sulphurylase, adenyl sulphate kinase (APS), Phospho-adenylylsulphate reductase (PAPS) and sulphite reductase. An O-acetyl-Lserine (thiol) lyase incorporates the sulphide forming the aminoacid cysteine. The complete process of assimilatory sulphate reduction supplies prototrophic organisms with reduced sulphur as requiered for the biosynthesis of sulphur containing metabolites.

The PAPS reductase gene from *E. coli* K12 was cloned and expressed in a PAPS reductase-deficient strain of *E.coli* . The gene product was precipitated after cell disruption using ammonium sulphate precipitation. The protein was purified in three chromatographic steps, Phenyl-Sepharose CL4-B, Blue, Sepharose CL-4B and Red Sepharose CL4B.

Protein crystallization was carried out at 4 C in 0.1 M citrate buffer pH 5.2, 0.2 M ammonium acetate and 13 % methyl PEG 5000. The crystals were orthorhombic, space group C222 and they difracct beyond 3 Å resolution.

Molecular Characterization of the Elsamicin Binding to the B and Z Conformations of DNA

J. Portugal[1], A. Vaquero[1], C. Alhambra[2], F.J. Luque[2] & M. Orozco[2]
[1]Centro de Investigación y Desarrollo. CSIC. & [2]Universidad de Barcelona. Barcelona (Spain)

The elsamicin A (an antitumour antibiotic) binding sites can be mapped in DNA regions which are rich in guanine+cytosine [1-3]. Elsamicin A inhibits the rate of digestion by NruI to a greater extent than it does for other restriction enzymes, thus evidencing the sequence-selective binding of elsamicin to GCG regions in DNA [2]. Since alternating CG sequences are among those required to observe the Z-form DNA, we considered whether the elsamicin binding to poly[d(G-C)] and poly[d(G-m5C)] affects to the presence of the B and Z conformations[4]. Both the rate and the extent of the B-to-Z transition are diminished by the antibiotic, as inferred by spectroscopic methods under ionic conditions that otherwise favour the left-handed conformation of the polynucleotides. Moreover, elsamicin converts the Z-form DNA back to the B-form. Circular dichroism data indicate that elsamicin binds to poly[d(G-C)] and poly[d(G-m5C)] to form right handed-bound elsamicin regions. The elsamicin A effect might be explained by a model in which the antibiotic binds preferentially to a B-form DNA, playing a role as an allosteric effector on the equilibrium between the B and Z conformations, thus favouring the right-handed one [4]. Molecular Dynamics studies [5] of the elsamicin A binding to two DNA fragments containing unique CpG sites (GATACGATAC or GATGCGATAC) allowed us to obtain a three-dimensional model of the elsamicin-DNA complex. The three-dimensional model explains the preference of this small ligand for binding to the B-form DNA. Furthermore, the roles played by the chromophore and the disaccharide moiety in the sequence-specific binding to GC-tracts can be determined.

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STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF LDL BY INFRARED SPECTROSCOPY.

J.L.R. Arrondo

Dept. Bioquímica; Univ. Pais Vasco; P.O. Box 644; E-48080 Bilbao; Spain.

Lipoproteins are a suitable example to apply infrared techniques. Low density lipoproteins (LDL) are constituted of a lipid core composed by triglicerides and cholesteryl esters and a surface layer with phospholipids, cholesterol, and a single protein, apolipoprotein B. The protein component of LDL is one of the largest protein known, and its structure and topology is not clear yet. Because of its large size and the lipid moiety, not all the techniques can be applied reliably. Use of prediction methods gives an α-helix component between 25 and 43% and ß-sheet values between 41 and 20%. The infrared spectra in H_2O and D_2O at 37 °C gives a structure of 24% α-helix, 26% ß-sheet, 11% ß-turns, 22% unordered structure, and 17% ß strands (non-sheet extended structures). Additionally, more information is obtained from some peculiar characteristics of the infrared spectra. Thus, a band at 1619 cm^{-1} represents a particular feature of lipoproteins. This band is not found at such low frequency in non-denatured proteins either in D_2O or in H_2O media. The band can be related to an extended chain interacting with the lipids and gives a picture of lipoprotein B as a long chain with different domains. The core undergoes thermal transitions assignables to the cholesteryl esters. Infrared spectroscopy shows that core modification is sensed in the surface, being the cholesteryl esters thermotropic event accompanied by conformational protein changes. These protein changes can be studied in the different bands assignable to protein conformations. The bands and the parameters arising from them follow different thermal patterns, indicating the distinct thermal sensitivity of the diverse conformations. The thermal effect also involves a protein irreversible denaturation at higher temperature. Ionic strength changes the temperature at which the protein "senses" the phase transition. Low ionic strength reduces the temperature from around 30 °C to 20 °C, and also the band corresponding to α-helix is affected by this change. Lipoprotein oxidation has been related with the process of atherioesclerosis. Examination of LDL after exposure to an oxidant agent shows changes in the conformation of the apolipoprotein concomitant with the exposure to the oxidant agents. Thus, short exposure to the oxidant agent does not produce significant changes in the thermal event at low temperature, but produces a decrease in the thermal denaturation temperature. Long exposures to the oxidant reveal significant changes in the infrared spectrum and differences in the sensitivity to the temperature in both the reversible and irreversible transitions.

COMPARISON OF SOLUTION STRUCTURES OF ω-CONOTOXIN GVIA AND ω-CONOTOXIN MVIID DETERMINED BY ^{1}H-NMR

C.Civera[1], A.Vazquez[1], J.M.Sevilla[1], F.Gago[2], A.G.Garcia[3], B.M.Olivera[4], P.Sevilla[1].

[1]Dpto.Quimica Fisica II, Facultad de Farmacia, Ins.Pluridisciplinar, Unidad RMN, UCM, 28040 Madrid. Spain.
[2]Dpto.Fisiologia y Farmacologia, Campus Universitario, 28871 Alcala de Henares, Madrid. Spain.
[3]Dpto.Farmacologia, Facultad de Medicina, UAM, 28029 Madrid. Spain.
[4]Department of Biology, University of Utah, UT84112. U.S.A.

Neurotoxins isolated from the venoms of marine snails *Conus geographus* block neurotransmitter receptors and ion channels (1). They all are small polypeptides (25-30 residues) and have multiple disulfide bridges. ω-Conotoxins (ω-Ctx's) that block calcium channels have a common pattern of Cys residues: C----C----CC----C----C. The DNA sequence that corresponds to the primary structure of ω-Ctx family has been isolated (2), and synthesis of different toxins and related analogs has been possible.Thus several studies for the activity of these molecules have appeared recently (3,4). But we are still far of knowing the mechanism of intermolecular recognition. A first approximation towards a better understanding of the molecular determinants of binding specificity in this class of compounds is the elucidation of their solution structure. We calculated the three-dimensional structure of the ω-Ctx GVIA (5) and recently the solution structure of other toxin ω-Ctx MVIIC (6) has appeared.

In this work we present the tertiary structure of ω-Ctx MVIID, calculated by ^{1}H NMR and we compare it with the one of ω-CTX GVIA we obtained previously.

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Crystal and Molecular Structure of a Dodecanucleotide in B form

Lourdes Urpí[1], Lucy Malinina[1], Tam Huynh-Dinh[2] and Juan A. Subirana[1]
[1]*Dept. Enginyeria Química, E.T.S.E.I.B., Universitat Politècnica de Catalunya, Av. Diagonal 647, 08028-Barcelona, Spain.*
[2] *Unité de Chimie Organique, Institut Pasteur, 28 Rue de Dr. Roux,75724 - Paris, France.*

The dodecadeoxynucleotide d(CGCTCTAGAGCG) has been synthesized and crystallized. This sequence is quite different from those previously studied. Its structure has been solved by the single crystal x-ray diffraction methods. The dodecamer has the B form. It has a new cell, with space group $P2_1$ and cell parameters: a= 35.05Å, b= 68.91Å, c= 26.15Å, β=91.16°. There are two duplexes in the asymmetric unit, so there are four chemically identical strands of DNA.

The structure has been solved by molecular replacement. The initial model was a standard B-DNA. It was necessary to split this model into fragments in order to reach a solution. It was refined by molecular dynamics simulated annealing and positional treatment. The refinement has been finished with an R factor of 15.2% for 3238 reflections with $F>2.5\sigma(F)$ in the resolution region 8.0 to 2.25 Å. During the process of refinement it has been possible to locate 70 molecules of water.

The more important features of the structure are:

1.- The two duplexes of the asymmetric unit are slightly curved in different directions. They are not related by non-crystallographic symmetry. Comparison of both conformations demonstrates the flexibility of this sequence.
2.- Both duplexes interact with neighbour molecules through the terminal guanines. This pattern of interaction has already been observed in all dodecamers ending by CG.
3.- The two duplexes of the asymmetric unit show an additional interaction through stacking of terminal cytosines.

Details of conformational parameters, base stacking patterns, intermolecular interactions and hydration will be presented.

STRUCTURE OF THE MAJOR ANTIGENIC LOOP OF FOOT-AND-MOUTH DISEASE VIRUS COMPLEXED TO A NEUTRALIZING ANTIBODY.

N.Verdaguer and I.Fita, Dept. Enginyeria Química ETSEIB-UPC, Diagonal 647 08028-Barcelona, Spain.
D.Andreu and E.Giralt, Dept. Química Orgànica, Universitat de Barcelona, 08028 Barcelona, Spain.
M.G.Mateu and E.Domingo, Centro de Biologia Molecular "Severo Ochoa" (CSIC-UAM), Universidad Autónoma de Madrid, 28049 Madrid, Spain.

A hallmark of RNA viruses is their population complexity, rapid antigenic variation, and hability to escape from neutralization by antibodies. Foot-and-mouth disease virus (FMDV) is a particularly relevant representative of the highly variable RNA viruses, since it is the economically most important animal patogen world-wide. The disease can be controlled by slaughter of affected animals or by regular vaccination in enzootic areas. However, the effectiveness of current, inactivated virus vaccines, and the development of new, synthetic vaccines against FMD have been hampered by the extensive antigenic variation of the virus. Seven distinct serotypes and multiple variants within each serotype have been described. A major, highly variable antigenic site (site A) of FMDV involved in virus neutralization is located within the G-H loop of capsid protein VP1 (1). This loop includes a conserved Arg-Gly-Asp (RGD) motif which mediates attachment to cells and virus infectivity (2). The conformation of the G-H loop could not be defined in any of the several crystal structures determined for native FMDVs (including isolates O1 BFS of serotype O and C-S8c1 of serotype C) (1,3). However, for this loop a structure was defined upon reduction of a disulfide bridge on the capsid of FMDV type O (4). In type C the loop appeared as disordered in the X-ray diffraction analysis, in spite of the disulfide bond being absent in this serotype (3). Multiple, naturally occurring substitutions at two hypervariable segments within the G-H loop of FMDV led to significant antigenic differences among variant viruses of this serotype. In adition, single amino acid replacements at one of few critical positions did severely affect the antigenic specificity of this site (5). Such antigenic differences could be faithfully mimiked with synthetic peptides wich include the relevant substitutions (6). Interpretation of the effect of aminoacid replacements in viral neutralization can be greatly aided by strucutral information of antigen-antibody complexes. We report here the crystal strucutre of a 15-amino acid peptide (A15; sequence YTASARGDLAHLTTT-NH2) which represents the antigenic site A of FMDV (CS8c1) complexed with the Fab fragment of a strongly neutralizing monoclonal antibody (MAb SD6) raised against the virus. The structure has been determined at 2.8 Å resolution with a crystallographic agreement factor of 18.2 %. The peptide shows a high degree of internal structure with a nearly cyclic conformation. The conserved RGD motif participates directly in the interaction with several complementarity-determinig regions of the antibody molecule. The Arg-Gly-Asp triplet shows the same open turn conformation found in the reduced form of FMDV (serotype O) and also in integrin-binding proteins. The observed interactions provide a molecular interpretation of the amino acid replacements observed to occur in mutants resistant to neutralization by this antibody. Also, the structure suggests a number of restrictions to variation within the epitope, which are imposed to keep the RGD motif in its functional conformation.

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A NOVEL STRUCTURAL MODEL FOR THE CYTOPLASMIC DOMAINS OF INTEGRIN TRANSMEMBRANE RECEPTORS . Michael J. Williams, Tom W. Muir, Stephen B.H. Kent and Mark H. Ginsberg, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

Integrins are heterodimers of α and β subunits that are characterised by short cytoplasmic tails, each linked by a transmembrane stretch to a single large extracellular domain. The cytoplasmic domains are key to many integrin functions. However, their study is limited by the difficulty of producing suitable recombinant or synthetic proteins with the correct conformation. To address this problem we have developed a novel protein mimic of integrin cytoplasmic domains that takes into account a number of features or assumptions about their structure. These include (a) the first residue of each tail can be predicted and thus the relative stagger of the two tails can be deduced, (b) the tails are connected to helical transmembrane stretches and (c) the tails are parallel, terminating with COOH-termini. Our model proteins are made by the chemoselective ligation of two individual polypeptides (mini-subunits) which each consist of an integrin cytoplasmic tail at the C-terminus, connected to a 28 residue stretch made of four heptad repeats and terminated with uniquely reactive groups at the N-terminus. These reactive groups allow the covalent linkage of the two mini-subunits in a parallel orientation and relative stagger defined by a helical coiled-coil structure formed between the heptad repeats. Thus the tails are brought together in a way that closely reflects the structural constraints within the intact receptor. Initial structural studies on the αIIbβ3 integrin model protein confirm that the coiled-coil structure is formed and suggest that the tails now adopt structures that are significantly different to isolated tail peptides. The biological activity of this model protein is presently under examination, in conjunction with high resolution structural studies.

List of Invited Speakers

Ad Bax	Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, MD. 20892 (USA). Tel.: 1 301 496 10 25 Fax : 1 301 496 08 25
Tom L. Blundell	ICRF Unit of Structural Molecular Biology, Department of Crystallography, Birkbeck College, Malet Street, London WC1E 7HF and Biotechnology and Biological Sciences Research Council, Central Office, Polaris House, North Star Avenue, Swindon SN2 1UH. (U.K.). Tel.: 44 171 580 66 22 Fax : 44 171 631 68 05
Miquel Coll	Centro de Investigación y Desarrollo, CSIC, Jordi Girona 18-26, 08034 Barcelona (Spain). Tel.: 34 3 400 61 00 Fax : 34 3 204 59 04
Christopher M. Dobson	Oxford Centre for Molecular Sciences, University of Oxford, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT (U.K.). Tel.: 44 86 527 59 16 Fax : 44 86 527 59 21
Philip R. Evans	MRC Laboratory of Molecular Biology, Hills Road, Cambridge (U.K.). Tel.: 44 22 340 22 11 Fax : 44 22 321 35 56
Stephen W. Fesik	Pharmaceutical Discovery Division, Abbot Laboratories, Abbot Park, IL. 60064 (USA). Tel.: 1 708 937 12 01 Fax : 1 708 938 24 78
Guillermo Giménez	Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 561 18 00 Fax : 34 1 562 75 18
Cornelis W. Hilbers	Nijmegen Son Research Center for Molecular Structure, Design and Synthesis, Laboratory of Biophysical Chemistry, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen (The Netherlands). Tel.: 31 80 65 21 60 Fax : 31 80 65 21 12
Thomas L. James	Department of Pharmaceutical Chemistry, University of California, 926 Medical Science, San Francisco, CA. 94143-0446 (USA). Tel.: 1 415 476 15 69 Fax : 1 415 476 06 88

Wolfgang Kabsch	Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstr. 29, 69028 Heidelberg (Germany). Tel.: 49 6221 486 276 Fax : 49 6221 486 437
Robert Kaptein	Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8,3584 CH Utrecht (The Netherlands) Tel.: 31 30 53 37 87 Fax : 31 30 53 76 23
George D. Markham	Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA. 19111 (USA). Tel.: 1 215 728 24 39 Fax : 1 215 728 35 74
Martín Martínez Ripoll	Departamento de Cristalografía, Instituto de Química-Física Rocasolano, CSIC, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Fax : 34 1 564 24 31
José M. Mato	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 50 54 Fax : 34 1 585 50 53
Dinshaw J. Patel	Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY. 10021 (USA). Tel.: 1 212 639 72 07 Fax : 1 212 717 30 66
Simon E.V. Phillips	Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT (U.K.). Tel.: 44 532 333 112 Fax : 44 532 33 31 67
Manuel Rico	Instituto de Estructura de la Materia, CSIC, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Ext. 1154 Fax : 34 1 564 24 31
David Stuart	Laboratory of Molecular Biophysics, Rex Richards Building, South Parks Road, Oxford OX1 3QU (U.K.). Tel.: 44 1865 27 53 67 Fax : 44 1865 27 51 82
Gabriele Varani	MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (U.K.). Tel.: 44 223 24 80 11 Fax : 44 223 21 35 56
Kurt Wüthrich	Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Hönggerberg, CH-8093 Zürich (Switzerland). Fax : 41 1 633 11 51

List of Participants

Fareed Aboul-ela	MRC Laboratory of Molecular Biology, Division of Structural Studies, Hills Road, Cambridge CB2 2QH (U.K.). Tel.: 44 223 24 80 11 Fax : 44 223 21 35 56
Lluís Bellsolell	Departamento de Biologia Molecular i Cel.lular, C.I.D., (CSIC), c/Jordi Girona 18-26, 08034 Barcelona (Spain). Tel.: 34 3 401 66 87 Fax : 34 3 204 59 04
Rodolfo Biekofsky	University-Industry Centre for Pharmaceutical Research and Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, 29-39 Brunswick Sq., London WC1N 1AX (U.K.). Tel.: 44 71 753 58 00 Fax : 44 71 278 19 39
Jerónimo Bravo	Departament d'Enginyeria Química, ETSEIB, Avda. Diagonal 647, 08028 Barcelona (Spain). Tel.: 34 3 401 66 85 Fax : 34 3 401 71 50
Ramón Campos-Olivas	Instituto de Estructura de la Materia, CSIC, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Fax : 34 1 564 24 31
Rafael Giraldo-Suárez	Departamento de Microbiología Molecular, Lab. 452, Centro de Investigaciones Biológicas, CSIC, c/Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 561 18 00 Ext. 4349 Fax : 34 1 562 75 18
Carlos González	Instituto de Estructura de la Materia, CSIC, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Ext. 1166 Fax : 34 1 564 24 31
M. Angeles Jiménez	Instituto de Estructura de la Materia, CSIC, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Ext. 1162 Fax : 34 1 564 24 31
Saulius Klimašauskas	Institute of Biotechnology, Graičiūno 8, Vilnius 2028 (Lithuania). Tel.: 370 2 641 887 Fax : 370 2 643 436
María J. Macias	European Molecular Biology Laboratory, Heidelberg (Germany). Fax : 49 6221 38 73 06

Rachid C. Maroun	Institut Pasteur, Immunologie Structurale,(CNRS URA 359), 25-28 rue du Docteur Roux, 75724 Paris Cedex 15 (France). Tel.: 33 1 45 68 86 05 Fax : 33 1 45 68 86 39
Manuel Martín	Grupo de Carbohidratos, Instituto de Química Orgánica General, CSIC, Juan de la Cierva 3, 28006 Madrid (Spain). Fax : 34 1 564 48 53
Guillermo Montoya	European Molecular Biology Laboratory (EMBL) Structures Programme, Meyerhofstr. 1, Postfach 10.2209, D-69012 Heidelberg (Germany). Fax : 49 6221 38 73 06
José Luís Neira	MRC Unit for Protein Function and Design, Cambridge University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW (U.K.). Tel.: 44 1223 33 63 57 Fax : 44 1223 33 64 45
Edward T. Olejniczak	Pharmaceutical Discovery Divison Abbott Laboratories, Abbott Park IL. 60064 (USA). Tel.: 1 708 937 02 98 Fax : 1 708 938 24 78
Miguel Ortíz Lombardía	Departamento de Biología Molecular y Celular, Centro de Investigación y Desarrollo, CSIC, Jordi Girona, 18-26 08034 Barcelona (Spain). Tel.: 34 3 400 61 00 Ext. 261 Fax : 34 3 204 59 04
Mª de los Angeles Pajares	Instituto de Investigaciones Biomédicas, CSIC, c/Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 40 00 Fax : 34 1 585 45 87
Antonio Pineda-Lucena	Centro de Investigaciones Biológicas, CSIC, c/Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 561 18 00 Ext. 4370 Fax : 34 1 562 75 18
Miquel Pons	Departamento de Química Orgánica, Universidad de Barcelona, Martí i Franquès 1-11, 08028 Barcelona (Spain). Tel.: 34 3 402 12 63 Fax : 34 3 339 78 78
José Portugal	Centro de Investigación y Desarrollo, CSIC, Jordi Girona 18-26, 08034 Barcelona (Spain). Tel.: 34 3 400 61 57 Fax : 34 3 204 59 04

José Luis R. Arrondo	Departamento de Bioquímica, Facultad de Ciencias, Universidad del País Vasco, Apartado 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00 Fax : 34 4 464 85 00
Antonio Romero	Instituto de Química Física "Rocasolano", CSIC, Departamento de Cristalografía, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Fax : 34 1 564 24 31
Juliana Sanz	Instituto de Química-Física "Rocasolano", CSIC, Departamento de Cristalografía, Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00 Fax : 34 1 564 24 31
Paz Sevilla	Departamento de Química Física II, Facultad de Farmacia, Instituto Pluridisciplinar, Unidad de RMN, Universidad Complutense, Paseo Juan XXIII, 1, 28040 Madrid (Spain). Tel.: 34 1 394 32 57 Fax : 34 1 394 32 45
Andrzej Stasiak	Laboratoire d'Analyse Ultrastructurale, Bâtiment de Biologie, Université de Lausanne, niveau 1, CH-1015 Lausanne-Dorigny (Switzerland). Tel.: 41 21 692 42 82 Fax : 41 21 692 41 05
Christopher J. Thorpe	Microbiology and Tumorbiology Centre, Karolinska Institute, S-17177 Stockholm (Sweden). Tel.: 46 8 728 62 35 Fax : 46 8 306 43 8
José Tormo	Department of Molecular Biophysics and Biochemistry and Howard Hughes Medical Institute, Yale University, 266 Whitney Avenue, New Haven, CT. 06520 (USA). Tel.: 1 203 432 57 95 Fax : 1 203 432 32 82
Lourdes Urpí	Department d'Enginyeria Química, ETSEIB, Universitat Politècnica de Catalunya, Avda. Diagonal 647, 08028 Barcelona (Spain). Tel.: 34 3 401 66 85 Fax : 34 3 401 71 50
Nuria Verdaguer	Department d'Enginyeria Química, ETSEIB-UPC Avda. Diagonal 647, 08028 Barcelona (Spain). Tel.: 34 3 401 66 88 Fax : 34 3 401 71 50 or 401 66 00
Michael J. Williams	The Scripps Research Institute, Department of Vascular Biology, 10666 North Torrey Pines Road, La Jolla, CA. 92037 (USA). Tel.: 1 619 554 71 15 Fax : 1 619 554 64 03

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The lectures summarized in this publication were presented by their authors at a workshop held on the 8th through the 10th of May, 1995, at the Instituto Juan March.

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