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Protein Structure and Protein Engineering

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Abstracts

W.P. Jencks

How Does ATP Make Work?

It is not obvious how to think about eq 1.

$$ATP \rightleftharpoons ADP + P_i + work \qquad (1)$$

This equation describes muscle contraction, active transport, and other processes in which the chemical energy of ATP hydrolysis is utilized to perform mechanical or osmotic work, or ATP is synthesized by ion transport. The coupling of ATP hydrolysis and work in such coupled vectorial processes can be defined by a set of *rules* that describe enzyme specificities that change in different states of the system. When these chemical and vectorial specificities are followed the system is fully coupled; to the extent that they are not followed, *in any step*, the reaction becomes uncoupled. In general, the rules serve to separate the chemical and vectorial processes into two or more parts that are inserted between each other, so that neither process can occur without the other. Some properties of the coupling by the calcium ATPase of sarcoplasmic reticulum and examples of known or possible coupling rules for several such reactions will be described.

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P.L. Privalov

Stability of Protein Structure and Hydrophobic Interactions

Analysis of the calorimetric studies of protein denaturation and of dissolution of non-polar substances in water shows that the positive contribution of the hydrophobic interactions in stabilization of the protein compact state is due to van der Waals interactions between the protein non-polar groups, while the contribution of water solvation by these groups, in spite of the widely spread opinion, appears to be always negative. This destabilizing action of water solvation on the protein increases as the temperature decreases, and at a significantly low temperature causes unfolding of the compact structure of protein, i.e. cold denaturation.

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A.R. Fersht

Dissection of Enzyme Structure and Activity

The hallmark of enzyme catalysis is the use of binding energy to control rate and specificity. To understand an enzyme reaction, one has to characterize the intermediates on the reaction pathway and determine the interaction energies between the enzyme and each of the intermediates as the reaction proceeds. Protein engineering is eminently suitable for such a task because it allows the systematic dissection of the active site of an enzyme to map out residues involved in catalysis and probe the energetics. The contributions of each residue in the active site of the tyrosyl-tRNA synthetase to binding and catalysis are being determined by making sensible mutations which remove defined interactions with the substrates. The free energy profiles for the reactions of mutant and wild-type enzymes may be measured. The difference in energy between wild-type and mutant enzymes gives the apparent binding energy of the mutated side chain in each complex. By this means, the following have been determined: the contributions of different types of hydrogen bonds to specificity; their roles in catalysis; the importance of enzyme-substrate versus enzyme-transition state versus enzyme-intermediate complementarity; the fine tuning of enzyme catalysis during "evolution". Prior to these studies, the mechanism of activation by the aminoacyl-tRNA synthetases was a reaction of totally unknown mechanism. It is now seen that catalysis results solely from the use of binding energy. There are residues that do not bind the substrates in the ground state but stabilize just the transi-

tion state. This is consistent with the classical ideas of Haldane and Pauling of enzyme-transition-state complementarity. There are, however, regions of the protein that bind the ribose ring more tightly in the intermediate tyrosyl adenylate than in the transition state. This is to stabilize the intermediate against dissociation and to increase its concentration relative to unreacted ATP and tyrosine. Most recently, portions of the enzyme apparently far removed from the substrates have been shown to participate in catalysis via movement of flexible loops. The interactions in the transition state were not obvious from examination of the crystal structure but was discovered from the effects of mutagenesis. The transition state structure of the reagents has been further probed by application of linear free energy relationships which give the fraction of available binding energy used to stabilize each state.

The crystalline enzyme is a symmetric dimer which binds two moles of substrates. The enzyme exhibits half-of-the-sites activity in solution, however. Experiments on heterodimers constructed from various combinants of mutant and wild-type subunits have revealed that tRNA binds to the enzyme by spanning both subunits, and that only one tRNA molecule binds during catalysis. The charging complex is, therefore, a symmetric. Further experiments on heterodimers reveal that the half-of-the-sites activity is a consequence of a preexisting asymmetry and that the enzyme in solution is asymmetric.

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Is there a Code for Protein Folding?

The term "protein folding" refers to both the spatial arrangement of the amino-acid residues in a functional protein and the mechanism by which the unordered (nascent) polypeptide chain achieves its native conformation in going from the one-dimensional to the corresponding three-dimensional structure[1]. Faced with the astronomically high number of possible conformers of a polypeptide chain of average length, a surprisingly small number of "folding topologies" has been deduced from well over 100 known high-resolution crystal structures solved so far. As taken from comparative studies on the globins or cytochrome *c*, a given tertiary structure may tolerate a great number of amino-acid substitutions without significantly changing its topology. From this we may conclude that the "code of protein folding" is highly degenerate. On the other hand, single amino-acid substitutions can lead to large alterations in the stability and folding, indicating that certain parts of the polypeptide chain contribute important interactions to overall stability. Compared to the number of possible amino-acid sequences, there exists only a small number of polypeptide chains yielding a well-defined stable structure. Obviously, the majority of possible sequences does not code for a defined functional protein.

In vitro folding experiments have shown that small single-domain proteins may undergo fully reversible-denaturation reactions. In multi-domain proteins, the acquisition of the native three-dimensional structure is determined by folding and merging of domains. To generate assembly structures, sequential folding and association must be coordinated such that specific recognition of "structured monomers" is achieved. In general, the overall kinetic mechanism may be quantitatively described by a sequence of uni- and bimolecular reactions[2].

The question as to whether folding in vivo occurs as a cotranslational or posttranslational event, is still under dispute. Domains apparently represent independent folding units. Evidence of the vectorial character of the folding process comes from incomplete reconstitution due to "wrong aggregation" or acquisition of non-native substates[3,4].

Macro-assemblies are determined either by intrinsic form-determining properties of the protomers, or by assembly programs such as genetically determined morphopoiesis, or vectorial cotranslational assembly gradients[1].

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K. Wüthrich

The Method of Protein Structure Determination by NMR in Solution: Initial New Insights Relating to Molecular Mobility

During the period 1979–82 methods were introduced for efficient, sequence-specific assignment of the proton nuclear magnetic resonance (NMR) spectra of proteins. This now provides the basis for determination of the three-dimensional structure of small proteins in solution[1], enabling for the first time a direct, detailed comparison of corresponding protein structures in single crystals and in noncrystalline environments. Knowledge of the extent to which molecular structures are capable of responding to changes in environment, i.e. to adapt in reversible ways to different thermodynamic equilibrium situations, is of fundamental importance for a deeper understanding of the relations between structure and function. In the context of this fundamental problem corresponding protein structures in the crystalline state and in aqueous solution, as well as in different noncrystalline states will be compared.

Sequence-specific NMR assignments enable one further to map out time fluctuations of protein structures which

are in thermodynamic equilibrium. This will be illustrated with data on aromatic ring flips and on the exchange kinetics of individual amide protons.

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G.M. Clore and A.M. Gronenborn

The Determination of Three-Dimensional Structures of Proteins in Solution by Nuclear Magnetic Resonance

The determination of 3D-structures of proteins in solution using NMR spectroscopy comprises three stages: (i) the assignment of proton resonances by 2D-techniques to demonstrate through-bond and through-space connectivities; (ii) the determination of a large number of short (< 5 Å) interproton distances using nuclear Overhauser effect (NOE) measurements; and (iii) the determination of the 3D-structure on the basis of these distances. Our approach for step (iii) has involved the use of restrained molecular dynamics and simulated annealing. The principles of the restrained dynamics and simulated annealing approach will be illustrated for model calculations on crambin[1,2] and examples from the set of 3D-structures in solution that we have determined to date will be presented: purothionin[3], phoratoxin[4], hirudin[5], the globular domain of histone H5[6], potato carboxypeptidase inhibitor[7], barley serine proteinase inhibitor 2[8], secretin[9] and growth hormone relasing factor[10].

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M. Levitt and R. Sharon

Accurate Simulation of Protein Dynamics in Solution

Simulation of the molecular dynamics of a small protein, bovine pancreatic trypsin inhibitor (abbreviated as BPTI), is found to be more accurate when water molecules are included than when in vacuum. The time-averaged structure in solution is much more like that observed in high resolution x-ray studies of BPTI (1.4 Å as opposed to 2.4 Å). The amplitudes of atomic vibration in solution are 40% smaller than in vacuum and are in good agreement with new estimates of the internal motion in protein crystals. There are fewer incorrect hydrogen bonds made in solution than in vacuum (two as opposed to seven).

A shell of water molecules of higher than normal density (1.25 g/m*l*) is found about 3.8 Å from the protein surface. The motion of the water molecules close to the protein surface is restricted, as the rate of diffusion is reduced by 50%. Water molecules in contact with non-polar atoms have their free energy increased by contact with the protein; a manifestation of the hydrophobic interaction. Twenty of the twenty-nine water molecules found to be well-ordered in both of the X-ray studies are also found to be well-ordered in the simulation.

Overall, this small protein is found to be more rigid and solid-like in solution than concluded from previous in vacuo simulations. Water improves the simulation not only by improving the accuracy of the representation but also by speeding the redistribution of energy. This work provides a sound basis for the realistic simulation of diverse properties of biological macromolecules in solution using simple atom-centered forces and classical mechanics.

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D. Alonso and K.A. Dill

Theory for the Thermal and Solvent Stability of Globular Proteins

The globular state of proteins is stabilized by the hydrophobic interaction and destabilized by the conformational entropy. We have developed a statistical thermodynamic theory to describe the dependence of this balance of forces on chain length and composition[1]. Here we discuss extension of that work to the prediction of effects of temperature and solvent character on stability of the globular state.

One long-standing puzzle is why proteins denature upon heating, since the hydrophobic effect strengthens with temperature. We show that "cold" denaturation is driven by the hydrophobic interaction, and ordinary denaturation is largely driven by the conformational entropy. We show that the nature of the unfolded state is responsible for important features of the temperature dependence of folding. In addition, comparison of theory with experiment shows that there is a driving force that causes the polar residues also to favor folding.

Our results are compared with experiments of Privalov et al.[2].

Solvents such as urea and guanidinium chloride can also cause protein denaturation. We compare predictions of the theory with experiments of Shortle et al.[3] and Pace[4].

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Crystallography of the trp *Repressor/Operator System: Molecular Flexibility in Genetic Regulation*

Two crystal forms of trp repressor and one of the inactive unliganded aporepressor have been refined to atomic resolution. Comparison of these structures reveals that the extensive inter-subunit interface of four intertwined α-helices from each subunit forms a rigid unified domain that resists tertiary structural changes. This 'central core' is flanked symmetrically by two flexible 'reading heads' that contain a bihelical motif. Two tryptophan molecules bind symmetrically to sites wedged between the core and the reading heads and force the reading heads apart so that they can penetrate successive major grooves of B-DNA. When unliganded, the reading heads collapse en bloc toward the dyad, diminishing their separation to the point where they cannot complement the contours of B-DNA. Comparison of the two repressor structures showed an unexpected degree of flexibility within the reading heads, i.e., the binding of the corepressor ligand does not create a unique and rigid complementary surface for the operator, but rather appears designed to allow repressor to adjust to the variable helical parameters and molecular contours in its 'one dimensional' search for the operator site.

The functional consequences of L-tryptophan binding have been further clarified by the refined crystal structure of a pseudorepressor formed with a competing desamino analog of L-tryptophan, indole propanoic acid, that produces a structure that is nearly isomorphous to repressor but cannot bind DNA. Progress in the high-resolution structure determination of a crystalline trp repressor/operator complex will be discussed.

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J.R. Knowles

The Evolution of Enzyme Function

Since the emergence of the first living organism more than three billion years ago, the catalytic effectiveness of many of the enzymes that mediate metabolism has been improved and refined to the point where the reaction rate of the enzymic process is limited only by the rate of diffusion of substrate to the active site[1]. Any change in the amino-acid residues of such an efficient enzyme must either be silent (and have no effect on catalytic function) or deleterious (and slow the catalysed rate below that of the diffusion limit). The methods of site-directed mutagenesis can therefore generate sluggish mutant enzymes[2]. If a method exists to select organisms that harbour enzymes of *higher* catalytic effectiveness out of pools of random mutants of the sluggish enzyme, we can search for pseudo-revertants, where changes at second sites partially compensate for the first damaging mutation[3]. This approach allows us to study the functional improvement of an enzyme's catalytic apparatus, and permits us to ask whether other ('unnatural') constellations of catalytic amino-acid residues exist that have a kinetic efficiency approaching that of the wild-type. The design of experiments of this kind will be described, and our results on one glycolytic enzyme, triosephosphate isomerase, will be discussed.

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Recent Studies of Ligand Binding to Carboxypeptidase A

In our recent single crystal X-ray diffraction studies the following substrate analogues have been bound to carboxypeptidase A:

1) 2-benzyl-3-formylpropanoic acid at 1.7 Å resolution; bound as gem-diol;
2) (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid at 1.54 Å; bound unchanged;
3) 2-benzyl-4-oxo-5,5,5-trifluoropentanoic acid at 1.7 Å; bound as gem-diol;
4) *N*-({[(benzyloxycarbonyl)amino]-methyl}hydroxyphosphinyl)-L-phenylalanine at 1.8 Å; hydrolyzed;
5) 5-benzamido-2-benzyl-4-oxopentanoic acid at 1.7 Å; bound as gem-diol;
6) 5-amino-(*N*-*t*-butoxycarbonyl)-2-benzyl-4-oxo-6-phenylhexanoic acid at 1.75 Å; bound as gem-diol;
7) Glycyl-L-tyrosine at 1.6 Å (− 9 °C); bound with carbonyl oxygen and NH_2 to Zn;
8) *N*-benzoyl-L-phenylalanine to 1.8 Å; Phe bound at S_1', *N*-B_z-Phe bound at S_1 and S_2.

In none of the pre-product cases was there nucleophilic addition of Glu^{270} to the carbonyl. In the four gem diol examples, the stabilization of a tetrahedral-like species by $Zn^{2\oplus}$ and Arg^{127} were seen, although whether these are selected from solution or produced by attack of $ZnOH_2$ or ZnOH is not decided. The substrate-product complex has the potential of behaving as a false indication of an intermediate at low temperatures and high substrate concentrations.

The results are consistent with the present view that nucleophilic attack occurs by $ZnOH_2$ or ZnOH, and that Glu^{270} can function as a proton transfer agent from $ZnOH_2$ to the NH group of the scissile peptide bond. Consistent with the observation by Rutter's group that the Phe^{248} mutant is almost fully functional, no essential catalytic role is assigned to Tyr^{248}.

We cannot exclude the possibility that some substrates, perhaps certain esters, proceed through the anhydride mechanism. Further studies are under way.

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W. Bode

Structural Requirements of Serine Proteinases to Achieve Substrate Specificity[1]

Trypsin-like serine proteinases are not only involved in general proteolytic cleavage reactions (protein digestion) but also in more specific and complex processes, such as activation of zymogens, regulation of zymogen cascades, release of hormones from precursor proteins, fertilization and differentiation. All these serine proteinases possess structurally similar reactive domains with identical catalytic residues but differing binding regions. The substrate peptide bond(s) actually being cleaved is (are) mainly determined by the amino acid preceding the scissile peptide bond(s); the preference of a proteinase for distinct amino-acid side chains is achieved by a sterically fitting primary binding site (called specificity pocket) and a suitable arrangement of hydrogen bonding partners and charged groups.

The more specific serine proteinases are capable of selectively complexing with extended peptide segments and cleaving only distinct peptide bonds within specific peptide environments (limited proteolysis); such a specificity requires a specific architecture of the primary and secondary binding sites by which productive binding of similar substrates is prevented but binding of proteins with correct sequences is supported[2].

Based on the spatial structure of bovine trypsin and chymotrypsin, of porcine pancreatic kallikrein and of porcine pancreatic and human leukocyte elastase[3], it will be shown how nature has designed proteolytic enzymes of distinct specificity.

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The Interplay Between Enzyme Mechanism, Protein Structure, and Inhibitor Design

The design of enzyme inhibitors has traditionally depended on a knowledge of the mechanism of the enzyme, as exemplified by the synthesis of so-called "transition-state analogs". Such compounds are intended to be stable analogs of the supposed transition state or a high-energy intermediate along the enzyme reaction pathway, and to take advantage of binding interactions that are not available to the ground state form of the substrate. As particular examples of transition state analogs, a series of phosphonic acid peptide derivatives have been synthesized and shown to be potent inhibitors of a variety of peptidases, such as thermolysin and carboxypeptidase A (zinc peptidases), pepsin and penicillopepsin (aspartic peptidases), and chymotrypsin and subtilisin (serine peptidases). The "transition state analogy" of some of these inhibitors has been evaluated, and structural information is available for several of their enzyme complexes.

With the increasing availability of protein crystal structures, a new approach to the design of inhibitors can be envisaged, namely one which depends more on knowledge of the three-dimensional structure of the enzyme active site and less on consideration of the specific reaction mechanism. Some preliminary work in this direction will be presented.

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Structure, Function and Evolution of Haemoglobin Studied by Protein Engineering and X-Ray Crystallography

The geometries of the Fe-O_2 and Fe-CO bonds in myoglobin and haemoglobin differ significantly from those in free porphyrin model compounds. It has been suggested that steric hindrance by the valine-E11 and histidine-E7 residues and a hydrogen between histidine-E7 and the oxygen would affect the geometry and electronic state of the Fe-ligand bonds and that these interactions may play an important role in controlling the ligand affinity. We have produced mutant haemoglobins in *Escherichia coli* in which Val(67β)E11 is replaced by Ala, Met, Leu and Ile and His(63β)E7 by Gln, Val and Gly and have studied the effect of these mutations on the equilibrium and kinetics of ligand binding. The conformation of these new side chains and their effect on the protein structure have been studied by X-ray crystallographic analysis. Some amino-acid residues in proteins are mutated in the co-ordinated manner during evolution. We have studied the effect of co-ordinated mutations on the structure and function of haemoglobin.

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R.A. Lerner and A. Tramontano

Catalytic Antibodies

Monoclonal antibodies have been elicited to haptens in which an organophosphorus moiety imparts the stereoelectronic properties of an esterolytic transition state for analogous carboxylic esters. Some of these were found to react specifically and stoichiometrically with activated esters that share certain structural features with the hapten. This is explained as a combining site directed acyl transfer, facilitated through the stabilization of a tetrahedral intermediate or transition state by antibody-ligand binding interactions. Studies on the effect of chemical modification of amino acids in the protein implicate the involvement of a histidine and a tyrosine residue of the combining site in the acyl transfer mechanism. One of these residues is presumed to be acylated in the reaction. These same antibodies are also shown to behave as enzymic catalysts with the appropriate ester substrates. These substrates are distinguished by the structural congruence of both hydrolysis products with haptenic fragments, as well as the correspondence of the acyl center with the phosphono group of the hapten. Mechanisms are proposed to account for the divergent chemical behavior of these esters with the antibodies. These are based on the difference in leaving group ability between the hydrolysis substrates and esters which covalently combine with the antibody. The antigenic phosphonates are potent inhibitors of this reaction in accord with their assigned role as transition state analogs. These experiments demonstrate that antibody-antigen binding may be directed to chemical processes, according to the prevailing theory which relates binding energy to enzyme function. The generation of artificial enzymes through transition state stabilization by antibodies has long been expected as a corollary to Pauling principle catalysis. These observations provide evidence towards the fulfillment of that prediction.

In continuing work, monoclonal antibody elicited to a transition state analog representative of an intramolecular six-membered ring cyclization reaction has been shown to act as a stereospecific, enzyme-like catalyst for the appropriate substrate. Formation of a single enantiomer of a δ-lactone from the corresponding racemic δ-hydroxyester was shown to be accelerated by the antibody ca. 170-fold permitting isolation of the lactone in an enantiomeric excess of ca. 94%. This finding has demonstrated the feasibility of catalytic antibody generation for chemical transformations that require stereochemical control.

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E.T. Kaiser

Design and Construction of Biologically Active Peptides and Proteins, Including Enzymes

We have developed a successful design approach which has been used in the construction of a host of biologically active peptides, including apolipoprotein models, hormones and toxins. In recent work we have turned to the re-design of the structural regions of several enzymes. In these studies we have employed both genetic engineering techniques and total synthesis. Our synthetic routes have made use of an oxime polymer for the rapid preparation of protected peptide segments, followed by coupling of the segments after they have been purified. With the latter methodoly we have focused on proteins which are in the vicinity of a hundred amino acids in length. We have used genetic engineering for the re-design of the structural regions of larger targets. The total synthesis approach has several advantages including the possibility of introducing spectroscopic probes at any desired location, the feasibility of employing unnatural amino acids and flexibility in allowing the incorporation of non-peptidic structural regions in the re-designed protein.

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E. Bayer

Conformational Organization and Substrate Interaction of Polymer-Supported Peptides

Polymer-supported synthetic peptides play an important role in various fields of biochemistry, like immunological studies, affinity chromatography, substrate-peptide interactions. Relatively little is known about matrix effects of the polymer upon the conformation because most methods of structure investigation cannot be applied. Only in the case of soluble polymers the spectroscopic methods like NMR and CD can be used.

Silicone- and Polyoxyethylene-supported peptides are step-wise synthesized with the liquid-phase peptide synthesis[1], and the sequence-dependent development of conformation determined in each step by NMR, CD, and eventually IR. Examples are given for Substance P, collagen and myoglobin sequences. Using polysiloxane peptides with different conformation, the discrimination and selectivity for optical antipodes of various classes of natural compounds and drugs is investigated, and correlated with the conformation of the diastereotopic transition state. Polyoxyethylene peptide mimicks for myoglobin and peroxidase are prepared and their oxygen binding investigated.

Since polyoxyethylene is especially compatible with peptides and shows no matrix effects immobilization of polyoxyethylene chains (3000 Da) to polystyrene has been achieved, and these beads investigated as support for peptides.

These PS-POE craft copolymers are hydrophilic and do not influence the conformation of peptides because the long POE spacer removes the terminally bound peptide from the cross-linked polystyrene matrix.

Extented studies of these polymer peptides by cross-polarization magic-angle spinning (CP-MAS)-NMR spectroscopy and correlations with solution NMR-spectra have been achieved, including 2D solid state NMR spectroscopy and ^{13}C-NMR relaxation-time studies.

The PS-POE polymers also have been applied for studies of peptide-nucleotide and peptide-peptide interactions.

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T.A. Jones

Computer Modelling with a Protein Database

My primary interst in computer modelling has been concerned with the protein crystallographers' problems first to construct a model then to improve it during refinement[1]. Our recent work[2] was made possible by hardware developments resulting in affordable 32-bit computers and high-performance colour displays. This work makes use of a skeletal representation of electron density[3] to present a much larger volume and colour to represent possible folding hypotheses. Taken together this helps the initial map interpretation. This aspect of the implementation has similarities to the Grinch system developed at the University of North Carolina. However, our skeleton can then be matching fragments from a data base of well refined proteins. Alternatively the fragments may be chosen from 170 five-residue building blocks, which are the result of a cluster analysis study (Jones and Levitt, in preparation). This technique, Proleg (PROtein LEGo), has affected our general attitude to molecular modelling, which then becomes a problem of picking the best fragment to fit our observations (crystallographic, NMR or structurally related proteins). Our future developments are aimed at a networked environment of work stations (of various capabilities) with cpu and data base servers. One of our goals is to produce a real space-fitted protein model[4,5] without manual intervention and to locate and correct the majority of conformational errors during refinement.

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A. Wada

Automated and High-Speed DNA Sequencing – Non-Biological Technology Promotes Biological Advances

Increasing the speed of genetic information analysis has become highly requisite in fields related to the study of living organisms. In this context, we have, since 1981, been exploring an automatic DNA-sequencing system. The aim of this project is to develope machines and other accessories which carry out a series of complex procedures in the DNA base-sequence analysis[1,2].

One might suppose that a project such as genetic analysis is totally biological in character. It is however worthy of mention that our group comprises also researchers and has access to industries *not* generally involved in the life-sciences and biotechnologies. In the cross-disciplinary work necessitated by genetic study, the resources of physics, computer science, electronic industries, plastics and textile industries should also be utilized.

This example demonstrates, I believe, that the development of biological science and technology ought not to be restricted to current biological methods alone.

In the 21st century, we foresee that highly sophysticated DNA super-sequencers will be set up in several countries and will become symbols of the effort of nations to broaden and build on human knowledge, taking their place beside other existing symbols such as large particle accelerators, giant telescopes, and space research programmes.

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J. Kraut

Exploring Enzyme Structure-Function with Mutagenesis and Crystallography

We have applied a combination of mutagenesis, X-ray crystallography and enzymology to answer some typical structure-function questions regarding two enzymes with highly refined x-ray structures, *E. coli* dihydrofolate reductase (DHFR, EC 1.5.1.3) and yeast cytochrome-*c* peroxidase (CCP, EC 1.11.1.5).

In DHFR a conserved Asp/Glu at position 27 hydrogen-bonds to the substrate's pteridine ring. This carboxyl group may be an essential proton donor, but seems to be too far from the pteridine N5, the ultimate proton acceptor. We have variously substituted Asp^{27} to ask if a proton donor at this position is necessary. Also, other sites were substituted to test alternative proton-donor locations. X-ray structures of these mutants show only minimal perturbations, mainly of bound water molecules. Steady-state kinetic data suggest that Asp^{27} is indeed involved in substrate protonation. However other proton donors, either at position 27 or nearby, can also function, but less efficiently.

Yeast cytochrome-*c* peroxidase is a ferri-heme enzyme. Reaction with peroxide gives an intermediate, Cpd I, exhibiting a radical-like EPR signal. Previously we had proposed that Trp^{51}, which contacts the 6th coordination site of the heme, carries the radical. However, a mutant with Phe at this position is fully functional and still gives a radical intermediate. Other possible radical sites, further from the heme, are suggested by small structural perturbations in Cpd I. These have now been tested by new mutants that exhibit drastic effects on enzyme function, but only small effects on the structure.

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Correlations Between Structure and Thermostability of Variant Phage T4 Lysozymes

The lysozyme of bacteriophage T4 has been used to investigate the structural basis of the thermodynamic stability of proteins. Temperature sensitive (ts) mutants were identified to locate residues that contribute to stability. The ts mutations exclusively alter amino acids with low crystallographic B values and low static solvent accessibility in the wild-type protein[1]. Many types of noncovalent interactions are affected, suggesting that different interactions can make comparable contributions to protein stability. The high-resolution X-ray crystal structures of six of the ts mutant proteins provide examples of both localized and dispersed structural changes in response to mutation.

Site-directed mutagenesis has been used to make multiple substitutions at selected sites to determine the relative magnitudes of specific interactions. For example, the thermal stabilities of X-ray crystal structures of lysozymes with 13 different substitutions for Thr^{157}[2] and 10 different substitutions for Pro^{86} were determined. Observed correlations between native structure and thermal stability suggest that interactions in the folded protein dominate the changes in the free energy of stabilization caused by mutations. One general conclusion from these structural studies of selected and site-directed mutants is that the ability to undergo conformational changes makes proteins surprisingly tolerant of amino-acid substitutions.

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Synthetic Antibodies with Known Three-Dimensional Structure

The particularly well studied antibody combining site of McPC603 is used by us as a model system for quantitatively investigating factors that contribute to efficient hapten binding, subunit interactions, as well as for the potential of stabilizing a transition state (i.e. chemical catalysis) through the controlled modification of the protein[1].

The genes encoding the variable domains (V_H and V_L) of the phosphorylcholine-binding antibody McPC603 were obtained by DNA synthesis. In addition, we constructed genes encoding the variable and the appropriate constant domains of each chain in order to express the exact F_{ab} fragment whose crystal structure has been determined. The design of the synthetic genes took into consideration the facile replacement of gene fragments (e.g. the hypervariable loops) as well as current knowledge about efficient expression[1].

We developed an expression system that allows for the first time assembly of completely functional antibody-binding domains in *E. coli* in vivo[2]. This F_v fragment was purified to homogeneity in a single step. The N-termini

of both chains of the F_v fragment were sequenced and found to be homogeneous and identical to the native antibody. The dissociation constant of the hapten – F_v-fragment complex was found to be identical to that of the native hapten – antibody complex. Both disulfide bonds were present in the Fv fragment. Thus, we can demonstrate unequivocally for the first time that an F_v fragment is fully functional and assembles by itself in *E. coli*.

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R. Huber

Flexibility and Rigidity in Proteins and Protein-Pigment Complexes

Proteins may be rigid or flexible to various degrees as required for optimal function. Flexibility at the level of amino-acid side chains occurs universally and is important for binding and catalysis. Flexibility of large parts of a protein which rearrange or move are particularly interesting[1]. We differentiate between certain categories of large-scale flexibility although the boundaries between them are diffuse: Flexibility of peptide segments, domain motions and order-disorder transitions of spatially contiguous regions. The domains may be flexibly linked to allow rather unrestricted motion or the motion may be constrained to certain modes. The polypeptide segments linking the domains show characteristic structural features. The various categories of flexibility will be illustrated with the following examples: a) Small protein proteinase inhibitors, which are rather rigid molecules and provide binding surfaces complementary to their cognate proteases, but also show limited segmental flexibility and adaptation[2,3]. b) Large plasma inhibitors, which exhibit large conformational changes upon interaction with proteases, probably for regulatory purposes[4]. c) Pancreatic serine proteases, which employ a disorder-order transition of their activation domain as a means to regulate enzymic activity[5]. d) Immunoglobulins in which rather unrestricted and also hinged domain motions occur in different parts of the molecule, probably to allow binding to antigens in different arrangements[6,7]. e) Citrate synthase, which adopts open and closed forms by a hinged domain motion to bind substrates and release products and to perform the catalytic condensation reaction, respectively[8–10]. f) Riboflavin synthase, a bifunctional multienzyme complex in which two enzymes (α and β) catalyse two consecutive enzymic reactions. The β-subunits form a shell in which the α-subunits are enclosed. Diffusional motion of the catalytic intermediates is therefore restricted. In addition, segmental rearrangement occurs in the assembly of the β-subunit[11,12]. In contrast, rigidity is the dominant impression provided by the recent structures of the light-harvesting complexes[13,14] and the reaction centres[15–17] involved in the photosynthetic light reactions. These are large protein pigment complexes in which the proteins serve as matrices to hold the pigments in the appropriate conformation and relative arrangement. Since motion would contribute to desactivation of the photo-excited states of the pigments and diminish the efficiency of light energy and electron transfer, a functional role for reduced flexibility is easy to rationalize for these proteins.

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