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Biological Functions of Proteinases

Edited by H. Holzer and H. Tschesche

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Biological Functions of Proteinases

Edited by
H. Holzer and H. Tschesche

With 142 Figures

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Prof. Dr. HELMUT HOLZER
Biochemisches Institut
der Albert-Ludwigs-Universität
Hermann-Herder-Straße 7,
7800 Freiburg/FRG

Prof. Dr. HARALD TSCHESCHE
Lehrstuhl für Biochemie
Fakultät für Chemie
der Universität Bielefeld
Universitätsstraße,
4800 Bielefeld/FRG

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Preface

Proteinases were among the first enzymes to be investigated biochemically, and purification and crystallization especially of proteolytic enzymes of the digestive tract has contributed much to our present knowledge of enzymic structure and mechanisms of catalysis. However, for a long time little has been known about the functional aspects of proteinases. The only exception from this have been the digestive tract enzymes responsible for extracellular catalysis of protein breakdown and supply of amino acids for new-protein assembly and nitrogen metabolism in the respective organs. The work of Schoenheimer, summarized for the first time in 1942 in a paper entitled "Dynamic state of body constituents", showed that continuous turnover of proteins takes place in cells. But scientists did not pay much attention to these findings at that time. The continuous accumulation of knowledge of a variety of intracellular proteolytic processes during the past decades has greatly stimulated research in this field. The central role of proteolysis in cellular regulation has become fully evident during recent years. It is the aim of the 30th Mosbach Colloquium to present an overview of our present knowledge of proteinase structure, function and control.

The relationship between globular protein structure of a proteinase and induction of enzymic activity will be discussed for trypsin and trypsinogen activation. One significant proteinase action is the total degradation of proteins to serve cellular needs under different conditions. Thus papers to be presented will touch on general protein turnover controlling steady-state concentrations of proteins and enhanced degradation of proteins for cellular adaptation under starvation and differentiation conditions in mammals and microorganisms. Other papers will deal with degradation of "nonsense" proteins to remove harmful protein waste, and with control of proteolysis by proteinase inhibitors in microorganisms and mammalian tissues under normal and pathological conditions.

Another important mode of proteinase action is the generation of numerous vital functions by limited proteolytic cuts. A variety of such limited proteolytic cleavages will be discussed such as secretion and transport of proteins across membranes, the assembly of viruses, blood coagulation and fibrinolysis, the control of blood pressure, fertilization, the defense reaction of the complement system and the "SOS-reaction" upon mutagenesis in *Escherichia coli*. Increased proteolytic activity is also found in transformed cells.

Even though progress in the field of proteolysis has been quite rapid in recent years, gaps in our knowledge still exist. It is hoped that the 30th Mosbach Colloquium, by presenting an overview of our knowledge of the biological functions of proteinases, will allow these gaps to be clearly recognized and then filled in by future research.

Acknowledgments. The organizers are grateful to the Gesellschaft für Biologische Chemie and its chairman Prof.Dr. K. Decker for their active support of the colloquium. Special thanks are due to Prof.Dr. E. Auhagen and Prof.Dr. H. Gibian for the technical organization of the meeting. The organizers are greatly indebted especially to the Deutsche Forschungsgemeinschaft and to all persons and institutions who provided the necessary funds and who helped to make this meeting a successful one.

October, 1979

HELMUT HOLZER
HARALD TSCHESCHE

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Conformational Flexibility and Its Functional Significance in Some Protein Molecules

R.Huber[1]

Introduction

The term "flexibility" in context with protein structures is used with a variety of meanings. For instance, both a protein molecule in random coil conformation and a molecule occurring as two different stable, but interconvertible conformers are named flexible. A precise definition of flexibility in a particular system requires determination of the number and geometry of the various conformers, their stability, the energy barriers separating the conformers, the kinetic parameters of interconversion and the thermal motion of the atoms within each conformer.

A complete analysis of the dynamic behavior of a large molecule, if possible at all, requires a variety of physicochemical studies by diffraction and spectroscopic methods.

X-ray diffraction provides a static, time-averaged picture of the molecule in a crystal lattice. Analysis of the crystallographic temperature factor enabled by the recent development of refinement methods (1,2), also gives some information about dynamic behavior. Spectroscopic methods, in particular NMR, ESR, Mössbauer- and fluorescence spectroscopy allow a much more detailed analysis in frequency in solution once the origin of the spectral signals is identified. This is a difficult problem. Recent theoretical approaches (molecular dynamics calculations) offer promising prospects of understanding the dynamic behavior of a protein molecule (3,4).

The pancreatic trypsin inhibitor (PTI) is presently the most thoroughly studied object by diffraction and spectroscopic techniques, as well as molecular dynamics calculations (5,6,7,3,4). There is little doubt that many of the phenomena observed in PTI are generally valid in protein structures, but larger protein molecules show more complex behavior. Unfortunately spectroscopic techniques, except perhaps Mössbauer and fluorescence spectroscopy, face serious problems with large molecules, so that X-ray diffraction is often the sole source of information about dynamic properties as well.

It is the intention of this article to describe the contribution of protein crystallography to the problem of flexibility and to describe some examples in which large-scale segmental flexibility has been discovered by X-ray diffraction which is apparently required for proper function, regulation, and catalysis.

The Temperature Factor in Protein Crystallography

As the energy of lattice vibrations (phonons) is very small compared to the energy of X-ray photons (about six orders of magnitude), lattice

[1]Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München/FRG

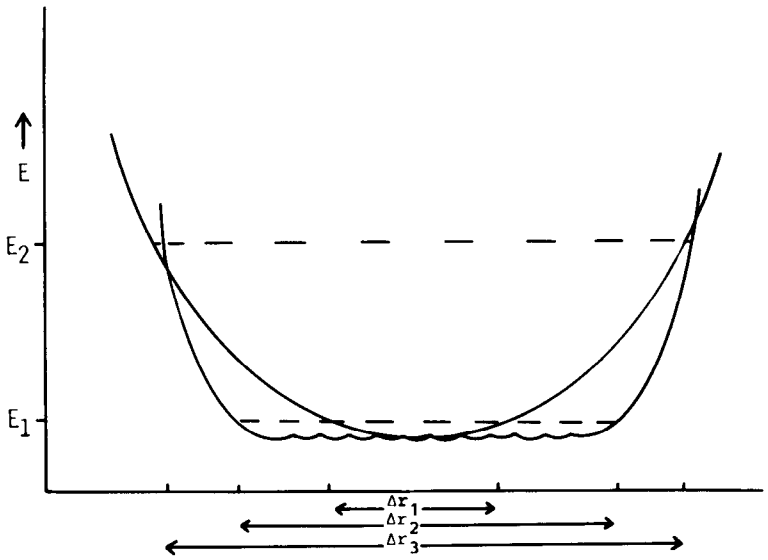

Fig. 1. Simplified, one-dimensional energy-conformation diagram of a molecule with a large number of rigid microstates compared to a single "soft" conformer. The space occupied, Δr_3, is similar at energy E_2 but becomes different at lower energy E_1: Δr_1 and Δr_2

vibrations cannot be observed by X-ray spectroscopy, but affect scattering of X-rays. The intensity of X-rays scattered by a crystal with thermal motion compared to a perfect static crystal is reduced by an exponential factor dependent on the scattering angle and the Debye-Waller factor, which is proportional to the mean square displacement of an atom (8a). As X-ray scattering is an instantaneous process compared to lattice vibrations, it is clear that the effect of a large number is closely related conformers (microstates), forming geometrically a quasi-continuum and randomly packed into a crystal lattice on the scattered X-rays is indistinguishable from that of a single vibrating conformer (Fig. 1). If the conformers are few and widely separated in space, the formalism of mixed crystals for X-ray diffraction holds (8a), producing a general decrease of the contributions from the atoms affected. Cooling is the obvious means to distinguish, as only the thermal vibrations are frozen out. This is general practice in small molecule crystallography.

Analysis of disorder in protein crystals, however, poses several problems: the experimental phases obtained by isomorphous replacement are rather inaccurate and do not allow reliable derivation of temperature factors. The finding that protein crystal structures can be refined (1,2) provided a means to determine temperature factors of individual atoms. The errors are still large compared with data obtained from small molecule crystal structures, but the values are physically reasonable, when averaged for rigid groups and smoothed along the chain. This is shown by the observation that external polypeptide loops have higher than average temperature factors, external long amino acid side chains show increasing temperature factors along the side chain, and – most objectively – molecules crystallized and analyzed in different lattices show the same trend in temperature parameters along the chain (2,5,9a).

The problem of decomposing the temperature factor observed into its contributions from static and dynamic disorder remains, however. Cooling is difficult as protein crystals contain a large proportion of solvent which must be prevented from freezing. Even in the most favorable case, where the protein crystal tolerates 70% methanol, a rather narrow temperature range between room temperature and about -70°C, may be attained (9b). A systematic study of the variation of the temperature parameters within this temperature limit is under way (10a,b). Shock-frozen myoglobin crystals at liquid nitrogen temperature have been investigated by Mössbauer spectroscopy. It has been deduced that a substantial part of the temperature factor observed is due to static disorder (11). The static disorder contributing to the temperature factor is composed of two factors: a component due to imperfect lattice formation of the molecules as rigid bodies (lattice defects) and a component due to slight differences in the structure of the individual molecules (microstates). Lattice defects are a crystal property and irrelevant for the molecules in solution. Whether microstates exist in protein molecules is a question presently under theoretical discussion (3,4). It appears that protein crystallography may contribute little to this issue.

The examples discussed in the following are characterized by large disorder of a substantial part of the molecule. In these cases drastic effects are observed with no significant electron density for the disorder segments, while the ordered domains are well defined. In the light of the previous discussion "invisibility" may be caused by two limiting situations: thermal mobility corresponding to a temperature factor or more than 50 Å^2 (corresponding to r.m.s. displacement of about 0.8 Å) or three or more different widely separated conformers. These conclusions are drawn from significance considerations in refined electron density maps. In less well-determined crystal structures the significance limits might be lower.

As the major parts of the molecules considered are well defined, lattice defects are excluded as the major source of disorder here. It is unclear in these cases which type of disorder (static or dynamic) prevails; posssibly, spectroscopic studies will provide insight into these problems in the future, even if the molecules are large.

As the disordered segments involve a substantial part of the molecules described, functional significance of the disorder is likely and clearly indicated in a number of cases.

The functional aspect of disorder is very different for the different systems discussed: in trypsinogen-trypsin, and presumably in other activatable serine proteases the disordered segments in the zymogen become ordered in the active enzyme and form the substrate binding site. The disorder-order transition serves a regulatory purpose here (12).

In citrate synthase a disordered N-terminal domain is probably the binding site of the co-enyzme A co-factor (13). Flexibility might mediate the transfer of an enzymatic intermediate (citryl-CoA) between two active sites, here. But there are indications that a rigid conformer exists also here, which might have regulatory function.

In antibody molecules segmental flexibility might allow binding to antigenic sites of different geometric arrangement and distance (14, 15).

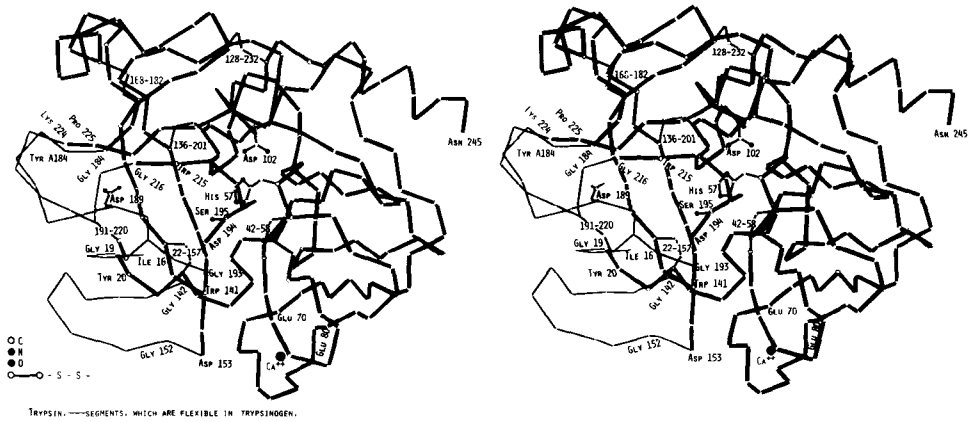

Fig. 2. Stereo drawing of the C^{α} carbon positions of trypsin. Residues linked by single lines are flexible in trypsinogen. Residues linked by double lines are well defined. Catalytic residues and hinge residues are indicated

In viral proteins such as tobacco mosaic virus protein (TMV) (16,17) and tomato bushy stunt virus (TBSV) (18) segments in contact with RNA are flexible. It is suggested (16,18) that structural variability is required to allow binding to different RNA nucleotide sequences. In intact TMV the RNA binding segments are stabilized (17).

Small polypeptide hormones appear to be flexible in most cases in tne sense of a random coil with some short-range order (19). It is clear that the interaction of a flexible hormone molecule with its receptor is less strong and specific than a rigid conformer could bind to an optimally complementary receptor. But the specificity inherent in a linear polypeptide sequence might suffice and dissociation from the receptor is easier when binding is weaker.

In the following the experimental background and the functional implications of flexibility are discussed in some detail for trypsinogen-trypsin, citrate synthase and antibody molecules.

Trypsinogen-Trypsin-Pancreatic Trypsin Inhibitor (PTI)

This system has been studied in great detail and the various crystal structures were refined at the highest resolution allowed by the crystalline order (12). The proenzyme trypsinogen is converted to trypsin by cleaving the N-terminal activation hexapeptide and liberating the new N-terminus Ile 16. This leaves both molecules virtually identical in about 85% of the polypeptide chain, but the rest is different. This consists of four segments tightly interdigitating in trypsin: The N-terminus to Gly 19, Gly 142 to Pro 152, GlyA 184 to Gly 193 and Gly 216 to Asn 223 (Fig. 2). This segments, named activation domain, are disordered in trypsinogen. Flexibility starts rather abruptly at single residues, usually glycines. Glycine which has no side chain to grab is a preferred candidate to mediate flexibility. A very distinct feature of the activation domain is in the complete lack of aromatic residues. It is interesting to note that this holds also for the switch and hinge region in antibody molecules – to be discussed later – and the flexible region in tobacco mosaic virus protein (16). Unfortunate-

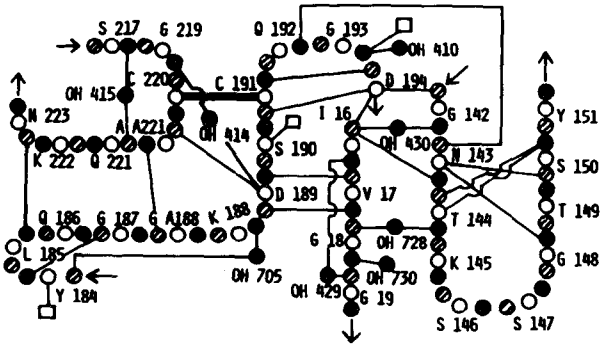

Fig. 3. The activation domain in trypsin. (-) hydrogen bonds between residues of the acitvation doman, (-) hydrogen bonds to residues outside the domain

● N
○ C^{α} + Side chain
● O

Hydrogen bonds made by residues of the activation domain.

Hydrogen bonds mediated by immobilized water,
Hydrogen bonds made with residues outside of the domain,
Disulfide bridge 191-220

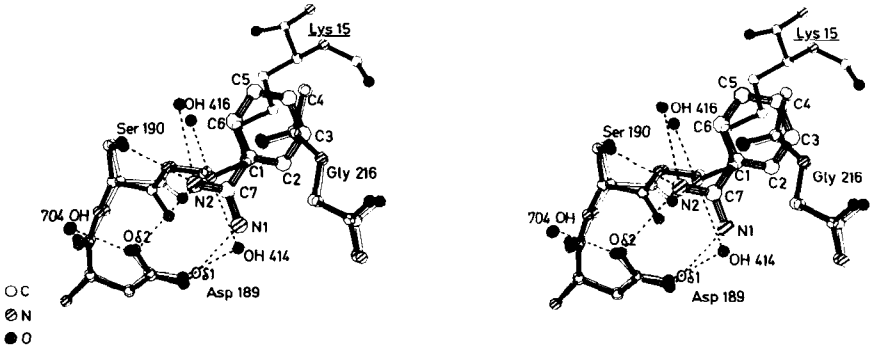

Fig. 4a. Stereo drawing of the Lys 15 in the specificity pocket of trypsin as seen in the PTI trypsin complex (=) overlaid with benzamidine (≡) as seen in bezamidine-inhibited trypsin (-). Residues of PTI are *underlined*

ly, the amino acid sequence is unknown in citrate synthase. It appears as if aromatic residues confer rigidity to protein molecules.

The chain segements which are flexible in trypsinogen form a tightly interdigitating structural unit in trypsin (Fig. 3). There are more than 20 hydrogen bonds cross-linking the segments of the activation domain in trypsin. The Asp 194 carboxylate Ile 16 ammonium ion pair appears to act as a clamp. It is conceivable that the absence of this ion pair in trypsinogen destabilizes the activation domain. The activation domain is cross-linked by a disulfide 191-220 which is reducible in trypsinogen (20) but not in trypsin. Segments of the activation domain form the substrate binding site well characterized from

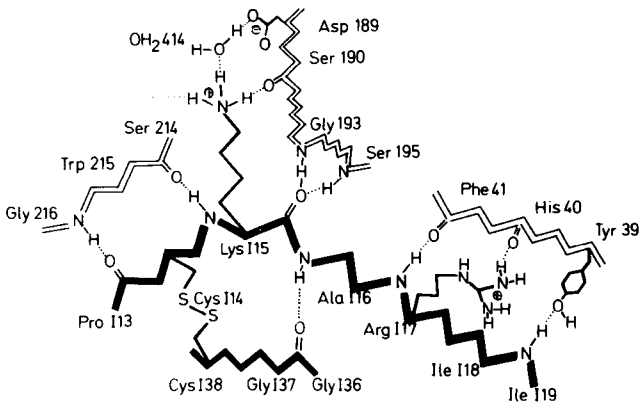

Fig. 4b. Scheme of PTI trypsin interaction seen in the complex. Residues of PTI are indicated by *I*

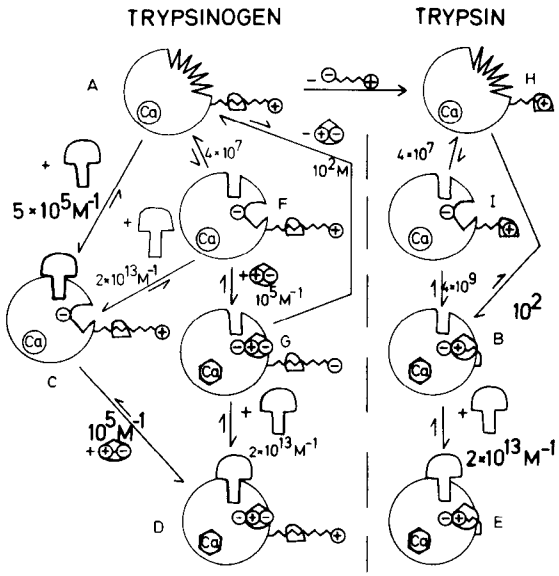

Fig. 5. Simplified equilibrium scheme for the trypsinogen, PTI, Ile-Val system *(left)* and the trypsin PTI system *(right)*. ( ) flexible segments, Ile-Val dipeptide. The various species observed crystallographically are *A*,*B*,*C*,*D*, and *E*. Equilibrium constants are experimentally determined *(large numbers)* or have been inferred *(small numbers)* on the assumption that the equilibria are identical for species with the same structural features [21]

the studies of the trypsin PTI complex (2) (Fig. 4a,b). A number of residues of the activation domain is directly involved in substrate binding. Asp 189 is of particular importance as it is responsible for the primary specificity of trypsin for basic residues. Asp 189 interacts with lysines or arginines of the substrate.

Binding of substrates to trypsinogen is impaired as trypsinogen lacks the complementary binding surface. But the flexibility of this binding surface suggests that transformation to a trypsin-like state should be possible by sufficiently strong ligands. This is the case in the trypsinogen PTI complex (9). Here trypsinogen is forced to adopt a trypsin-like conformation at the cost of some association energy. In the trypsinogen PTI complex the binding pocket for the Ile 16 N-terminus is formed, but empty. Exogenous Ile-Val di-peptide is bound by the complex with considerable energy (21). The ternary complex is virtually indistinguishable from the trypsin PTI complex. The Ile-Val pep-

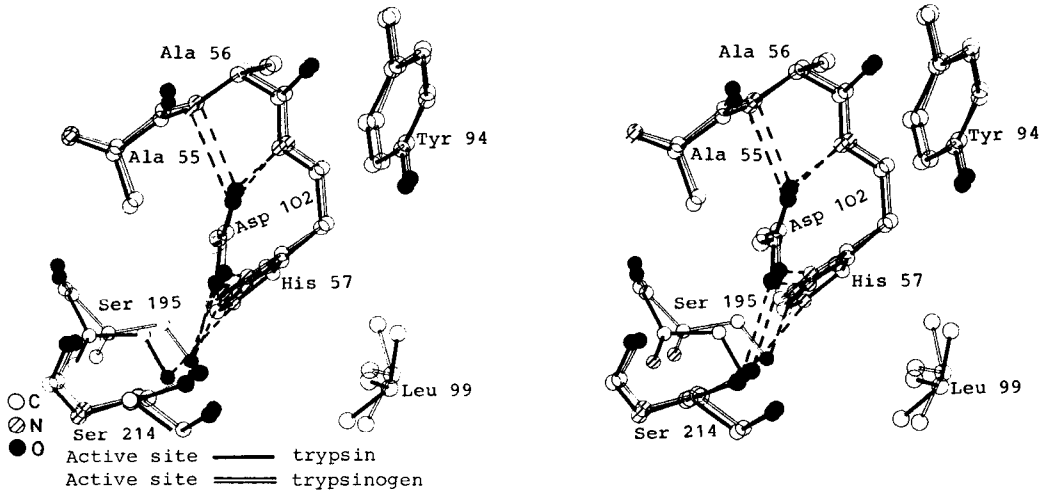

Fig. 6. Stereo-drawing of the catalytic residues in trypsinogen (=) and trypsin (-) overlaid

tide acts as an effector and can induce the transformation to a trypsin-like state also in the presence of much weaker ligands than PTI (22). These structural and thermodynamic data allow the construction of a scheme shown in Figure 5. It is interesting to note that trypsinogen in a trypsin-like state is considerably less stable than native trypsinogen. Rigidification and shaping of the activation domain without the supporting effect of the endogenous N-terminal Ile 16 is very costly in energy. Rigidification and shaping of the activation domain may be regarded as a folding process: The activation domain folds around the Ile-Val N-terminus. However, it is clear that the activation domain is covalently sevenfold linked to the rigid body of the molecule and its conformational freedom must be very limited. Folding of the activation domain is therefore more a final shaping than the folding of a random coil poly-peptide chain and it is a comparatively fast process (23).

The difference between trypsin and trypsinogen discussed so far concern the substrate binding site. Functional data show that the inactivity of trypsinogen is indeed due to inability to bind substrate (24). There is very little difference in the arrangement of the catalytic residues (Fig. 6) in free trypsin and trypsinogen. A discussion of the subtle differences in the free molecules, if they are significant, seems irrelevant. The relevant species is an enzyme substrate complex. The trypsin PTI and trypsin STI (2,25) complexes have many characteristic of such a species.

In the trypsin PTI complex the schematic arrangement of catalytic residues and the scissile peptide (Lys 15-Ala 16) is shown in Figure 7. The seryl 195 O^{γ} forms a "half" bond to the carbon of the scissile peptide Lys 15. The carbonyl group is tetrahedrally distorted. In the context of this article attention should be focused on His-57 which is hydrogen-bonded to Ser 195, but at some stage of the catalytic sequence must protonate the leaving NH group of Ala 16. This requires a conformational change leading to the imidazole hydrogen bonded to NH of Ala 16. But we know from NMR experiments in free PTI (7) that

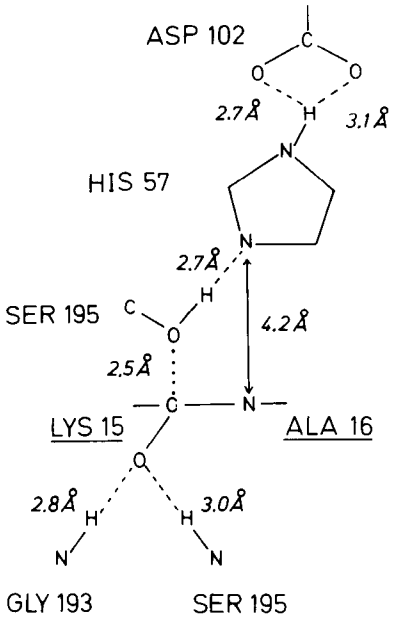

Fig. 7. Scheme of the arrangement of the catalytic residues and the scissile peptide as seen in the complex. Residues of PTI are *underlined*

even tightly packed, internal aromatic residues flip at a considerable frequency of a few thousand times per second. The proton transfer by His 57 in serine proteases is one of many examples of similar activities in other enzymes. Limited side chain flexibility appears essential here. Enzymes might even be designed so that particular vibrational modes enabling transfer are energetically favored.

Proton transfer over short distances as here appears mechanistically simple compared to the transfer of large intermediates over large distances in multi-enzyme complexes (26). We begin to see and study some features of an enzyme with large-scale transfer at the molecular level at the example of citrate synthase.

Citrate Synthase

Citrate synthase, the condensing enzyme, catalyzes the formation of citrate from oxaloacetate and acetylcoenzyme A. The enzyme from pig heart is a dimer with a molecular weight of about 100,000. The folding of this globular molecule is characterized by the absence of sheet conformation and the predominance of helices, some of which are largely buried in the interior of the molecule (13). It was remarkable to observe that the electron density per monomer accounts for only about 360 residues of the 430 required by molecular weight. The invisible part is disordered and is N-terminal and probably involved in binding of co-enzyme A (Fig. 8a,b). This conclusion is drawn from the absence of difference density when crystals with and without CoA are compared. Although this analysis provides no information about the conformation of the N-terminal segment, CoA binding properties should be associated with a rigid fold. The functional significance of disorder here is unclear. There are indications of two active sites in citrate synthase (13). CoA sitting on a flexible protein arm might transfer the intermediate (citryl-CoA) from one site to the other. Acyl carrier proteins

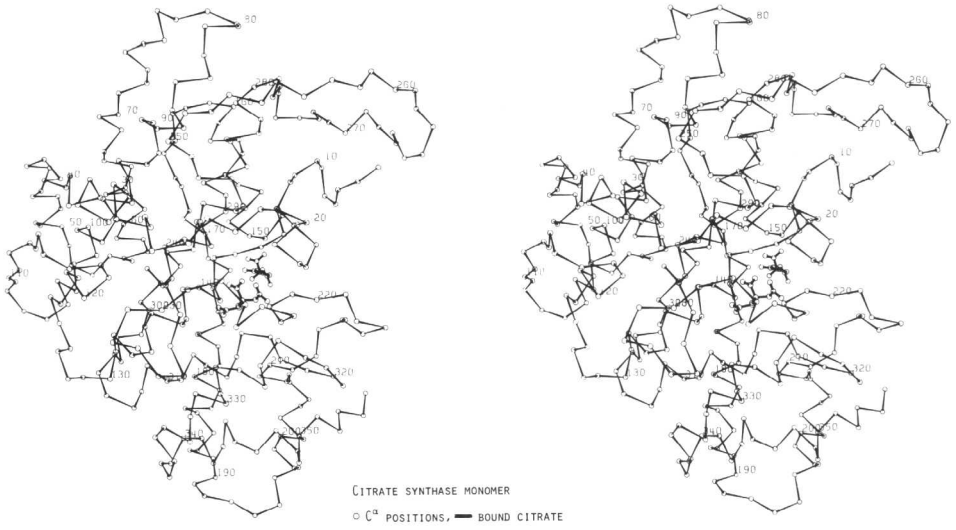

Fig. 8a. Stereo-drawing of chain-folding of a monomeric subunit of citrate synthase (13). The disordered N-terminal segment must continue to the right from residue 1 on

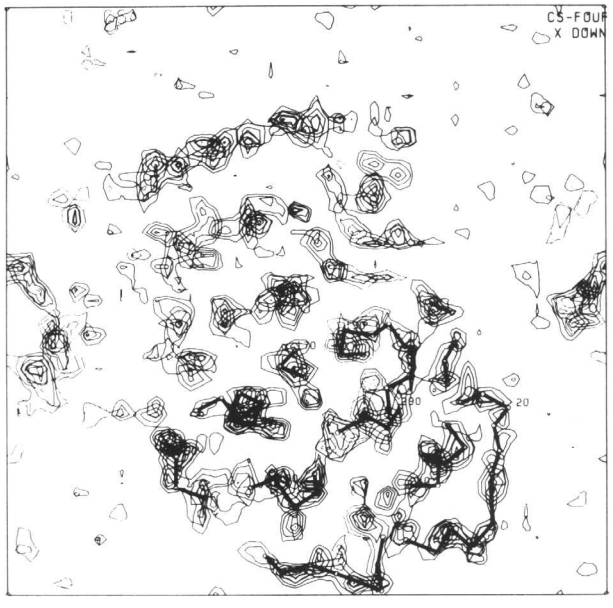

Fig. 8b. Several sections through the electron density map around residue 1. There is ample space in the crystal lattice for the disordered domain

as separate subunits are common in large multi-enzyme complexes where they serve this purpose (26). But we have found and are presently analyzing a different crystal form which, according to several quite preliminary lines of evidence, has the CoA binding domain defined.

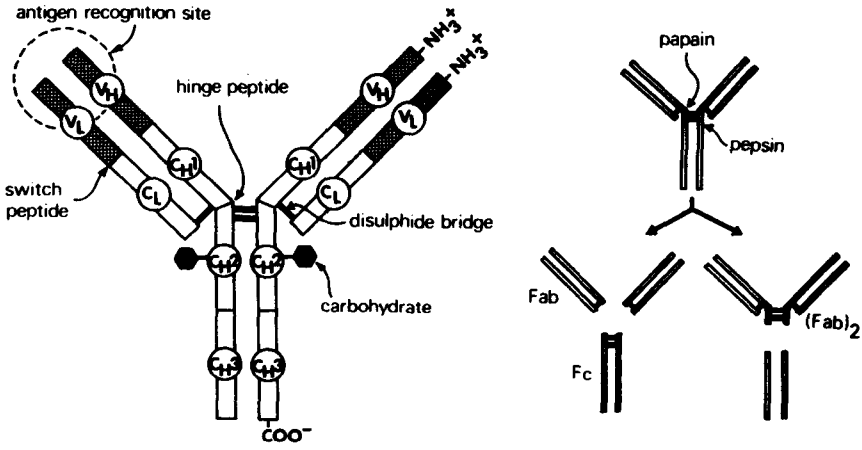

Fig. 9. Structure of antibody (IgG) molecule and enzyme cleavage products. V_L-variable half of light chain, C_L-constant half of light chain, V_H-variable part of heavy chain, C_H1, C_H2, C_H3 — the three constant homology regions of the heavy chain. Fab-antigen-binding fragment, consisting of light chain and half of the heavy chain (V_L, V_H, C_L, C_H1), Fc-C-terminal half of the heavy chain with the interheavy chain disulfide bond intact. Hinge peptide: the segment connecting C_H1 and C_H2 and containing the inter-heavy chain disulfide linkage; switch peptides: the segments connecting V and C parts comprising residues at 110 (light chain) and 119 (heavy chain)

The change of crystal modification possibly reflecting the change in molecular conformation is caused by a slight pH change. Perhaps the disorder-order transformation serves regulatory properties here as in trypsinogen. The citrate synthase problem requires certainly more structural studies to define clearly what function the disorder serves. The building principle of a small N-terminal domain flexibly attached to the main body of the molecule shows possibly more than superficial resemblance to such diverse proteins as TBSV protein (18) or lac repressor (27a,b). The common function in all three cases is nucleotide binding!

Antibody (Fig. 9)

The structure of the intact IgG molecule Kol provided the first example of large-scale segmental disorder observed in crystals. The Fc part with a molecular weight of 50,000 representing the stem of the Y-shaped molecule showed no significant electron density while the Fab arms were well ordered (28). Recently the same phenomenon was discovered in a different intact IgG molecule Nie. No detailed structure analysis is yet at hand here, but as crystals of the intact molecule and its $(Fab)_2$ fragment are isomorphous and the diffraction pattern very similar, the Fc part must be disordered (29). The situation is different with the IgG molecule Dob, where the Fc part is ordered. But Dob is chemically abnormal as it lacks the hinge region (30). These data suggest a correlation between the presence of a hinge region and Fc disorder. Alternatively, crystallizability and disorder might be correlated in the cases of chemically normal IgG molecules.