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Cryoenzymology: The Study of Enzyme Mechanisms at Subzero Temperatures

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Enzymes are very efficient catalysts. Typically rate enhancements of 10^{12} or greater over the corresponding noncatalyzed reaction and of 10^6–10^9 in the analogous acid- or base-catalyzed reaction are found.[1] Many factors have been proposed to account for the efficiency of enzyme catalysis,[1] including strain,[1,2] entropy of binding,[3] acid–base catalysis,[4] complementarity to the transition-state complex,[5] and proximity and orientation effects.[6] A major goal in enzyme mechanism studies is the elucidation of the nature of the interaction between enzyme and substrate during the dynamic processes of the catalysis. The main obstacle to the achievement of this goal stems from the very rapidity of the reaction which makes it of such interest. It is generally accepted by enzymologists that, after the initial productive binding of substrate to the active site of the enzyme (at an essentially diffusion-controlled rate[7]), a series of intermediates (eq 1) and transition-state complexes occur, leading to the eventual release of products and free enzyme. In essence, the problem reduces to one of identifying and characterizing these intermediates and transition states.

Underlying Basis of Cryoenzymology

This Account concerns a relatively new approach for investigating enzyme mechanisms, namely cryoenzymology,[8] which possesses the potential to overcome the above-mentioned problem. In particular, as will be illustrated, the method is well-suited for the provision of structural information about enzyme–substrate intermediates at atomic resolution, as well as kinetic and thermodynamic parameters associated with such intermediate transformations. Most of our current insights into the details of enzyme–substrate interactions have come from studies with pseudosubstrates or inhibitors.[9] It seems probable that in such cases the very slow rate of reaction is due to improper orientation of enzyme catalytic groups and the substrate, and consequently such studies provide misleading information as far as the catalytic mechanism is concerned. On the other hand, the most specific (kinetically) substrate can be used in analogous cryoenzymological studies.[10] Similarly, in spite of great advances, particularly through the use of rapid-reaction[11] and relaxation techniques,[12] kinetic studies of enzyme-catalyzed reactions usually provide little or no insight into the structure of intermediates.[11]

The theoretical basis of cryoenzymology has been presented in detail elsewhere.[13–15] Briefly the method utilizes the fact that different steps in the overall enzyme-catalyzed reaction usually have different free energies and enthalpies of activation. Thus if the reaction is initiated by mixing enzyme and substrate at a suitably low temperature[16] only the first step, the

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(16) Experience suggests that in many cases a temperature of the order of −100 °C would suffice.

initial complexation to form ES, the noncovalent Michaelis complex, will occur, there being insufficient energy available for overcoming the energy barrier to the subsequent intermediate. If the temperature is then gradually raised, a point will be reached where ES transforms into a subsequent intermediate, I_2 (eq 1).

$$E + S \rightleftarrows ES \rightleftarrows I_2 \rightleftarrows (I)_n \rightleftarrows EP \rightleftarrows E + P \quad (1)$$

If the temperature is then kept constant or lowered, I_2 may be trapped. Further raising of the temperature will eventually result in transformation of I_2 into a subsequent intermediate, which also may be stabilized by lowering the temperature. This process may be repeated until a temperature is reached at which turnover occurs.

Any intermediate for which $k_i \gg k_{-i} + k_{(i+1)}$ (see eq 2) can be accumulated in this manner. Note that in-

$$\underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} I_i \xrightarrow{k_{(i+1)}} \quad (2)$$

termediates may exist, for example between ES and I_2 in the aforementioned case, which may not be detected because this condition does not hold. The concentration of the stabilized intermediate is dependent on the relative magnitude of k_{-i} and may be stoichiometric with that of the enzyme,[13] as has been shown for several acyl-enzymes[17-20] and intermediates in peroxydase catalysis.[21] The concentrations of trapped intermediates obtained in this manner will be much greater than those attainable in freeze-quenching-type experiments. Reductions in rates by factors as large as 10^{12} can be obtained using cryoenzymology. Several cases have been examined in which intermediates with normal lifetimes of milliseconds are stable for hours at low temperatures.[8,20]

The major advantages which are unique to cryoenzymology stem from the potential to accumulate essentially all of the enzyme in the form of a particular intermediate. For example, if the trapped intermediate can be stabilized for a period of hours to days it is possible to use x-ray crystallography to obtain structural information to very high resolution.[10] Furthermore, the large rate reductions allow the most specific substrates to be used. A wide variety of physical and chemical techniques may be used to study an accumulated intermediate and also to provide information about the kinetics and thermodynamics of its transformations. As will be discussed shortly in more detail, the technique is admirably suited for detecting intermediates some of which may not even be present in detectable amounts under normal conditions.[8,22,23]

The main limitations of the low-temperature technique are the need to use aqueous organic cryosolvent to avoid the inherent rate-limiting enzyme–substrate diffusion of frozen solutions and the possibility that the potential energy surface for the reaction may be such that conditions in which an intermediate accumulates cannot be attained. Difficulties may also arise in ascertaining whether an intermediate which is observed at subzero temperatures is also on the productive catalytic pathway under normal conditions. In spite of these limitations it is clear from recent investigations that cryoenzymology, particularly utilizing crystallographic techniques,[10,17,24] will play a very valuable and important role in unravelling the finer details of enzyme catalytic mechanisms.

The General Experimental Approach

If the data obtained at subzero temperatures and in aqueous organic cryosolvents are to be of value in furthering our understanding of the dynamic processes of enzyme catalysis, it is necessary to demonstrate their relevance to the reaction in normal conditions, i.e., that the catalytic reaction pathway is unchanged. Consequently the initial enzyme systems we chose for study by cryoenzymology were ones which had already been extensively studied and for which a wealth of data was available for comparison. Further the first step which should be taken in any such investigation is to demonstrate that the enzyme and reaction pathway are unchanged by subzero temperatures and the aqueous organic solvent system.

In my laboratory we have developed the following general procedure for the study of enzyme–substrate intermediates: (1) selection of a suitable cryosolvent and demonstration that no adverse effects on the catalytic or structural properties of the enzyme occur;[8,18,20,25] (2) detection of intermediates at subzero temperatures by using appropriate spectral probes in the substrate or intrinsic to the enzyme;[8,20,23,26,27] (3) characterization of these intermediates with respect to the kinetics and thermodynamics of their transformations;[8,20,23,28] (4) determination of suitable conditions to obtain a particular intermediate stabilized in the crystalline state;[17] and (5) the application of x-ray diffraction to obtain a high-resolution structure of the intermediate.[10]

By such an approach, combining cryoenzymology, kinetic isotope effects, and x-ray crystallography, we hope to obtain sufficient information to be able to postulate a mechanism which fully accounts for the observed efficiency of enzyme catalysis. In the remainder of this article this general approach will be illustrated with selected examples to demonstrate the unique potential of cryoenzymology.

Choice of Cryosolvent. Douzou and co-workers[29,30] have carried out extensive physicochemical studies on the properties of the most commonly used cryosolvents. In general the viscosity, pH*,[30] and dielectric constant increase with decreasing temperature. At present the selection of a suitable solvent system is empirical. The main factors involved are the desired freezing point, viscosity, and possible cosolvent participation in the

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reaction.[31] Superimposed is the effect of the cryosolvent on the protein structure. Although many studies of protein–cosolvent interactions have been carried out, very few have been sufficiently rigorous to provide unambiguous information concerning the nature of the interactions. Part of our recent effort has therefore been directed at this question. We find, for example, that as the cosolvent concentration is increased the midpoint (T_m) for the cooperative, reversible, native $\rightleftarrows$ denatured thermal transition decreases in a complex (nonlinear) fashion.[32] For many of the enzymes we have studied, e.g., the serine proteases, papain, and ribonuclease, we have observed that in the cosolvent concentration usually employed (60–80%) the values of T_m are usually in the 10 to 20 °C range. Thus the enzyme will be stable and active at subzero temperatures, but denatured at room temperature. For other enzymes we have investigated, however, the T_m is below 0 °C for such cosolvent concentrations (e.g., β-galactosidase). Consequently it is usually necessary to conduct experiments in which the suitability of the cryosolvent is being determined at temperatures of 0 °C or below. Such experiments are exemplified by a recent study of papain.[20]

Incubation of the enzyme in a variety of potential cryosolvents at 0 °C followed by periodic assay for catalytic activity indicated dimethyl sulfoxide and ethanol systems to be very promising (no loss of activity over extended periods). Experiments were then carried out to determine the effect of increasing cosolvent concentration on the (kinetic) catalytic parameters of the reaction of papain with a specific substrate and on the intrinsic spectral properties of the enzyme (UV absorption, fluorescence, and circular dichroism).[20] The pH and temperature dependence of the acylation and deacylation reactions were also determined. The results of these studies indicated that 60% aqueous dimethyl sulfoxide and 80% aqueous ethanol were satisfactory solvents.[20] In other enzyme systems we have also used nuclear magnetic resonance[33] and x-ray diffraction[10] to examine the effects of cosolvent and low temperatures on the structure of the enzyme.

Detection of Intermediates. As previously indicated, a major advantage of cryoenzymology is the potential to reveal intermediates which are not readily detectable at ambient temperatures. The ability to use low temperatures to stabilize enzyme–substrate intermediates, previously only discerned using rapid-reaction methods, was elegantly demonstrated by Douzou and co-workers[21] using horseradish peroxidase. Intermediate complex I, formed by reaction of peroxidase and hydrogen peroxide, could be obtained in 98% yield and was quite stable at temperatures below −40 °C. The addition of a hydrogen donor then resulted in transformation to complex II which could also be stabilized at such temperatures. The spectral (absorption, circular dichroism, and electron spin resonance) and kinetic properties of these intermediates were consistent with data obtained in aqueous solution using rapid-flow techniques.[21]

There have been several recent reports in which cryoenzymology has permitted the detection of previously undetected intermediates. For example, a rapid release of 1 equiv of *p*-nitrophenol per enzyme in the reaction of β-glucosidase with *p*-nitrophenyl β-D-glucoside at low temperatures indicated the existence of a glucosyl-enzyme whose breakdown was rate limiting.[34] A subsequent rapid-reaction study under normal conditions confirmed the low-temperature results.[35] More recent studies at subzero temperatures with methyl, *p*-nitrophenyl, and methyl umbelliferyl glucoside substrates have revealed evidence for an intermediate occurring between the initial Michaelis complex and the glucosyl-enzyme.[36] We have also observed similar results in an investigation of β-galactosidase.[37] Our current interpretation concerning the nature of the intermediate preceding the glycosyl-enzyme is that it involves a substantial conformational change in the enzyme. Such an hypothesis is in accord with studies by Sinnott[38] who has proposed such an intermediate on the basis of kinetic studies.

Makinen et al.[22] have observed biphasic kinetics for the reaction of carboxypeptidase A with a specific ester substrate at subzero temperatures in a ternary ethylene glycol–methanol–water cryosolvent. From a study of the temperature dependence of the reactions they conclude that an intermediate is present whose rate of formation is rate limiting at temperatures above −10 °C and whose breakdown is rate determining at lower temperatures. Thus the intermediate, postulated to be a covalent mixed-anhydride acyl-enzyme, is not detectable under normal conditions.

Other systems in which cryoenzymology has resulted in the detection of previously undetected intermediates include ribonuclease A with 2′,3′-cytidine cyclic phosphate,[32] glucose oxidase with glucose,[39] chymotrypsin,[8] trypsin,[25] papain,[40] and bacterial luciferase.[41] This latter system is of interest because the relatively long-lived intermediate involving an oxygenated flavine mononucleotide, which can be isolated by low-temperature gel filtration, was attributed to the 4a-hydroperoxy derivative.[41] A subsequent model compound was shown to have similar spectral and chemical characteristics.[42]

Characterization of Intermediates

The potential to probe the interactions of different regions of the substrate with the enzyme, and vice versa, has been used in studying several hydrolytic enzymes. This approach, which is made feasible by the incorporation of "probes" into various domains in the substrate, without affecting the specificity for the modified substrate, will be demonstrated for chymotrypsin.

That part of the substrate which binds in the hydrophobic pocket[43] of the enzyme was probed using

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nitrotyrosine substrates;[23] the acylamino binding locus was investigated using *N*-(2-furyl)acryloyl substrates (Fink and Feldman, unpublished results), and the leaving-group site was studied using a *p*-nitroanilide substrate.[8] In addition, the role of the enzyme's tryptophan side chains was examined using the near-UV-transparent substrate *N*-acetyl-L-phenylalanine methyl ester.[26,27] In essentially all the systems we have examined with chymotrypsin in the 0 to −100 °C region we have observed a consistent pattern, regardless of the cryosolvent, substrate, or means of monitoring the reaction. There is an initial very fast transient, attributable to substrate binding, followed by one or two pH-independent reactions, ascribed to substrate-induced changes in the spatial orientation (and possibly solvation state) of enzyme and substrate groups in the active site.[8,23,27] Subsequently a pH-dependent reaction, involving the active-site imidazole, and probably not the formation of a tetrahedral intermediate,[8] is observed, followed by the pH-dependent acylation reaction in which the acyl-enzyme is formed.[23,27] Hydrolysis of the acyl-enzyme (deacylation) then completes the catalytic reaction.

The interpretations of these time-dependent spectral changes are based in part on observations of the following type. The initial very rapid reaction was attributed to binding on the basis of kinetic calculations and the nature of the spectral changes occurring when nitrotyrosine substrates were used. These show that the nitrophenol moiety of the substrate enters an area of lower polarity (the so-called hydrophobic pocket[43]) at this stage of the reaction.[23] If modified enzyme was used in which the active-site imidazole was methylated, then the first three reactions were observed but not the subsequent ones.[8,23] The small size of the methyl group would not be expected to prevent substrate binding, but would prevent any catalytic action by the imidazole. If chymotrypsin is modified so that substrate binding cannot occur, then none of the reactions was observed.[8,23] With the *p*-nitroanilide substrate the spectrum of the product of the first pH-dependent reaction was indicative of sp^2 hybridization at the adjacent carbonyl, thus eliminating the possibility of its being a tetrahedral intermediate.[8] Using specific *p*-nitrophenyl ester substrates it is possible to stop the reaction after formation of the acyl-enzyme.[17-19] The amount of *p*-nitrophenol liberated provides a ready measure of the acyl-enzyme concentration and can be used as the basis of a low-temperature active-site normality assay.

The fact that for most of the substrates investigated changes in the monitoring probe are observed, regardless of which part of the substrate the probe is located in, suggests that changes in the local environment surrounding the whole substrate occur in each step in the reaction. These changes are also reflected in the intrinsic spectral character of the enzyme.[27] That the observed intermediates are on the productive catalytic reaction pathway, or in rapid reversible equilibrium with it, may be inferred from the knowledge that, for each accumulated intermediate, turnover will occur when the temperature is subsequently raised sufficiently.[8,27] We hope to obtain more specific information from x-ray diffraction studies of the crystalline intermediates.

It is not so surprising that these intermediates preceding the acyl-enzyme are not readily detected at 25 °C. When the rates of their transformations are extrapolated to 25 °C and corrected for the cryosolvent effect on K_s, values of 10^3 s^{-1}, or greater, are obtained.[8,27] In fact there have been a few studies at 25 °C which suggest the existence of at least two intermediates between the Michaelis complex and the acyl-enzyme for chymotrypsin.[44]

Kinetic Analysis of Intermediate Transformations

Kinetic analyses of enzyme-catalyzed reactions may be considerably simplified in the low-temperature method because in many cases each intermediate transformation can be followed separately, i.e., appropriate conditions of temperature, pH*, and substrate or enzyme concentration can be chosen so that only one step of the overall reaction occurs. The expressions relating the observed rates with the underlying microscopic rate constants have recently been derived,[13] as have the relationships between the concentration of an accumulated intermediate and the underlying rate constants.[13] By determining the effect of substrate concentration on the observed rate for each subreaction it is possible, in principle at least, to determine all the individual rate constants. We have recently carried out such detailed studies for chymotrypsin[28] and papain;[40] one of the noteworthy points is that the equilibrium between the initial enzyme–substrate complex and the subsequent intermediate strongly favors the latter.

From the dependence of observed reaction rate on temperature it is possible to obtain values for $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$. In general we find that the values of $\Delta H^{\ddagger}$ increase for successive steps in the overall reaction and those for $\Delta S^{\ddagger}$ become more positive (but are still usually quite negative).[8]

Mechanism of Action of the Serine Proteases

It seems appropriate at this point, in the light of our recent cryoenzymology studies, to briefly outline our current view of the mechanism of the serine proteases. Chymotrypsin will be used as a representative example of these enzymes. More details concerning the mechanism of chymotrypsin catalysis may be found in recent reviews.[45-49] Key features include a specificity-determining "hydrophobic pocket" into which the substrate side chain fits,[43] a covalent acyl-enzyme intermediate involving the side chain of Ser-195, an essential hydrogen-bonded aspartate–imidazole couple in the active site, and a putative tetrahedral intermediate in the formation and hydrolysis of the acyl-enzyme.[46]

On the basis of cryoenzymological investigations with a number of specific ester and amide substrates in this laboratory, we have arrived at the following general

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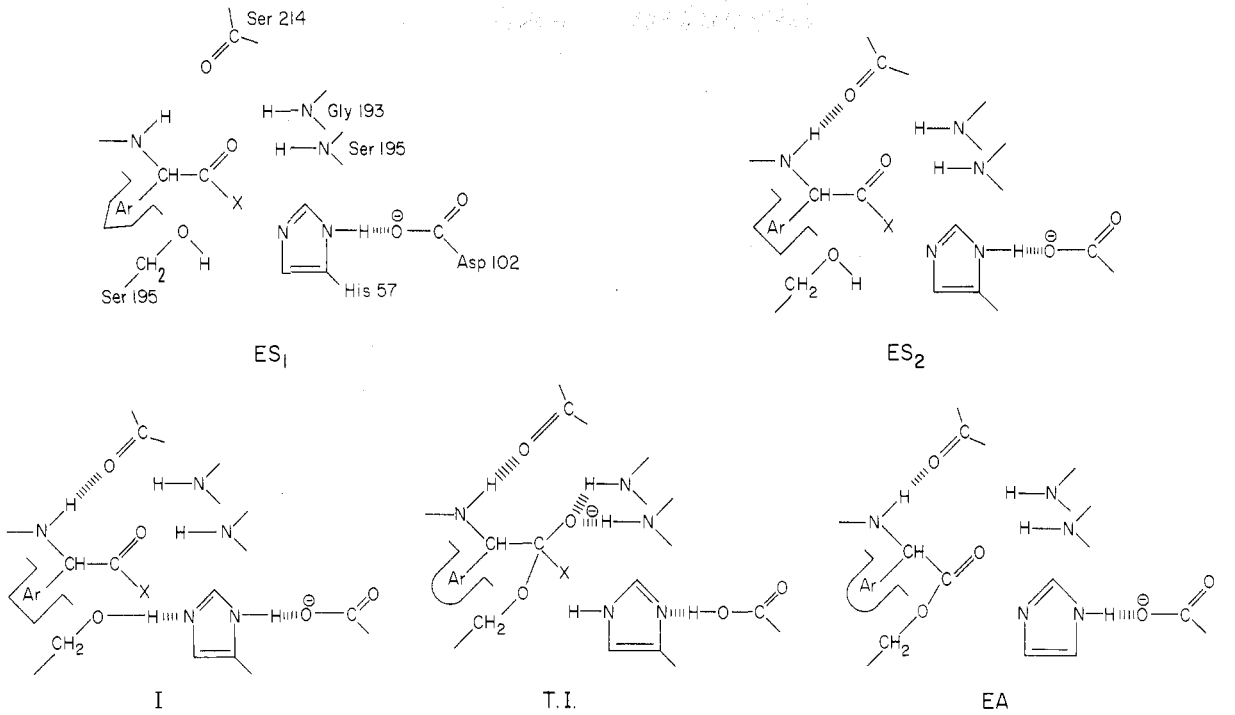

Figure 1. Schematic outline of the major features in the postulated mechanism of acylation of chymotrypsin. (a) The ES_1 complex. (b) ES_2, in which the major changes include an H bond to Ser-214, the relative positions of the substrate and the hydrophobic pocket, and the positioning of the peptide bonds of Gly-193,195. (c) Complex I, in which changes in the imidazole and Ser-195 positions lead to H-bond formation, and partial distortion of the substrate carbonyl toward sp^3 hybridization is concomitant with movement of the peptide bonds (193, 195). (d) The tetrahedral intermediate, involving stabilization of the oxyanion by H bonding, the transfer of the proton from Ser-195 to Asp-102, and relative movement of the substrate toward the serine. (e) The acyl-enzyme formed by loss of XH. The imidazole, serine, and peptide bonds (193, 195) have returned to similar positions as in ES_1.

scheme for the reaction pathway in chymotrypsin catalysis:[8,23,27]

$$E + S \overset{1}{\rightleftarrows} ES_1 \overset{2}{\rightleftarrows} ES_2 \overset{3}{\rightleftarrows} I \overset{4}{\rightleftarrows} TI \overset{5}{\rightleftarrows} EA \overset{6}{\rightarrow} E + P_2 \quad (3)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad + \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad XH$$

Our interpretation of the mechanism of catalysis of the enzyme in terms of this scheme follows. A highly schematic representation of the major features is given in Figure 1. It is convenient to visualize the reaction as proceeding by a series of states: (1) association–activation,[50] steps 1–3; (2) the initial covalent bond breaking–making step, 4, in which a covalent tetrahedral species is formed; (3) departure of the leaving group X and collapse of the tetrahedral intermediate to the acyl-enzyme, step 5; and (4) hydrolysis of the acyl-enzyme, involving the reverse series of reactions in which X is replaced by OH of water.

The term association–activation has been coined by Schultz[50] to encompass the binding of substrate and subsequent changes in substrate and enzyme conformation and orientation which lead to a lowering of the free energy of activation for the bond breaking–making steps(s). In the case of the serine proteases, substantial evidence in addition to that from cryoenzymology supports the notion of discrete steps in the association–activation process. For example, the binding of competitive inhibitors appears as a two-step process.[50-54]

In addition, a number of studies with specific substrates have shown the existence of one or more intermediates between ES_1 and EA.[44]

We believe the initial step involves the formation of a "loose" noncovalent complex, ES_1, in which both enzyme and substrate are in essentially the same conformation as they were prior to complexation. This reaction involves the displacement of some active-site water molecules and is essentially diffusion controlled. As shown in Figure 1a the O^{γ} of Ser-195 and the $N^{\epsilon 2}$ of His-57 are too far apart, and incorrectly oriented, for a hydrogen bond to exist between them.[47,55]

The next step in the reaction involves a "tightening" of the complex to give ES_2 (Figure 1b), resulting from possible further water displacement, and movement of the substrate further into its binding cleft, possibly accompanied by changes in the size and shape of the hydrophobic pocket.[9a,23,46,47] In addition, changes in the acylamino binding locus involving the formation of a strong hydrogen bond between the carbonyl of Ser-214 and the amide H occur.[9a,47] The substrate carbonyl is still trigonal at this stage.

Both cryoenzymology and conventional studies indicate the existence of a pH-dependent step in the association–activation process.[8,23,27,44] In the former, reaction 3, the conversion of ES_2 to I, has a pK of approximately 6 and is prevented by methylation of the imidazole of His-57. Binding energy obtained from the first two steps is used in this third step to bring about partial substrate distortion with concomitant changes in the position of the imidazole of His-57 and probably of Ser-195 (Figure 1c). These latter movements may

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(54) The reaction of *N*-acetyl-D-phenylalanine methyl ester with chymotrypsin in 65% aqueous dimethyl sulfoxide at −45 °C shows a slower step following the rapid initial binding of the inhibitor (unpublished observations of C. Stimpson and A. L. Fink).
(55) J. J. Birktoft, J. Kraut, and S. T. Freer, *Biochemistry*, **15**, 4481 (1976).

result in the formation of a hydrogen bond between Ser-195 and His-57, if the imidazole acts as a base catalyst in the subsequent nucleophilic attack by the serine O^{γ} on the substrate carbonyl. This last step of the association activation process can only occur if $N^{\epsilon 2}$ of the imidazole is free, i.e., if protonated or methylated, reaction 3 will not occur. The 10^4-slower reaction observed with $N^{\epsilon 2}$-methyl-His-57-chymotrypsin,[56] in which ES_2 must transform directly into TI, thus reflects the rate enhancement contributed by the $ES_2 \rightarrow I$ step, and possibly therefore the contribution of the imidazole acting as a base catalyst, and a shuttle for the proton to the aspartate. Whether or not an actual hydrogen bond is formed to the serine, this third step would involve movement of the imidazole closer to the serine and substrate. In addition, partial distortion of the substrate carbonyl toward tetrahedral geometry, caused at least in part by movement of the peptide bonds of Gly-193 and Ser-195, might occur.

The next step (4) in the formation of the acyl-enzyme involves the key chemical reaction, namely nucleophilic attack by the O^{γ} of Ser-195 to yield a tetrahedral intermediate. Since the transition-state complex in this reaction is expected to be very similar in structure to the tetrahedral intermediate, and from the principle of maximum binding of the transition-state complex to the enzyme, there will be maximum complementarity between enzyme and substrate at this stage.[47] The driving force for reaction 4 includes "pressure" by the active-site groups to distort the substrate into the tetrahedral configuration, i.e., the much tighter fit of the tetrahedral form, as well as probable assistance of the base catalysis by the imidazole. Two key features in this step are the formation of the so-called "oxyanion hole",[47,56] involving the peptide bonds of Gly-193 and Ser-195 and their hydrogen bonding to the O^- of the tetrahedral species, and the transfer of the proton from the $N^{\delta 1}$ position of the imidazole to the aspartate (Figure 1d). Formation of the covalent bond between enzyme and substrate is probably accompanied by movement of the substrate, particularly the side-chain portion, toward the serine. The preponderance of evidence indicates that formation of the tetrahedral intermediate is usually slower than its breakdown.[57] The absence of the oxyanion hole in chymotrypsinogen has been suggested as a major reason for the lack of catalytic activity of the zymogens.[58]

The collapse of the tetrahedral intermediate, step 5, Figure 1e, to the acyl-enzyme involves transfer of the proton from Asp-102, via the imidazole, to the leaving group X and its subsequent departure; the rehybridization of the substrate C-1 to sp^2; and the relaxation of the imidazole and serine back to the positions they occupied in ES_1.[10] Existing evidence for movements of His-57 during association–activation is quite good.[46,47] That Ser-195 makes significant movements is less clear.[46,47] In fact, since the Fourier difference electron density map for (*N*-carbobenzoxy-L-alanyl-elastase)-(elastase) at −55 °C shows no changes in the serine position within the limits of resolution (probably <0.3 Å), any such movements in association–activation or tetrahedral intermediate formation must be reversed in the collapse of the tetrahedral intermediate to the acyl-enzyme.[10]

The preceding description of events leading to acyl-enzyme formation is based mostly on indirect evidence and must be considered largely hypothetical at present. Cryoenzymological experiments currently under way, from which we expect to obtain high-resolution structures of at least two of the intermediates prior to the acyl-enzyme, however, should enable us to obtain much more direct evidence for the catalytic events. Our current understanding thus suggests that much of the efficiency of the catalysis is derived from a combination of small but critical changes in the spatial orientation and positioning of active-site groups and substrate. Energy obtained from the binding process is then used to distort the substrate to the key transition-state structure, in which there is maximum complementarity or tightness of binding to the enzyme.

X-Ray Crystallographic Studies at Subzero Temperatures

A "marriage" of x-ray diffraction and cryoenzymology offers the potential to obtain detailed structural information about enzyme–substrate intermediates. Previous crystallographic studies of enzyme–"substrate" intermediates have, of necessity, used pseudosubstrates or inhibitors in order to have a sufficiently stable species for the essentially static diffraction technique.[9] A major advantage of cryoenzymology is that it permits the use of the most specific of substrates in the production of stabilized intermediates. The significance of this point should not be overlooked, since structural studies of intermediates formed from nonspecific substrates produce details about *nonproductive* intermediates. X-ray crystallography at subzero temperatures offers a number of advantages, including decreased radiation damage to the crystal.[15,24]

Recent studies we have carried out in collaboration with Professor G. A. Petsko at Wayne State University have demonstrated the feasibility of obtaining the structures of productive intermediates trapped at subzero temperatures.[10,17] Since the advantages and limitations of the technique are considered in detail elsewhere,[15] only the results and their implications will be mentioned here.

Acyl-enzymes of the mammalian serine proteases can be readily accumulated, isolated, and stabilized from specific nitrophenyl ester substrates at subzero temperatures.[17-19] By starting with crystals of the enzyme, rather than the dissolved enzyme, and allowing the substrate to diffuse into the crystal at the low temperature, we were able to show that at temperatures below −50 °C and in the pH-optimum region very high active-site occupancies could be obtained for the crystalline productive acyl-enzymes of γ-chymotrypsin, trypsin, and elastase.[17] Furthermore, these crystalline intermediates were stable for several days at the low temperature, but underwent deacylation when the temperature was raised.[17]

By using a flow-cell it was possible to repeat these experiments on a diffractometer.[10] For example, using elastase and its specific substrate *N*-CBZ-L-alanine

(56) R. Henderson, *Biochem. J.*, **124**, 13 (1971).
(57) E. C. Lucas and M. Caplow, *J. Am. Chem. Soc.*, **94**, 960 (1972); M. L. Bender and M. Philip, *ibid.*, **95**, 1665 (1973); A. R. Fersht and Y. Requena, *ibid.*, **93**, 7079 (1971); A. Frankfater and F. J. Kézdy, *ibid.*, **93**, 4039 (1971); M. W. Hunkapiller, M. D. Forgac, and J. H. Richards, *Biochemistry*, **15**, 5581 (1976).
(58) M. A. Kerr, K. A. Walsh, and H. Neurath, *Biochemistry*, **15**, 5566 (1976).

p-nitrophenyl ester, it was possible to compare the room-temperature structure of the native enzyme in the sodium sulfate mother liquor with that in 70% aqueous methanol at −55 °C. The Fourier difference map indicated no changes in the polypeptide backbone and only changes on the surface, ascribed to immobilization at the low temperature of "floppy" surface residues. The formation of the acyl-enzyme intermediate could be monitored by changes in the intensity of selected reflections when the substrate was flowed in at −55 °C. A Fourier difference map of the acyl-enzyme minus the native enzyme at −55 °C showed the absence of the nitrophenyl group, the alanyl residue covalently attached to the active-site Ser side chain, the alanyl side chain in van der Waals contact with the Val-216, and the planar CBZ group in the anticipated location. No evidence of movement in the active-site His, Asp, and Ser residues was detectable at the resolution of the experiment.[10] No loss of acyl group was detected when cryosolvent was used to wash the crystalline acyl-enzyme at −55 °C for 4 days. When the temperature was raised to −10 °C the acyl group disappeared at the rate predicted for deacylation. For the crystalline enzyme both acylation and deacylation rates appeared decreased by a factor of 10–20 over the corresponding reactions with dissolved enzyme.[17]

In addition to the demonstration that it is possible to obtain high-resolution structural data of productive enzyme–substrate intermediates in this manner, this investigation established that, in the crystal at least, the structure (and function) of the enzyme is essentially the same at subzero temperatures in aqueous organic solvent as under normal conditions, and that the conformation of the enzyme in the free and acylated forms is very similar. The not unexpected implication of the latter observation is that those features responsible for the great efficiency of enzyme catalysis are to be found, in the case of the serine proteases, in the conversion of enzyme into acyl-enzyme, and acyl-enzyme into free enzyme. Experiments are now under way to extend these crystallographic studies to higher resolution (≤2.0 Å), to trapped intermediates prior to the acyl-enzyme, to polypeptide substrates, and to the enzymes papain, subtilisin, ribonuclease A, and γ-chymotrypsin. The significance of the anticipated results from these experiments should need no elaboration. Suffice it to say that we would appear to be on the threshold of an era when it will be possible to obtain a series of time-lapse "pictures" as the catalytic reaction proceeds.

Utilization of Isomeric Aminoacyl Transfer Ribonucleic Acids in Peptide Bond Formation

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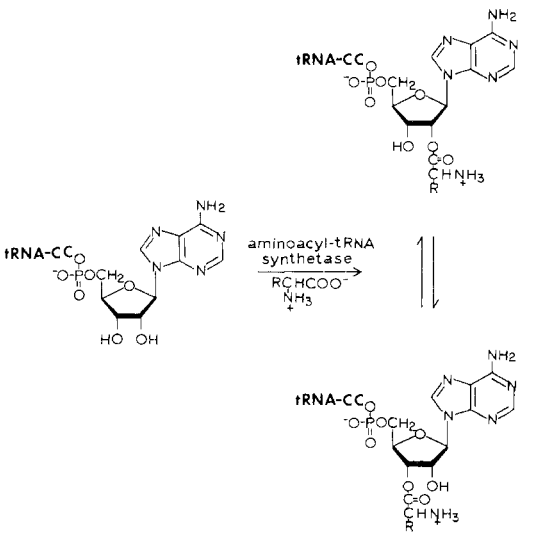
The biosynthesis of proteins from their component amino acids is one of the most important and intensively studied biological processes. Peptide bond formation is mediated via the intemediacy of aminoacylated transfer RNAs (tRNAs), which contain specific amino acids covalently bound as activated esters.[1] At least one tRNA corresponds to each amino acid (Figure 1, for example, depicts *E. coli* $tRNA^{Met}_{f}$[2]), and a cognate aminoacyl-tRNA synthetase catalyzes the formation of the activated ester from the tRNA and its amino acid. Aminoacylation occurs on the 2′- or 3′-OH group of the adenosine at the 3′ end of tRNA (eq 1), and the ester initially formed equilibrates rapidly ($t_{1/2} \simeq 0.2$ ms)[3] between the vicinal hydroxyl groups. Although it has seemed likely that each aminoacyl-tRNA synthetase utilizes only a single tRNA OH group for aminoacylation, the rapid equilibration between the two isomers of aminoacyl-tRNA has made it difficult to determine the initial site of aminoacylation.



(1)

After an amino acid is attached to its cognate tRNA, the position of insertion of the amino acid into a

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(1) J. Lucas-Lenard and F. Lipmann, *Annu. Rev. Biochem.*, **40**, 409 (1971).

(2) $tRNA^{Met}_{f}$ denotes the (formylatable) tRNA specific for methionine; methionyl-$tRNA^{Met}_{f}$ denotes the corresponding aminoacylated tRNA.

(3) B. E. Griffin, M. Jarman, C. B. Reese, J. E. Sulston, and D. R. Trentham, *Biochemistry*, **5**, 3638 (1966).