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Multiple Conformational Changes in Enzyme Catalysis†

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ABSTRACT: Understanding the molecular mechanisms of enzyme catalysis and allosteric regulation has been a primary goal of biochemistry for many years. The dynamics of these processes, approached through a variety of kinetic methods, are discussed. The results obtained for many different enzymes suggest that multiple intermediates and conformations are general characteristics of the catalytic process and allosteric regulation. Ribonuclease, dihydrofolate reductase, chymotrypsin, aspartate aminotransferase, and aspartate transcarbamoylase are considered as specific examples. Typical and maximum rates of conformational changes and catalysis are also discussed, based on results obtained from model systems. The nature and rates of interconversion of the intermediates, along with structural information, can be used as the bases for understanding the incredible catalytic efficiency of enzymes. Potential roles of conformational changes in the catalytic process are discussed in terms of static and environmental effects, and in terms of dynamic coupling within the enzyme−substrate complex.

Understanding enzyme catalysis on a molecular basis has fascinated biochemists for more than half a century. Two primary reasons exist for this fascination: the importance of enzymes in physiological processes and the incredible catalytic efficiency of enzymes. Enzymatic reactions typically proceed at rates more than millions of times faster than the corresponding uncatalyzed reactions. In this review, the molecular basis of the catalytic efficiency will be explored from the viewpoint of the macromolecular nature of enzymes, with particular emphasis on the shared aspects of many different enzymatic reactions. The general concepts that are postulated will be illustrated with specific examples and consideration of the reaction rates that are involved. No effort has been made to be comprehensive; this would require a very large treatise. However, as a result of the investigation of many enzyme reactions by a variety of methods, particularly kinetics and structure determinations, some general characteristics of enzyme mechanisms can be delineated.

First, a macromolecule is required for efficient catalysis, generally with a molecular weight of at least approximately 5000−10000. Many man-hours have gone into making model catalysts of low molecular weight and degrading enzymes to a minimal size, but thus far, significantly smaller molecules have not been found that approach the catalytic efficiency of fully active enzymes.

Second, for enzymes where comprehensive mechanisms have been established, multiple intermediates are almost always detected. Dividing a chemical reaction into multiple steps of relatively low activation energy, as contrasted to a single step of high activation energy, appears to be a general feature of the catalytic process and is an obvious way to enhance the rate of reaction. In some cases, this involves the formation of stable covalent enzyme−substrate intermediates, whereas in other cases, the intermediates are retained as noncovalently bound species on the enzyme.

Finally, multiple conformational changes appear to be a general feature of enzymatic mechanisms. In fact, the ability to have multiple conformations is a unique feature of

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macromolecules. The role of these conformational changes in the catalytic process is not fully understood, but several possibilities are apparent. For example, the structure can be optimized for each step in the reaction sequence, usually by subtle cooperative conformational changes. The binding of the substrate can be enhanced by a change in the enzyme structure, as exemplified by the "induced fit model" (*1*), and the substrates can be oriented very precisely for acid−base or electrostatic catalysis. Also, the reaction environment can be markedly altered: water can be squeezed out of the active site, or a limited number of water molecules can be retained for catalysis. This effectively creates a low-dielectric medium where electrostatic interactions are enhanced, and the p*K*s of ionizable groups can be significantly altered (*2*). The enzyme may also play a more dynamic role in the catalytic process by directly coupling conformational changes throughout the macromolecule to the catalytic process. Probably the first explicit model for this role was proposed by Eyring and Lumry (*3*) as the "rack", where the enzyme functions like the medieval rack and pulls the substrate apart. Along a similar line, a model in which noncovalent bond breaking and making within the macromolecule is coupled to the catalytic event has been postulated as a dynamic mechanism for lowering the activation energy (*4*). In any event, the conformational adaptability of the macromolecule clearly is essential for enzyme function.

These general principles are illustrated below by discussion of specific enzyme mechanisms and consideration of the elementary steps involved in the reaction mechanisms.

Enzyme Mechanisms with Multiple Intermediates and Conformations

Ribonuclease A was one of the first enzymes to be extensively studied by kinetic and structural methods (cf. ref *5*). It is relatively small, consisting of 124 amino acids with a molecular weight of 13 683, and is very stable. It catalyzes the breakdown of RNA in two steps. First, the diester bond is broken, and a pyrimidine 2′,3′-cyclic phosphate is formed. The enzyme greatly prefers pyrimidines in this position on the RNA chain. The cyclic phosphate is then hydrolyzed to give a terminal pyrimidine 3′-phosphate. The reactions of ribonuclease with dinucleosides and cytidine and uridine 2′,3′-cyclic phosphates have been studied (cf. refs *6* and *7*). The steady state turnover numbers are in the range of 2−3000 s^{-1}, and the Michaelis constants are 1−5 mM. Extensive transient studies of the enzyme using temperature jump and stopped-flow temperature jump methods have shown that the second-order rate constants for the binding of substrates approach the value expected for a diffusion-controlled reaction and that a conformational change accompanies the binding of substrates (*2*). The rate constants for the conformational change are $\sim 10^3$−10^4 s^{-1}, depending on the substrate and reaction conditions. The minimal reaction mechanism for the hydrolysis of a dinucleoside is shown in Figure 1. Multiple intermediates and conformations are clearly involved.

This mechanism can be related to the structure of the enzyme, which is shown in Figure 2. The molecule is kidney-shaped with a long N-terminal helix and shorter helices that are packed against a central β-pleated sheet. The overall structure is stabilized by four disulfide bonds, and large

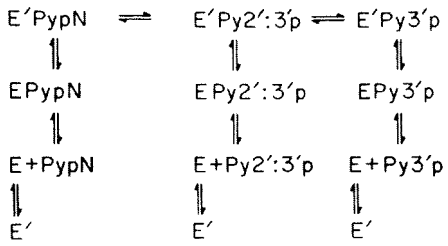

FIGURE 1: Schematic representation of the minimal reaction scheme for the hydrolysis of a dinucleoside by ribonuclease A. PypN is a pyrimidine dinucleoside; Py2′:3′p is a pyrimidine cyclic phosphate, and Py3′p is a pyrimidine 3′-phosphate. The primed enzyme species are different enzyme conformations. The specific rate constants have been determined in several instances (*7*).

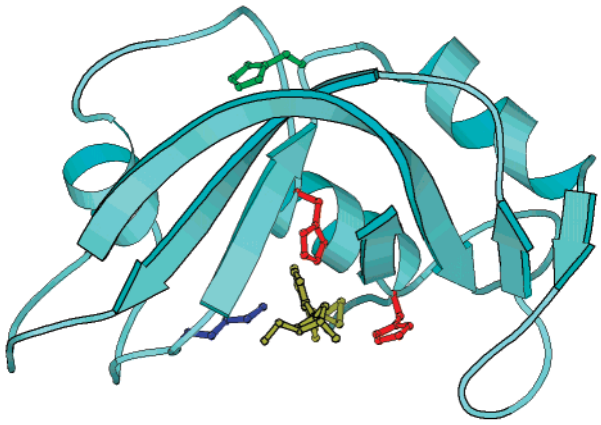

FIGURE 2: Schematic structure of ribonuclease A with deoxycytidyl 3′,5′-guanosine (yellow) bound to the active site (PDB entry 1RCA). The hinged structure is readily seen. The residues in red are histidines 12 and 119; the residue in blue is lysine 41, and the residue in green is histidine 48. These residues are discussed in the text.

solvent-exposed loops are present. A groove in the enzyme structure is the binding pocket for substrates, and very specific interactions occur between the enzyme and substrates, as would be expected. High-resolution structures of the enzyme and enzyme−substrate analogue structures indicate considerable flexibility exists around the active site, for example, histidine 119 and lysines 7, 41, and 66, but all of the structures have the same overall characteristics (cf. refs *8*−*10*). Structural studies of the enzyme and its complexes at low temperatures have led to the development of a structural model for the reaction (*11*). Conformational differences among the structures, if they exist, are apparently quite small.

Nevertheless, the kinetic measurements suggest that the enzyme exists in dynamic equilibrium between two forms that differ in the structure of the groove. The enzyme can be envisioned as a hinge that opens and closes, with the active site between the two halves of the hinge. The opening and closing has characteristic rate constants of ∼1000−3000 s^{-1}, and the equilibrium between the open and closed states is pH-dependent (*2*). When the substrate binds, the hinge closes, creating a hydrophobic environment. The reaction mechanism then proceeds through a series of proton transfer reactions involving histidine 12 and histidine 119 which stabilize the postulated pentacoordinated intermediate. The hinge opens to release the products, thus completing the enzymatic cycle. Lysine 41 appears to participate in the binding of substrates, and the enzyme is inactivated when it

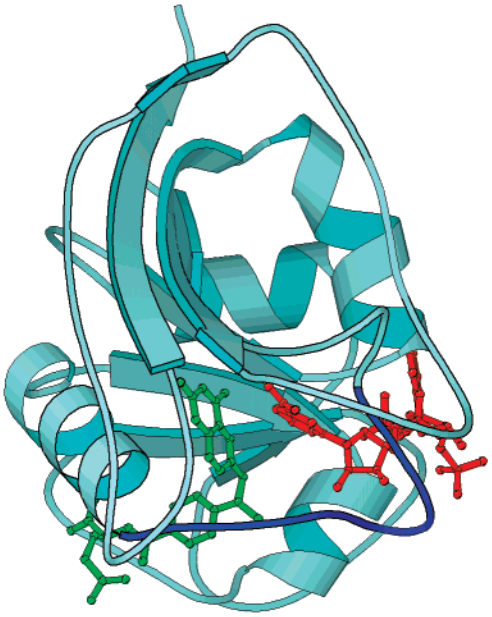

FIGURE 3: Schematic structure of dihydrofolate reductase with methotrexate (green) and NADPH (red) bound (PDB entry 1RH3). This structure is postulated to be a model for the transition state. The loop (residues 14−25) that closes over the active site is shown in blue.

is dinitrophenylated. If tyrosines that are not near the active site are iodinated, the catalytic properties of the enzyme are altered (*12*). Also, the p*K* of histidine 48, which is not located at the active site, is altered as the hinge opens and closes, and the protonation state of this group influences the rate of the conformational change. This is because it is located at the base of the hinge so that its environment is altered by the conformational change. The above results indicate the importance of the integrity of the entire macromolecule and of communication within the macromolecule, even relatively distant from the active site.

Dihydrofolate reductase catalyzes the reduction of 7,8-dihydrofolate by NADPH to form 5,6,7,8-tetrahydrofolate. Tetrahydrofolate is essential for the biosynthesis of purines, thymidylate, and several amino acids, so the enzyme has been the target of drug therapy for cancer. The structure of the enzyme from different sources has been determined (cf. ref *13*), and many steady state and transient kinetic studies have been carried out (cf. ref *14*). The structure of the *Escherichia coli* enzyme, 159 amino acids, is shown in Figure 3. Again the substrates are in pockets, and a flap (shown in dark blue) appears to close over the bound substrates.

A kinetic scheme has been proposed that accounts for all of the data and is reproduced in Figure 4 (*14*). The detailed mechanism is quite complex. In addition to the many intermediates that have been detected, the enzyme exists in two conformational states with an equilibrium constant of ~1 at pH 7.0. The mechanism, at neutral pH, follows a pathway in which tetrahydrofolate dissociation occurs after NADPH replaces NADP in the ternary complex. This dissociation, presumably coupled to a conformational change, is rate-limiting in the overall catalytic cycle. At high pH (>8.4), hydride transfer becomes rate-limiting. Conformational changes obviously play an important role in this mechanism. For example, closing of the flap over the active site has a rate constant of ~20 s^{-1} (*15*). Structural studies

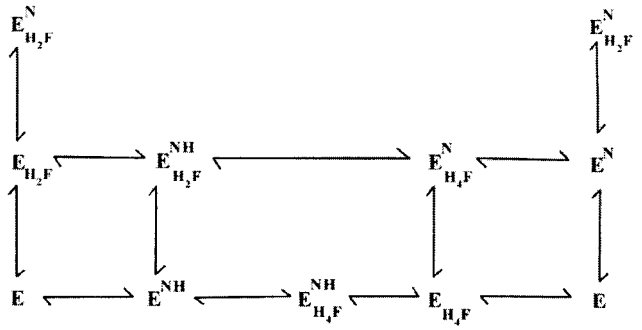

FIGURE 4: Complete kinetic scheme for the dihydrofolate reductase reaction (*14*). In this scheme, H_4F is tetrahydrofolate, H_2F is dihydrofolate, NH is NADPH, and N is $NADP^+$. In addition, the unliganded enzyme is known to exist in two different conformations. The specific rate constants have been determined for the native enzyme and mutated enzymes.

of the enzyme and five intermediates (Michaelis complex, ternary product complex, tetrahydrofolate binary complex, tetrahydrofolate−NADPH complex, and methotrexate−NADPH complex, a transition state model) have been used to assemble a structural movie of the reaction (*12*). Significant domain rotation and changes in interactions within structural loops occur as the reaction proceeds (*13*, *16*).

An extensive number of mutants have been well characterized with different rate constants for the individual steps in the catalytic cycle and different conformations stabilized along the reaction pathway (*16*, *17*). The rate constants of the individual unimolecular steps are in the range of 10−10^3 s^{-1}, and the individual bimolecular rate constants are in the range of 10^6−10^7 M^{-1} s^{-1}. Furthermore, a mutation far from the active site (Gly121Val) has been shown to alter significantly the kinetic and conformational properties of the enzyme (*17*). The backbone dynamics of this glycine residue also indicate large conformational fluctuations on the nanosecond time scale that are altered by mutation (*18*). Thus, dihydrofolate reductase clearly displays multiple intermediates and conformations, as well as communication between the active site and residues distant from the active site.

Chymotrypsin is the prototypic enzyme utilizing a covalent enzyme−substrate intermediate in its mechanism of action. It is an endopeptidase with a molecular weight of approximately 25 000 and has been intensively studied with various structural and kinetic tools (cf. refs *19*−*21*). Although much of the work was done many years ago, a complete understanding of the molecular mechanism of action still is being actively pursued. In brief, the enzyme catalyzes the hydrolysis of peptides and esters through the formation of an acyl intermediate covalently bound to a serine residue. In the case of most esters, hydrolysis of the acyl enzyme is rate-limiting so that the covalent intermediate can be isolated. In the case of amides and peptides, formation of the covalent intermediate is usually rate-limiting. A histidine residue at the active site is crucial for the reaction and acts as a general base catalyst in the mechanism. A study of the hydrolysis of an amide substrate using transient techniques revealed that the formation of the initial enzyme−substrate complex is followed by a conformational change, which in turn is followed by formation of the acyl enzyme which hydrolyzes to generate the free enzyme and product. Again, multiple intermediates are clearly present and are rapidly interconverted, with typical rate constants in the range of 0.1−100

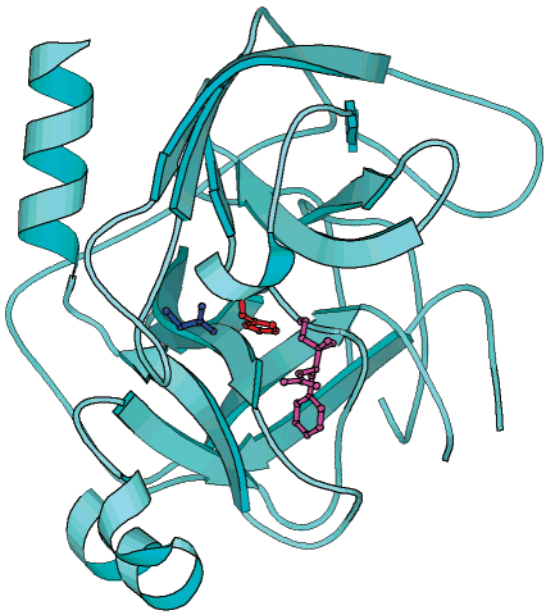

FIGURE 5: Schematic structure of γ-chymotrypsin with *N*-acetylphenylalanine trifluoromethyl ketone bound to serine 195 (red) at the active site (PDB entry 1GG6), a model for the transition state. Also shown are aspartate 102 (blue) and histidine 57 (red) at the catalytic site.

s^{-1}. The reaction of chymotrypsin with a substrate has also been studied at sub-zero temperatures in a water/dimethyl sulfoxide mixture (*22*). The formation of the acyl enzyme involves four steps, and the acylated enzyme is so stable that its breakdown could not be studied. In this case, some of the steps could involve solvation reactions, but again multiple intermediates are seen.

Structural studies of the enzyme reveal a well-defined pocket for the aromatic side chain of specific substrates, as shown in Figure 5. The pocket is lined with nonpolar side chains of amino acids and is very hydrophobic. Up to three amino acids coupled to the N-terminus of the aromatic amino acid interact with the enzyme. A hydrophobic environment is also present for the amino acid attached to the C-terminus, which explains why this is an endopeptidase. The serine−histidine−aspartate catalytic triad can be seen in the structure. Chymotrypsin, of course, is but one example of a serine protease, all of which have the same general structure, namely, two domains with the catalytic triad at the interface of the domains. The structures of many different forms of chymotrypsin, with various inhibitors bound, have been obtained (cf. refs *23* and *24*). A tetrahedral intermediate has been postulated to be an integral part of the mechanism, although it does not accumulate sufficiently for direct detection. However, structures of transition state analogues bound to chymotrypsin have revealed considerable detail about the interactions between the enzyme and substrate and the detailed chemical mechanism (cf. refs *23* and *24*). For the purposes of this discussion, the primary point to be noted is that multiple steps and conformations are involved. The binding site appears to be more complementary to the transition state than to the substrate.

A more modern example of a similar mechanism is protein tyrosine phosphatase, an important enzyme in signal transduction. This class of enzymes hydrolyzes phosphates that have been attached to protein tyrosines. In this case, a phosphorylated cysteine is the intermediate formed, and site specific mutagenesis played an important role in establishing the mechanism (cf. refs *25* and *26*). Structures of various forms of the enzyme also have been obtained. Both chymotrypsin and protein tyrosine phosphatase illustrate the importance of multiple intermediates and conformations in the catalytic process, and of the occurrence of a stable covalent intermediate.

$$\mathbf{Asp + E'_A \rightleftarrows E'_AAsp \rightleftarrows E_AAsp \rightleftarrows EAld \rightleftarrows}$$

$$\mathbf{EQui \rightleftarrows EKet \rightleftarrows E_NOa \rightleftarrows E'_NOa \rightleftarrows E'_N + Oa}$$

$$\mathbf{E'_N + Kg \rightleftarrows E'_NKg \rightleftarrows E_NKg \rightleftarrows EKet \rightleftarrows}$$

$$\mathbf{EQui \rightleftarrows EAld \rightleftarrows E_AGlu \rightleftarrows E'_AGlu \rightleftarrows E'_A + Glu}$$

FIGURE 6: Schematic mechanism for the transamination reaction catalyzed by aspartate transaminase, where Asp represents aspartate, Oa is oxalate, Glu is glutamate, Kg is ketoglutarate, E-Ald is the substrate−pyridoxal phosphate aldimine, E-Quin is the substrate−pyridoxal phosphate quinoid, E-Ket is the substrate−pyridoxal phosphate ketimine, E_A is the pyridoxal phosphate−enzyme (internal Schiff base), and E_N is the pyridoxamine phosphate−enzyme. The primed enzyme indicates the open conformation.

The final enzyme to be considered is aspartate aminotransferase. This enzyme catalyzes the transfer of an amino group from aspartate to ketoglutarate to give oxalacetate and glutamate. The enzyme utilizes enzyme-bound pyridoxal phosphate as a coenzyme, and the reaction proceeds by transferring the amino group from the amino acid to pyridoxal phosphate to give pyridoxamine phosphate. The amino group is transferred to the keto acid from the pyridoxamine in a second series of reactions. The enzyme from many different sources has been intensively studied with a variety of different kinetic, spectroscopic, and structural techniques (cf. refs *27* and *28*). This discussion will concern the mitochondrial enzyme, although the structures and mechanisms of action of all the enzymes are very similar.

Transient kinetics and spectroscopic measurements have shown that the reaction proceeds by formation of an aldimine between the amino acid and pyridoxal phosphate. The aldimine forms a quinoid structure that breaks down into a ketimine, which then decomposes into pyridoxamine and the ketoacid. The second half of the reaction follows the reverse of this process, with the pyridoxamine reacting with the ketoacid ultimately to give the pyridoxal−enzyme and amino acid. A temperature jump study of the reaction of the mitochondrial enzyme with the substrate analogue *β-erythro*-aspartic acid has permitted a detailed mechanism to be developed and determination of all of the associated rate constants, as well as the spectral properties of the intermediates (*29*). In brief, the initial formation of the enzyme−substrate complex is followed by a conformational change. The aldimine is then formed, followed by the quinoid and the ketimine. A conformational change then occurs, and the product is released. Thus, seven intermediates have been observed and characterized for half of the transamination reaction. The overall mechanism is shown schematically in Figure 6.

The structures of the enzyme from many different sources have been determined, as well as structures of the enzyme complexed with various inhibitors and substrates (cf. ref *28*). The enzyme is a homodimer with a subunit molecular weight

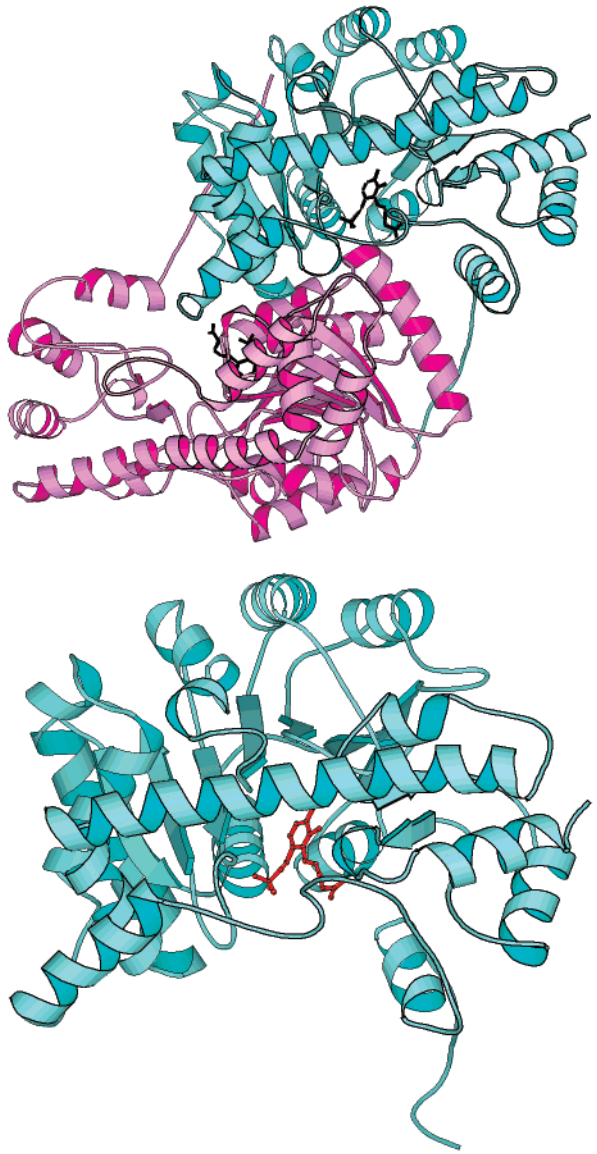

FIGURE 7: (Top) Structure of the aspartate aminotransferase with aspartate bound to pyridoxal phosphate (black). The two identical polypeptide chains are shown in cyan and magenta. The two identical catalytic sites are formed from two different polypeptide chains, and the two domains associated with each site can be readily seen. The open conformation is shown (PDB entry 1OXO). (Bottom) Closed conformation of one of the catalytic sites with aspartate bound to pyridoxal phosphate (red) at the catalytic site (PDB entry 1OXP).

of approximately 45 000. The structure, shown in Figure 7, consists of two domains, one of which is considerably larger than the other. The two active sites are formed at the interface of the dimer and the domains. The coenzyme resides at the bottom of the active site pocket and forms a Schiff base with lysine 258. Based on the structural work and kinetic studies, a mechanism has been proposed in terms of the structure (*30*). The mechanism parallels that outlined above, but now the interactions of the enzyme with the substrate can be described in detail. A significant conformational change occurs upon substrate binding; namely, the domains close around the substrate. The open conformation is shown in the top panel of Figure 7 and the closed conformation in the bottom panel of Figure 7. This closed conformation sequesters the substrate from the solvent, and the aldimine−quinoid−ketimine interconversions occur in the environment that has been created. Lysine 258 plays a crucial role as an acid−base catalyst in these reactions. The domains then open to permit dissociation of the product. The crystal structures of the mitochondrial enzyme with aspartate and glutamate bound have been determined and confirm this reaction sequence (*31*).

Thus, this coenzyme-utilizing enzyme follows the pattern of the other enzymes that have been discussed; namely, multiple intermediates and conformational changes are inferred by a variety of different methods. It is also worth noting that in the case of the *E. coli* enzyme, residues outside of the active site have been shown to modulate enzymatic activity (*32*).

Elementary Steps in Enzyme Catalysis

As we proceed with the discussion of the role of conformational changes in enzyme catalysis, it is worth considering the rates of individual reactions involved in conformational changes and enzyme catalysis. Of course, in principle all of the elementary reactions could occur within the time of single molecular vibrations, which would predict a maximum turnover number of $\sim 10^{12}$ s^{-1} for enzymes, but this is not a reasonable estimate of the maximum rates to be expected. Conformational transitions in proteins involve hydrogen bonding, water structure rearrangements (including "hydrophobic bonds"), and electrostatic interactions. The maximum rates of such reactions are well-known. In the case of hydrogen bonding, the formation of hydrogen-bonded dimers in nonaqueous solvents is diffusion-controlled (*33*). This implies that the rate of formation of the hydrogen bond after the reactants have diffused together is much faster than the rate at which the reaction partners can diffuse apart. This sets the rate constant for formation of a single hydrogen bond in the range of $10^{11}-10^{12}$ s^{-1}. Simple electrostatic interactions such as ion pair formation involve similar rate constants. However, these are not the relevant rate constants to discuss with regard to conformational changes involved in enzyme catalysis. Instead, the rates of cooperative, or "coordinated", conformational changes must be considered.

The breakdown of water structure around a simple polymer such as polyethylene glycol occurs with a specific rate constant of $\sim 10^{8}$ s^{-1} (*34*). This involves multiple water molecules and is one of the simplest "organized" processes that can be envisaged. More relevant rate constants to consider are those associated with simple helix coil transitions in polypeptides (cf. refs *33* and *35*−*37*) which lie in the range of $10^{7}-10^{8}$ s^{-1}. The rate constants for the formation and breakdown of small β-sheet structures are somewhat slower, $\sim 10^{6}$ s^{-1} (*38*). A related cooperative process involving noncovalent interactions, the stacking and unstacking of bases in nucleic acids, has rate constants in the range of $10^{6}-10^{7}$ s^{-1} (*39*). The reason that the rate constants for the cooperative formation and breakdown of these structures are significantly less than the rate constants for the elementary steps of hydrogen bond formation and breakdown is that nucleation or initiation of the reaction, in contrast to propagation, is rate-limiting. Thus, more complex cooperative conformational transitions in proteins are unlikely to occur on a time scale significantly shorter than microseconds. This is in accord with the turnover numbers and the rates of conformational changes that have been observed for enzymatic reactions (*2*).

The actual catalytic events involve chemical bonds breaking and forming. Even for enzyme catalysis, significant energy barriers are involved so that rates faster than microseconds are unlikely. For example, most enzymatic reactions utilize acid−base catalysis. The rate constant for the transfer of a single proton can be as fast as 10^{12} s^{-1}, but it must be remembered that enzyme catalysis involves returning the enzyme to its original protonation state. Thus, even if proton transfer occurs at its maximum rate in the forward reaction, this is unlikely to be true for the reverse reaction. Although conformational changes can alter p*K*s significantly, it is unlikely that acid−base catalysis can occur with a specific rate constant that is greater than $\sim10^6$ s^{-1} (*40*). If water with a normal p*K* is involved in the catalytic process, the maximum overall rate is $\sim10^3$ s^{-1} (*40*). This is in accord with current knowledge: with rare exception, enzymes have turnover numbers that are less than $\sim10^6$ s^{-1}.

The conclusion of this brief consideration of elementary reactions is that the rate of conformational transitions and overall catalytic rates are unlikely to exceed $\sim10^6$ s^{-1}. This is not an ironclad rigorous rule, but all experimental evidence points to the correctness of this working hypothesis.

Allosteric Enzymes

Allosteric enzymes are well-documented examples of the regulation of enzyme catalysis by events quite distant from the catalytic site. An extensive discussion of these interesting enzymes would stray too far from the central theme of this discourse, but a prototypic example, aspartate transcarbamoylase, is briefly considered.

Aspartate transcarbamoylase is a key enzyme in pyrimidine biosynthesis. It is subject to inhibition by CTP and to activation by ATP. The binding of aspartate to the enzyme displays the typical sigmoidal dependence of the extent of binding on substrate concentration. The binding of CTP makes the binding isotherm more sigmoidal, and the binding of ATP makes it less sigmoidal (*41*). Moreover, the binding isotherms of the regulatory ligands, CTP and ATP, display negative cooperativity. This enzyme has been extensively studied by a variety of different methods (cf. refs *42* and *43*), and its three-dimensional structure is known (*44*). The enzyme consists of two trimers of identical catalytic polypeptide chains (molecular weight of approximately 33 000/chain) held together by three dimers of identical polypeptide chains (molecular weight of approximately 17 000/chain), as shown in Figure 8. The binding of ATP or CTP causes the enzyme to shift between two distinct conformations of the enzyme, with ATP favoring the active conformation (R state) and CTP the inactive conformation (T state). The binding of aspartate also favors the active conformation. As seen in Figure 8, the trimers are closer together in the inactive state: the detailed differences between these two conformations have been documented (cf. ref *44*).

Kinetic studies of the enzyme, as well as many other physical measurements, suggest that this major conformational change can be described by the concerted model of Monod, Wyman, and Changeaux (*42*, *43*, *45*). The time scale for this conformational change is milliseconds. However, negative cooperativity cannot be accommodated by the Monod−Wyman−Changeaux model, and other conformational changes can be observed that involve many different

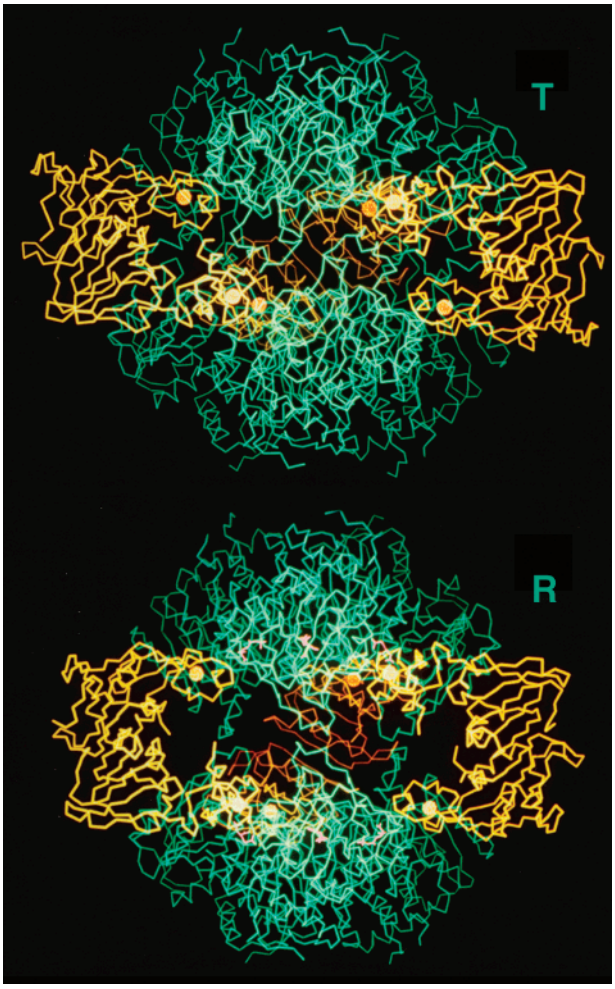

FIGURE 8: Backbone structures of the R (bottom) and T (top) states of aspartate transcarbamoylase. The two catalytic trimers, in green, are closer together in the T state. The three yellow regulatory dimers are at the interface of the catalytic trimers. Adapted from ref *44*.

parts of the molecule (*43*, *46*). Thus, the regulatory process involves multiple conformational changes, and it is the coupling between these many conformational changes, which involve the entire macromolecule, that regulates the enzyme activity. This multiplicity of conformational changes provides a versatile and exquisite mechanism for regulating the enzyme activity. The analogy to catalysis by less complex enzymes is apparent.

Role of Conformational Changes in Catalysis

The discussion above, which is only representative of a vast amount of data in the literature, establishes that protein conformational changes are the rule in enzyme mechanisms. It also sets limits for the rates of conformational changes, based on their associated elementary steps. Proteins are complex molecules, and the motions of individual residues and atoms have been observed by a variety of techniques, including magnetic resonance, fluorescence, and infrared spectroscopy. The time range of these motions is as short as picoseconds and nanoseconds. These motions occurring on very short time scales, less than a microsecond, reflect the integrity of the protein structure, and in fact are an integral part of the protein structure in solution. Moreover, the rates of these motions would be expected to change as the protein undergoes conformational changes. However, on the basis

of the rates observed for cooperative conformational changes and the rates of other elementary steps in enzyme catalysis, they are very unlikely to be *directly* coupled to the catalytic process. Instead, they are an indirect reflection of the conformation of the macromolecule. Conformational changes directly coupled to the catalytic event would be expected to occur on time scales longer than approximately a microsecond.

Still, even with the establishment of the time scale for conformational changes directly coupled to catalysis, we are left with the question of whether the protein plays a static or dynamic role in catalysis. A static role implies that the conformational changes prepare the catalytic groups on the enzyme for the next step. Included in this static picture would be increasing the effective concentration of the catalytic groups on the enzyme, restricting the configuration of the substrate, and the entropic advantage of having multiple catalytic groups assembled at the active site. Indeed, such effects are capable of explaining the tremendous catalytic efficiency of the enzyme, and undoubtedly play a significant role. Deliberately excluded from this list are descriptions such as "destabilization of the ground state" and "stabilization of the transition state". These terms are useful as a language in discussing enzymatic reactions, but do not provide a detailed molecular explanation of what occurs. Indeed, thermodynamics requires that the transition state be more tightly bound to the enzyme than the substrate, and the equilibrium between the enzyme−substrate complex and the transition state obviously is a reflection of the catalytic process. However, as is well-known, molecular descriptions are not an intrinsic part of thermodynamics.

An alternative way to view enzyme catalysis is that when a substrate binds to the enzyme, it becomes an integral part of the macromolecule. The subsequent dynamics of the macromolecular conformational changes are then the catalytic process itself. This view of catalysis means that the making and breaking of noncovalent bonds within the structure are part of the catalytic process, and that these events can occur both close to and far from the catalytic site. The advantage of having hundreds of intramolecular interactions dynamically involved in catalysis is that the energetics of the reaction can be easily manipulated to produce catalysis and extremely fine tuning is provided by the hundreds of intramolecular interactions. This mechanism could be viewed as a "gear shift" mechanism: the conformational transitions are analogous to shifting gears, and the interactions between the enzyme and substrate correspond to the gear coupling mechanism. Asking what "drives" the reaction is not terribly meaningful, as the essence of cooperative processes is that many events are occurring essentially simultaneously.

Although the circumstantial evidence supporting the coupling of conformational changes and enzyme catalysis is persuasive, in legal circles this would be viewed as a preponderance of the evidence, rather than as beyond a reasonable doubt. Experiments that bear most directly on this question are the effects of mutations on the dynamics of catalysis and enzyme structure since areas both near and far from the catalytic site can be probed (cf. refs *16*−*18*). The study of single-molecule enzyme kinetics offers the possibility of more direct observation of the coupling of enzyme catalysis and conformational changes (cf. ref *47*). Nuclear magnetic resonance measurements of relaxation times in the microsecond and millisecond time regime also can provide important information about the relationship between catalysis and conformational fluctuations (cf. ref *48*). Finally, developments in theory are providing new insight into enzyme catalysis, especially when coupled directly to experimental observations such as the effects of mutagenesis on catalysis (cf. ref *49*). For example, fresh insight into the mechanism of dihydrofolate reductase has been provided by recent theoretical calculations (*49*). Although current computer capabilities only permit relatively short time trajectories to be calculated, typically tens of nanoseconds or less, complete free energy profiles can be calculated for complex reactions. The time could not be more propitious for further development of an atomic level understanding of enzymatic reactions.

REFERENCES

1. Koshland, D. E., Jr. (1960) *Adv. Enzymol. 22*, 45−97.
2. Hammes, G. G. (1982) *Enzyme Catalysis and Regulation*, Academic Press, New York.
3. Lumry, R. (1959) in *The Enzymes* (Boyer, P., Ed.) 2nd ed., Vol. 1, p 157, Academic Press, New York.
4. Hammes, G. G. (1964) *Nature 204*, 342−343.
5. Richards, F. M., and Wyckoff, H. W. (1971) in *The Enzymes* (Boyer, P., Ed.) 3rd ed., Vol. 4, pp 647−806, Academic Press, New York.
6. Witzel, H. (1963) *Prog. Nucleic Acid Res. Mol. Biol. 2*, 221−258.
7. Hammes, G. G. (1968) *Adv. Protein Chem. 23*, 1−57.
8. Campbell, R. L., and Petsko, G. A. (1987) *Biochemistry 26*, 8579−8584.
9. Borkakoti, N. (1983) *Eur. J. Biochem. 132*, 89−94.
10. Gilliland, G. L. (1997) in *Ribonucleases: Structure and Function* (D'Alessio, G., and Riordan, J., Eds.) pp 305−341, Academic Press, New York.
11. Douzou, P., and Petsko, G. A. (1984) *Adv. Protein Chem. 36*, 245−361.
12. Hammes, G. G., and Walz, F. G., Jr. (1970) *Biochim. Biophys. Acta 198*, 604−606.
13. Sawaya, M. R., and Kraut, J. (1997) *Biochemistry 36*, 586−603.
14. Fierke, C. A., Johnson, K. A., and Benkovic, S. J. (1987) *Biochemistry 26*, 4085−4092.
15. Kitahara, R., Sareth, S., Yamada, H., Ohamae, E., Gekko, K., and Akasaka, K. (2000) *Biochemistry 39*, 12789−12795.
16. Miller, G. P., and Benkovic, S. J. (1998) *Biochemistry 37*, 6336−6342.
17. Cameron, C., and Benkovic, S. J. (1997) *Biochemistry 36*, 15792−15800.
18. Epstein, D. M., Benkovic, S. J., and Wright, P. E. (1995) *Biochemistry 34*, 11037−11048.
19. Hess, G. P. (1970) in *The Enzymes* (Boyer, P., Ed.) 3rd ed., Vol. 3, p 213−248, Academic Press, New York.
20. Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145−152.
21. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, W. H. Freeman, San Francisco.
22. Fink, A. L. (1973) *Biochemistry 12*, 1737−1742.
23. Brady, K., Wei, A., Ringe, D., and Ables, R. H. (1990) *Biochemistry 29*, 7600−7607.
24. Neidhart, D., Wei, Y., Cassidy, C., Lin, J., Cleland, W. W., and Frey, P. A. (2001) *Biochemistry 40*, 2439−2447.
25. Lohse, D. L., Denu, J. M., Santoro, N., and Dixon, J. E. (1997) *Biochemistry 36*, 4568−4575.
26. Hammes, G. G. (2000) *Thermodynamics and Kinetics for the Biological Sciences*, Wiley-Interscience, New York.
27. Christen, P., and Metzler, D. F., Eds. (1985) *Transaminases*, John Wiley and Sons, New York.
28. Jansonius, J. N., and Vincent, M. G. (1984) in *Biological Macromolecules and Assemblies* (Jurnak, F. A., and McPherson, A., Eds.) Vol. 3, pp 187−285, John Wiley and Sons, New York.
29. Hammes, G. G., and Haslam, J. L. (1969) *Biochemistry 8*, 1591−1598.

30. Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., and Christen, P. (1984) *J. Mol. Biol. 174*, 497−525.
31. Malashkevich, V. N., Toney, M. D., and Jansonius, J. N. (1993) *Biochemistry 32*, 13451−13462.
32. Gloss, L. M., Spencer, D. E., and Kirsch, J. F. (1996) *Proteins: Struct., Funct., Genet. 24*, 195−208.
33. Hammes, G. G. (1968) *Adv. Protein Chem. 23*, 1−57.
34. Hammes, G. G., and Lewis, T. B. (1966) *J. Phys. Chem. 70*, 1610−1614.
35. Hammes, G. G., and Roberts, P. B. (1969) *J. Am. Chem. Soc. 91*, 1812−1817.
36. Williams, K., Causgrove, T. P., Glilmanshen, R., Fang, K. S., Callendar, R. H., Woodruff, W. H., and Dyer, R. B. (1996) *Biochemistry 35*, 691−697.
37. Thompson, P. A., Eaton, W. A., and Hofrichter, J. (1997) *Biochemistry 36*, 9200−9210.
38. Munoz, V., Thompson, P. A., Hofrichter, J., and Eaton W. A. (1997) *Nature 390*, 196−199.
39. Dewey, T. G., and Turner, D. H. (1979) *Biochemistry 18*, 5757−5762.
40. Eigen, M., and Hammes, G. G. (1963) *Adv. Enzymol. 25*, 1−38.
41. Gerhart, J. C., and Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891−896.
42. Howlett, G. J., Blackburn, M. N., Compton, J. G., and Schachman, H. K. (1977) *Biochemistry 16*, 5091−5100.
43. Hammes, G. G., and Wu, C.-W. (1974) *Annu. Rev. Biophys. Bioeng. 3*, 1−33.
44. Libscomb, W. N. (1994) *Adv. Enzymol. 68*, 67−152.
45. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88−118.
46. Sakash, J. B., and Kantrowitz, E. R. (2000) *J. Biol. Chem. 275*, 28701−28707.
47. Xie, X. S., and Lu, H. P. (1999) *J. Biol. Chem. 274*, 15967−15970.
48. Eisenmesser, E. Z., Boco, D. A., Akke, M., and Kern, D. (2002) *Science 295*, 1520−1523.
49. Agarwal, P. K., Billeter, S. R., Rajagopalan, R., Benkovic, S. J., and Hammes-Schiffer, S. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 2794−2799.

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