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Understanding the Rates of Certain Enzyme-Catalyzed Reactions: Proton Abstraction from Carbon Acids, Acyl-Transfer Reactions, and Displacement Reactions of Phosphodiesters[†,‡]

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ABSTRACT: The structural factors responsible for the rapid rates (k_{cat}s) of enzyme-catalyzed reactions are not well understood. In this paper, we outline an analysis that we believe can provide a *quantitative* understanding of the k_{cat}s of three types of reactions: abstraction of the α-protons from carbon acids, acyl-transfer reactions, and displacement reactions of phosphodiesters. We propose that these reactions proceed *via* the formation of intermediates in which negative charge develops on the carbonyl or phosphoryl oxygens. Our analysis is based on Marcus formalism that separates the activation energy barrier for conversion of bound substrate to the intermediate, $\Delta G^{\ddagger}$, into contributions from a thermodynamic barrier, ΔG°, and an intrinsic kinetic barrier, $\Delta G^{\ddagger}_{int}$. We propose that one (or more) general acid catalyst positioned adjacent to the carbonyl or phosphoryl oxygens of the substrate is primarily responsible for reducing both ΔG° and $\Delta G^{\ddagger}_{int}$ from the values that characterize nonenzymatic reactions. The proton donors (1) stabilize the intermediates *via* the formation of short, strong hydrogen bonds (the pK_as of the protonated intermediates and the general acid catalysts are matched), thereby reducing ΔG°, and (2) stabilize the transition states for formation of the intermediates by negating the developing charge on the oxygens without the requirement for significant structural reorganization, thereby reducing $\Delta G^{\ddagger}_{int}$. The possible reductions in ΔG° and $\Delta G^{\ddagger}_{int}$ are sufficient to understand the rapid k_{cat}s of these reactions.

Enzymologists are both fascinated and perplexed by the mechanisms and rapid rates of enzyme-catalyzed reactions. Significant progress has been made in elucidating the chemical details of reaction mechanisms, including (1) the identity and sequence of bond-making and bond-breaking processes, (2) the spatial relationships between the bonds that are made and broken, and (3) covalent bond formation between the substrate and an active site functional group.

In contrast, the structural factors that are responsible for the rapid rates (in particular, the k_{cat}s) are less well understood (Jencks, 1975). Since both the identities and positions of active site functional groups must be known to understand the magnitudes of the k_{cat}s, progress understandably has been slow. However, high-resolution structures that reveal both the identities and positions of these groups are now available for several families of mechanistically related enzymes. Using

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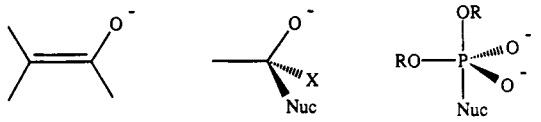
these structures, we believe that the k_{cat}s of some types of enzyme-catalyzed reactions now can be understood quantitatively.

In this paper we analyze the k_{cat}s of three types of reactions: (1) proton abstraction from carbon atoms adjacent to carbonyl and carboxylic acid groups (α-protons of carbon acids) (structurally characterized examples include mandelate racemase, triose-phosphate isomerase, citrate synthase, aconitase, glycolate oxidase, and thymidylate synthase), (2) acyltransfer reactions (structurally characterized examples include various proteases and esterases), and (3) displacement reactions of phosphodiesters (structurally characterized examples include metal ion-dependent and -independent DNases and RNases).

While these reactions may not appear initially to be mechanistically similar, we note that they do share a common property, the potential for formation of an intermediate in which (additional) negative charge develops on the carbonyl or phosphoryl oxygens: an enolate anion as the α-proton is abstracted (**1**), an anionic tetrahedral intermediate as a nucleophile adds to a carbonyl group (**2**), or a dianionic phosphorane as a nucleophile adds to a phosphoryl group (**3**).



1 **2** **3**

An important consequence of the increase in charge is that the anionic intermediate is significantly more basic than the substrate. In the absence of enzymes, **1**, **2**, and **3** are so unstable that the reactions in which they occur are extremely slow. We propose that the structural strategies by which active sites stabilize **1**, **2**, and **3** and lower the $\Delta G^{\ddagger}$s for their formation are identical.

A Quantitative Description of Reaction Profiles

The factors that contribute to the energy of the rate-limiting transition state for an enzyme-catalyzed reaction must be delineated if the magnitude of the k_{cat} is to be understood. We consider the energy of the transition state for formation of intermediates **1**, **2**, or **3** from bound substrate, since the k_{cat} can be no larger than the rate of formation of the intermediate.

A useful quantitative description of the energy profile of a reaction (free energy diagram) is provided by Marcus formalism (Cohen & Marcus, 1968; Marcus, 1969). Marcus formalism describes a reaction profile using the equation for an inverted parabola that specifies the free energy, G, at any point, x, on the reaction coordinate ranging from bound substrate ($x = 0$) to bound intermediate ($x = 1$):

$$G = -4\Delta G^{\ddagger}_{int}(x - 0.5)^2 + \Delta G^{\circ}(x - 0.5) \qquad (1)$$

where ΔG° and $\Delta G^{\ddagger}_{int}$ are independent parameters. Although the complete reaction profile is described by eq 1, only three points can be determined experimentally, the relative energies of the substrate and product (ΔG°) and of the transition state ($\Delta G^{\ddagger}$).

G reaches a maximum at the transition state. From eq 1

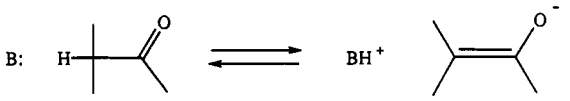
$$\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}(1 + \Delta G^{\circ}/4\Delta G^{\ddagger}_{int})^2 \qquad (2)$$

Equation 2 separates $\Delta G^{\ddagger}$ into the thermodynamic barrier, ΔG°, and a second energy that describes the difference between $\Delta G^{\ddagger}$ and ΔG° and is associated with $\Delta G^{\ddagger}_{int}$. From eq 2, $\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}$ when $\Delta G^{\circ} = 0$; for this reason, $\Delta G^{\ddagger}_{int}$ is termed the intrinsic kinetic barrier. If the magnitudes of $\Delta G^{\ddagger}_{int}$ differ, reactions that have identical ΔG°s occur at different rates.

From eq 2, $\Delta G^{\ddagger}$ for formation of **1**, **2**, or **3** is determined by both ΔG° and $\Delta G^{\ddagger}_{int}$. Since $\Delta G^{\ddagger}$ for an enzyme-catalyzed reaction is less than that for its nonenzymatic counterpart, we propose that the more rapid rate of an enzyme-catalyzed reaction can be understood by reductions in both ΔG° and $\Delta G^{\ddagger}_{int}$ from those that describe the nonenzymatic counterpart. Since $\Delta G^{\ddagger}$s for most enzyme-catalyzed reactions range from 13 to 17 kcal/mol (k_{cat}s range from 10^1 to 10^4 s^{-1}), the reductions in both ΔG° and $\Delta G^{\ddagger}_{int}$ often must be substantial. The functional groups in the active site must provide the interactions that allow these reductions to occur.

Proton Abstraction from Carbon Acids: The Difficulties in Forming Enolic Intermediates

Many enzyme-catalyzed reactions are initiated by abstraction of the α-proton of a carbon acid by a general base catalyst. The obvious kinetic problem confronted by enzymes is that the α-protons of carbon acids are not very acidic and the general base catalysts are not very basic, so ΔG° for general base catalyzed formation of **1** is too large to allow the observed k_{cat}s to be understood. The pK_as of the α-protons of aldehydes,



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ketones, and thioesters are 18–20, of carboxylic acids are 22–25, and of carboxylate anions are 29–32 (Kresge, 1991; Gerlt et al., 1991). The pK_as of the general base catalysts are usually $\leq$7 (Gerlt et al., 1991). If the pK_as of the α-proton and the basic catalyst differ by 25 pK_a units (e.g., 32–7), ΔG° = 35 kcal/mol, i.e., $\sim$20 kcal/mol greater than the observed $\Delta G^{\ddagger}$s for the enzyme-catalyzed reactions (13–17 kcal/mol). We conclude that active sites *must* be able to reduce ΔG° for proton abstraction from carbon acids.

A more subtle but real problem is that protons dissociate from carbon acids more slowly than from acids of equal pK_a in which the proton is bonded to a heteroatom (a normal acid; Kresge, 1975). In the Marcus formalism, the slower dissociation of a proton from a carbon acid is described by a larger $\Delta G^{\ddagger}_{int}$. The $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton of a carbon acid is $\sim$12 kcal/mol (Gerlt & Gassman, 1993); the $\Delta G^{\ddagger}_{int}$ for abstraction of the proton from a normal acid is $\leq$3 kcal/mol (Kresge, 1975). From eq 2, $\Delta G^{\ddagger}$ can be approximated by ΔG° for normal acids. For carbon acids, the larger $\Delta G^{\ddagger}_{int}$ requires that $\Delta G^{\ddagger}$ is larger than ΔG° and that the proton transfer rate is slower than that for a normal acid of equal acidity. However, a consequence of eq 2 is that the numerical contribution of $\Delta G^{\ddagger}_{int}$ to $\Delta G^{\ddagger}$ decreases as the formation of the intermediate from bound substrate becomes more endergonic.

Proton Abstraction from Carbon Acids: The Strategy for Reducing $\Delta G^{\ddagger}$

Triose-phosphate isomerase catalyzes the tautomerization of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; mandelate racemase catalyzes the racemization of the

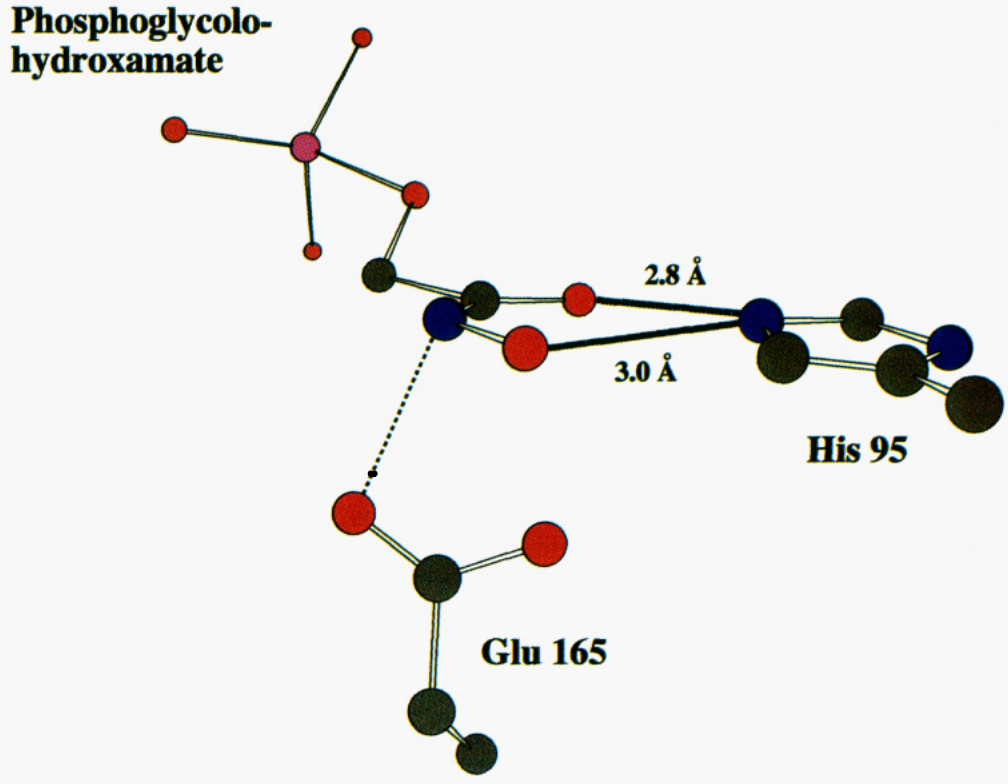


FIGURE 1: Active site of wild-type triose-phosphate isomerase complexed with the competitive inhibitor phosphoglycolohydroxamate (Davenport et al., 1991). The interaction of Glu 165 with the α-carbon of the substrate (the hydroxamate nitrogen of the inhibitor) is shown by (- - -); the hydrogen bonds between His 95 and the oxygens of the substrate (the oxygens of the inhibitor) are shown by (—).

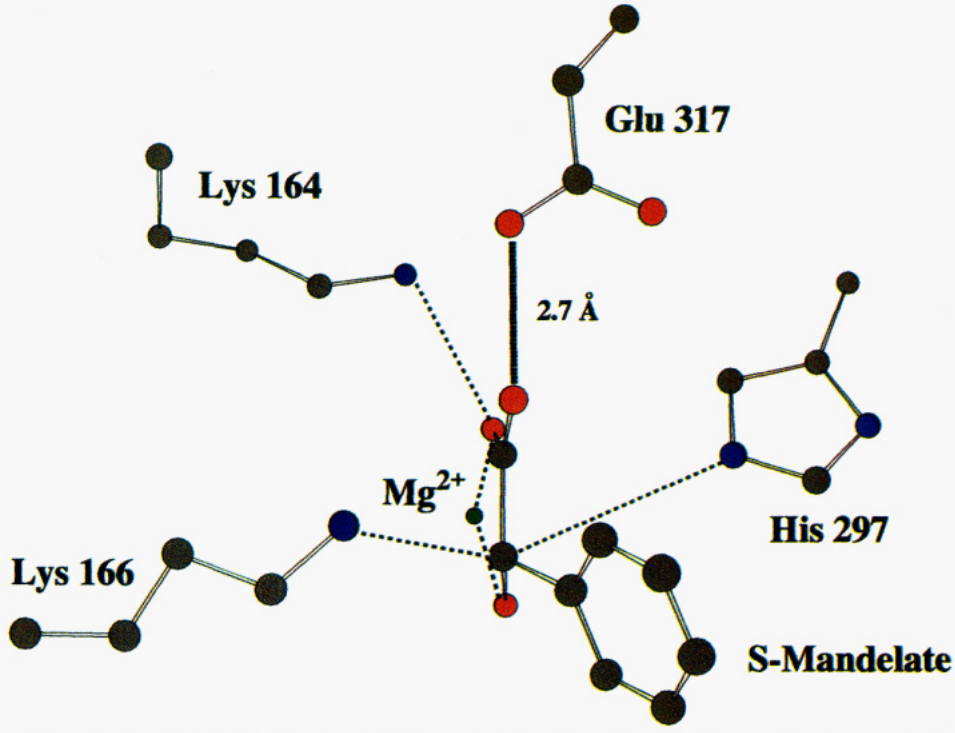
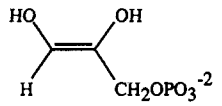


FIGURE 2: Active site of wild-type mandelate racemase with (*S*)-mandelate in the active site. The coordinates were derived from the structure of mandelate racemase inactivated by alkylation of the ϵ-amino group of Lys 166 with the affinity label (*R*)-α-phenylglycidate (Landro et al., 1993). The interactions of Lys 164, Lys 166, His 297, and Mg^{2+} with the substrate are shown by (- - -); the hydrogen bond between Glu 317 and the carboxylate oxygen of the substrate is shown by (—).

enantiomers of mandelate. In triose-phosphate isomerase, the general base catalyst is Glu 165 (Knowles, 1991; Figure 1). In mandelate racemase, two general base catalysts are present, Lys 166 and His 297 (Gerlt et al., 1992; Figure 2). Both reactions are initiated by proton abstraction by a general base catalyst to form an enolic intermediate (*cis*-enediol **4** for triose-phosphate isomerase and *gem*-enediol **5** for mandelate racemase). Protonation of the intermediate generates the product. The evidence for formation of an intermediate is the exchange of a substrate proton with a solvent proton during the reaction (triose-phosphate isomerase, Fisher et al., 1976; mandelate racemase, Landro et al., 1991).



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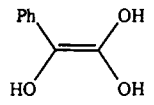

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Our proposal for understanding the k_{cat}s of these reactions is based on the realization that the active sites also contain a general acid catalyst *adjacent to the carbonyl group of the substrate*. Accordingly, the general acid catalyst transfers a proton *toward* the carbonyl group as the α-proton is abstracted by the general base catalyst. In triose-phosphate isomerase, the general acid catalyst is the *neutral* imidazole of His 95 (Nickbarg et al., 1988; Komives et al., 1991). In mandelate racemase, the general acid catalyst is the carboxylic *acid* of Glu 317 (Mitra et al., 1993). *We propose that the general acid catalyst is responsible for reducing both* $\Delta G°$ and $\Delta G^{\ddagger}_{int}$.

The Reduction in $\Delta G°$. In triose-phosphate isomerase and mandelate racemase, the pK_as of the general acid catalysts are closely matched to the pK_as of the neutral enol intermediates (**4** and **5**) that would be formed if abstraction of the α-proton were accompanied by complete proton transfer to the carbonyl group. Both X-ray (Davenport et al., 1991) and NMR (Lodi & Knowles, 1991) studies indicate that His 95 in triose-phosphate isomerase is present as the neutral imidazole; the NMR studies further suggest that the pK_a of the neutral imidazole to yield the imidazolate anion is ~11. Although the pK_a of **4** has not been measured, it should be similar to those of the enols of simple aldehydes and ketones, ~10–11 (Kresge, 1991). X-ray studies indicate that Glu 317 in mandelate racemase is present as the carboxylic acid (Landro et al., 1993); although the pK_a of this group is not yet known, it is likely to be ~6. The pK_a of **5** is 6.6 (Chiang et al., 1990). The pK_a of the general acid catalyst is also matched to the pK_a of the neutral enolic intermediate in other enzymes of this reaction type (Gerlt & Gassman, 1993).

We hypothesize that the matched pK_as of the general acid catalyst and the neutral intermediate permit $\Delta G°$ to be reduced by formation of a type of hydrogen bond that is unfamiliar to many biochemists (Hibbert & Emsley, 1990). In the gas phase, crystals, and nonaqueous solvents, a very strong hydrogen bond (a "short, strong hydrogen bond") is formed when a proton is shared by bases whose conjugate acids have equal pK_as. These hydrogen bonds are as short as 2.45 Å as compared to >2.60 Å for "normal" hydrogen bonds. Their strengths can be *≥20 kcal/mol* in contrast to those of normal hydrogen bonds (≤2 kcal/mol) that are formed in aqueous solution (Stahl & Jencks, 1986). Cleland (1992) recently discussed the importance of these hydrogen bonds in kinetic isotope effect studies.

Enzymologists apparently have overlooked the importance of short, strong hydrogen bonds for the reason that they are not formed in bulk aqueous solvent. However, active sites are not located in bulk solvent. Instead, active sites are frequently separated from bulk solvent by a flap that closes when the substrate binds. Such flaps are present in both triose phosphate isomerase and mandelate racemase. Within the sequestered active sites, *ordered* water molecules are observed. Crystallographic studies (M. Kreevoy, personal communication) indicate that ordered water molecules (in contrast to disordered water molecules in bulk solvent) do not disrupt short, strong hydrogen bonds to which they are hydrogen bonded (*via* normal hydrogen bonds). Thus, we conclude that active sites provide a suitable environment for the formation of short, strong hydrogen bonds.

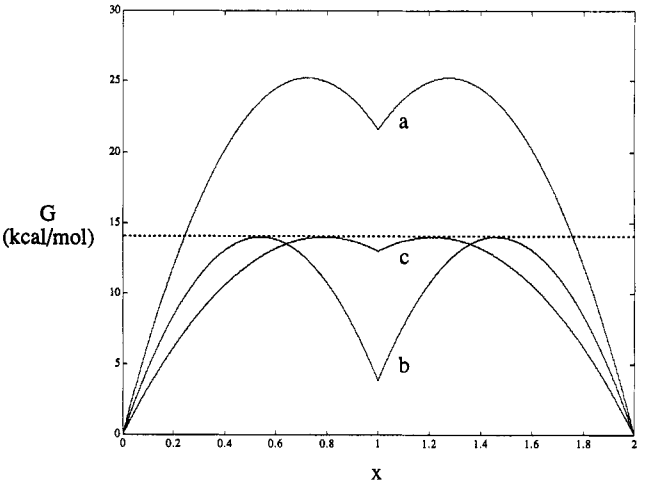


FIGURE 3: Reaction profiles illustrating the effects of reductions in $\Delta G°$ and $\Delta G^{\ddagger}_{int}$. The value of G is normalized so that $G = 0$ when $x = 0$. This normalization is accomplished by adding the G when $x = 0$ ($\Delta G^{\ddagger}_{int} + \Delta G°/2$) to the G calculated with eq 1. The bound substrate enantiomer is at $x = 0$, the enolic intermediate is at $x = 1$, and the bound product enantiomer is at $x = 2$. The reaction profiles were calculated using the following values for $\Delta G°$ and $\Delta G^{\ddagger}_{int}$: profile a, $\Delta G° = 22.4$ kcal/mol, $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, and $\Delta G^{\ddagger} = 25.8$ kcal/mol; profile b, $\Delta G° = 3.8$ kcal/mol, $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, and $\Delta G^{\ddagger} = 14.0$ kcal/mol; profile c, $\Delta G° = 13$ kcal/mol, $\Delta G^{\ddagger}_{int} = 5.6$ kcal/mol, and $\Delta G^{\ddagger} = 14.0$ kcal/mol.

Since the pK_a of the conjugate acid of the substrate (the protonated carbonyl group; ≤–4) is much less than that of the neutral enol intermediate (6–10), the strength of the hydrogen bond between the substrate and the general acid catalyst (a normal hydrogen bond: ≤2 kcal/mol) will be much less than the strength of the hydrogen bond between the intermediate and the general acid catalyst (a short, strong hydrogen bond: >20 kcal/mol). While interactions involving other functional groups in the active site and the substrate and the intermediate also may contribute to differential stabilization of the intermediate, we propose that formation of at least one short, strong hydrogen bond between the intermediate and the general acid catalyst dominates the stabilization of the intermediate, thereby reducing $\Delta G°$ sufficiently to allow the k_{cat} for the reaction to be understood.

We propose that the formation of a short, strong hydrogen bond between an otherwise too unstable intermediate and a general acid catalyst is a *general strategy for reducing* $\Delta G°$.

The Reduction in $\Delta G^{\ddagger}_{int}$. Physical–organic chemists have studied why protons dissociate from carbon acids more slowly than they dissociate from normal acids of equal pK_a. The consensus is that changes in solvation and hybridization that occur as the carbon acid is converted to the enolate anion are responsible for the large $\Delta G^{\ddagger}_{int}$ (Bernasconi, 1992). While the carbon acid must undergo the same changes in hybridization in an enzyme-catalyzed reaction as in its nonenzymatic counterpart, we hypothesize that the active site can decrease the contribution of solvation to $\Delta G^{\ddagger}_{int}$.

As the α-proton is abstracted, negative charge develops on the carbonyl oxygen. The contribution of solvation to $\Delta G^{\ddagger}_{int}$ is thought to result from changes in the orientations of solvent dipoles as the charge develops on the oxygen so that $\Delta G^{\ddagger}_{int}$ is dominated by a negative entropic contribution associated with solvent ordering. Since $\Delta G^{\ddagger}_{int} = \Delta H^{\ddagger}_{int} - T\Delta S^{\ddagger}_{int}$, $\Delta G^{\ddagger}_{int}$ can be reduced if $\Delta S^{\ddagger}_{int}$ can be increased toward 0. In nonenzymatic reactions, $\Delta G^{\ddagger}_{int}$ is reduced from 12 kcal/mol toward 3 kcal/mol, the value that describes proton dissociation from normal acids, by negating the charge that develops on

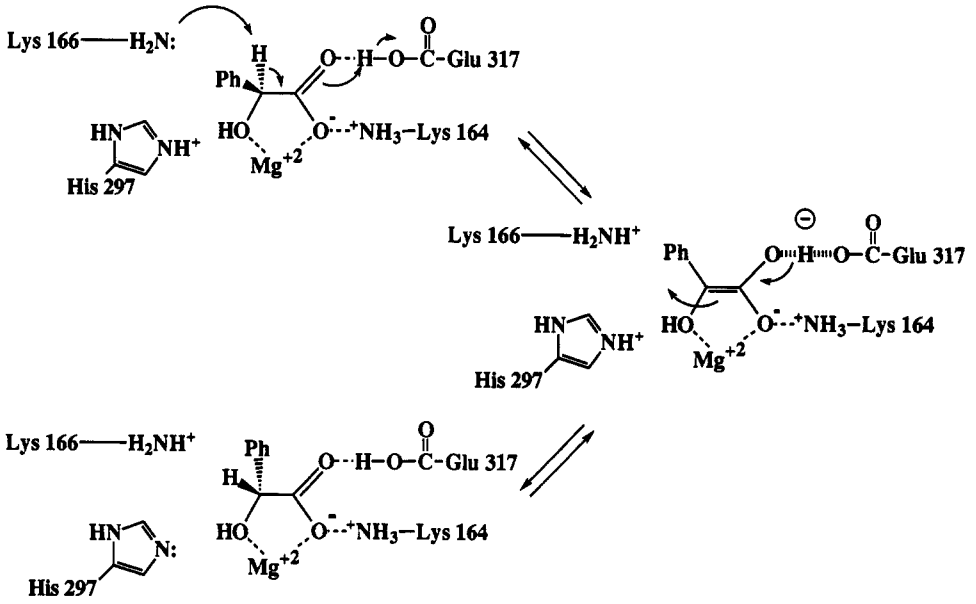


FIGURE 4: Mechanism of the reaction catalyzed by mandelate racemase with concerted general acid-general base catalyzed formation of an enolic intermediate stabilized by a short, strong hydrogen bond (hash marks) with Glu 317. The negative charge (⊖) is dispersed in the hydrogen bond and not localized on the heteroatoms or the bridging proton.

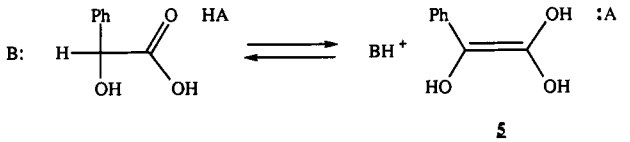
the carbonyl oxygen as the α-proton is abstracted (Gerlt & Gassman, 1993).

We propose that in active sites the general acid catalyst (positioned adjacent to the carbonyl group of the substrate) is also responsible for the reduction in $\Delta G^{\ddagger}_{int}$. The general acid catalyst is hydrogen bonded to the carbonyl group of the bound substrate. As negative charge develops on the carbonyl oxygen as the α-proton is abstracted, it can be stabilized by partial proton transfer from the general acid catalyst that does not require significant reorientation of the catalyst. Thus, when compared to the nonenzymatic reaction in disordered aqueous solution, the positioning of the general acid catalyst adjacent to the carbonyl group of the bound substrate in the active site should reduce $\Delta G^{\ddagger}_{int}$. Important consequences of reducing $\Delta G^{\ddagger}_{int}$ are (1) $\Delta G^{\ddagger}$ can be approximated by ΔG° and (2) the transition state for the reaction can resemble the stabilized intermediate.

We propose that the positioning of a general acid catalyst adjacent to the substrate is a *general strategy for reducing* $\Delta G^{\ddagger}_{int}$.

The k_{cat}s for Formation of Enolic Intermediates. We apply these proposals to understand the k_{cat} of the reaction catalyzed by mandelate racemase. The same conclusion is reached for other reactions, including that catalyzed by triose-phosphate isomerase (Gerlt & Gassman, 1993). The $\Delta G^{\ddagger}$ is 14 kcal/mol (k_{cat} = 700 s^{-1}; Landro et al., 1991). In the active site (Figure 2), one of the carboxylate oxygens of the substrate is coordinated to an essential Mg^{2+} and also hydrogen bonded to Lys 164. We propose that these interactions allow the substrate to react as if it were a carboxylic *acid* rather than a carboxylate *ion*.

Assuming concerted general acid-general base catalysis for formation of the neutral enol intermediate (**5**), the ΔG° can



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be calculated from that for the enolization of the mandelic acid, 21.6 kcal/mol (Chiang et al., 1990), and the pK_as of BH^+ and HA:

$$\Delta G^{\circ} = 21.6 \text{ kcal/mol} + (1.4 \text{ kcal/mol})[pK_a(HA) - pK_a(BH^+)]$$

where BH^+ is the conjugate acid of Lys 166 or His 297 (pK_as $\approx$ 6; Landro et al., 1991) and HA is Glu 317 (p$K_a \approx$ 6). With these values, ΔG° is 21.6 kcal/mol.

Since effective molarities are close to unity in general base catalyzed enolization reactions (Kirby, 1980), this calculated ΔG° need not be corrected for intramolecular catalysis (Jencks, 1975). From eq 2, if ΔG° = 21.6 kcal/mol and $\Delta G^{\ddagger}_{int}$ = 12 kcal/mol, $\Delta G^{\ddagger}$ = 25.2 kcal/mol for the formation of **5** (profile a in Figure 3). According to our proposal, the general acid catalyst, Glu 317, can reduce $\Delta G^{\ddagger}$ by 11.2 kcal/mol to achieve the observed k_{cat}.

The reduction in $\Delta G^{\ddagger}$ could be achieved by reducing ΔG° to 3.85 kcal/mol without any reduction in $\Delta G^{\ddagger}_{int}$ (profile b in Figure 3). This amount of stabilization of the intermediate relative to the substrate, 17.75 kcal/mol, is within the known strengths of short, strong hydrogen bonds (Hibbert & Emsley, 1990). However, we believe that the positioning of Glu 317 adjacent to the carboxylate group should reduce $\Delta G^{\ddagger}_{int}$. A more modest reduction in ΔG° from 21.6 kcal/mol to 13 kcal/mol and a partial reduction in $\Delta G^{\ddagger}_{int}$ from 12 kcal/mol to 5.6 kcal/mol will achieve the same reduction in $\Delta G^{\ddagger}$ (profile c in Figure 3). This reduction in $\Delta G^{\ddagger}_{int}$ almost eliminates its numerical contribution to $\Delta G^{\ddagger}$, so the transition state for formation of the enolic intermediate can resemble the intermediate (Gerlt & Gassman, 1993). At present, the concentration of the stabilized intermediate in the active site of mandelate racemase (or any enzyme of this reaction type) is unknown, so the actual amounts by which ΔG° and $\Delta G^{\ddagger}_{int}$ are reduced in active sites cannot be specified. Nevertheless, the amounts by which ΔG° and $\Delta G^{\ddagger}_{int}$ can be reduced are sufficient to account for the observed k_{cat}.

We propose that the mechanism of the reaction catalyzed by mandelate racemase involves concerted general acid-general base catalyzed formation of an enolic intermediate (Figure 4) that is facilitated by the formation of a short, strong

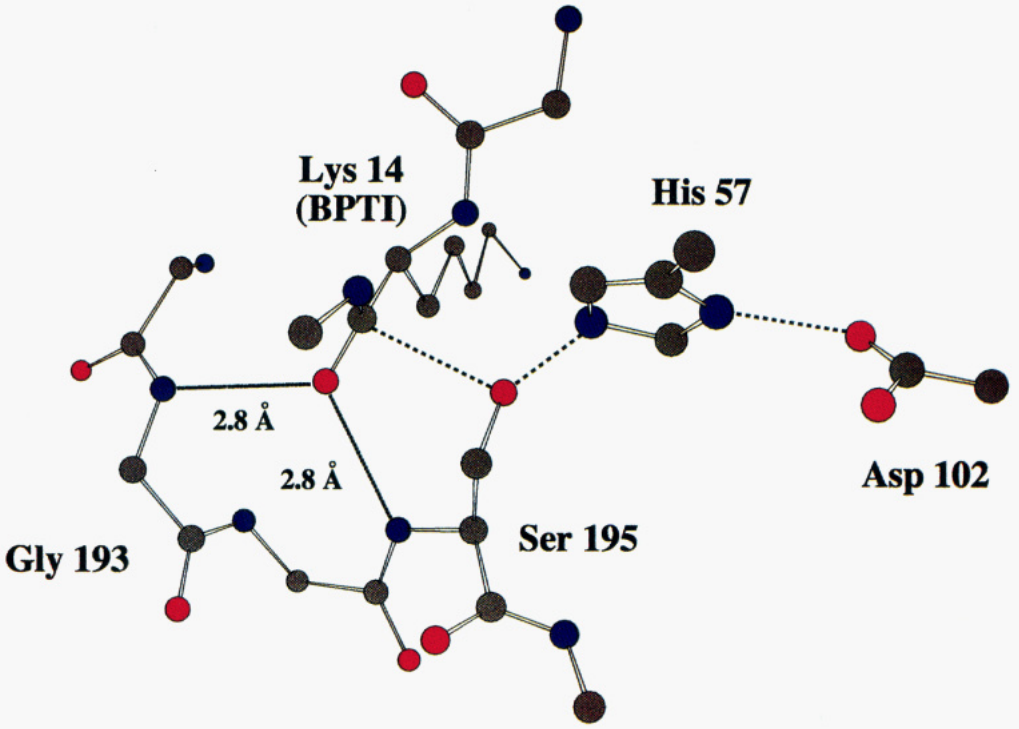


FIGURE 5: Active site of trypsin with BPTI bound in the active site (Marquart et al., 1983). The interactions among Asp 102, His 57, Ser 195, and the carbonyl group of Lys 14 of BPTI are shown by (- - -); the hydrogen bonds between the peptidic NHs of Gly 193 and Ser 195 and the carboxyl oxygen are shown by (—).

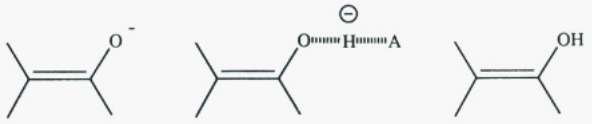
hydrogen bond (represented by hash marks). The mechanisms and k_{cat}s of reactions catalyzed by other enzymes catalyzing abstraction of the α-protons of carbon acids, including triose phosphate isomerase, can be similarly understood.

Implications of Our Proposals for Reducing $\Delta G^{\ddagger}$

We note several important implications of our proposals for reducing $\Delta G^{\ddagger}$:

(1) The essential general acid catalyst *does not and cannot* have a low pK_a. While this may seem counterintuitive, the matching of its pK_a to that of the weakly acidic neutral intermediate dominates the necessary reduction in ΔG°.

(2) The intermediate is neither an enolate anion (**1**) nor a neutral enol (**7**). Both of these species are too unstable. Instead, the intermediate is the strongly hydrogen bonded (stabilized) complex of the enol and the conjugate base of the active site general acid catalyst (or, equivalently, the enolate anion and the active site general acid catalyst), i.e., an enolic intermediate (**6**). We use "enolic intermediate" rather than "carbanion,"



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"enolate," or "enol" to describe the intermediate since "enolic intermediate" describes the structure and properties of the intermediate without any misleading mechanistic implications.

(3) Although our proposal for reducing $\Delta G^{\ddagger}$ focuses on reducing ΔG° and $\Delta G^{\ddagger}_{int}$, the active site must, as a corollary, reduce the pK_a of the α-proton in the transition state for proton abstraction. A reduction in this pK_a follows from the conclusion that the proton from the general acid catalyst is approximately half-transferred to the carbonyl oxygen in the transition state for formation of the enolic intermediate, since the transition state resembles the strongly hydrogen bonded enolic intermediate. Protonation of the carbonyl oxygen of a carbon acid reduces the pK_a of the α-proton by 15 pK_a units (Gerlt et al., 1991). If half-protonation of the carbonyl oxygen reduces the pK_a of the α-proton by $\sim$7.5 pK_a units and $\Delta G^{\ddagger}$ is approximated by ΔG°, the k_{cat}s for proton abstraction can be understood.

(4) The conversion of bound substrate to bound product is expected to be a stepwise rather than a concerted process. Our proposal for increasing k_{cat}s is based on the *observed* matching of the pK_as of the general acid catalyst and the neutral intermediate in several active sites. While a partial increase in charge on the carbonyl oxygen would occur in a nonsynchronous concerted reaction and this could allow stabilization of the transition state by hydrogen bonding, the available structural evidence suggests that a stepwise mechanism is the preferred strategy for reducing $\Delta G^{\ddagger}$. We previously have discussed the mechanisms of β-elimination reactions (Gerlt & Gassman, 1992, 1993).

(5) The most effective transition-state analog inhibitors will mimic not only the geometry but also the pK_a(s) of the neutral intermediate. The portion of the reduction in $\Delta G^{\ddagger}$ that is associated with $\Delta G^{\ddagger}_{int}$ cannot be realized in the binding of transition-state analogs since this contribution is a kinetic rather than a thermodynamic effect.

(6) Albery and Knowles (1976) predicted that the equilibrium constant for formation of the enolic intermediate from bound substrate is unity. However, we predict that this equilibrium constant will be less than unity (profile c in Figure 3). This prediction is based on (1) the matching of the pK_a of the general acid catalyst and the neutral intermediate and (2) the expected reduction in $\Delta G^{\ddagger}_{int}$ by the general acid catalyst.

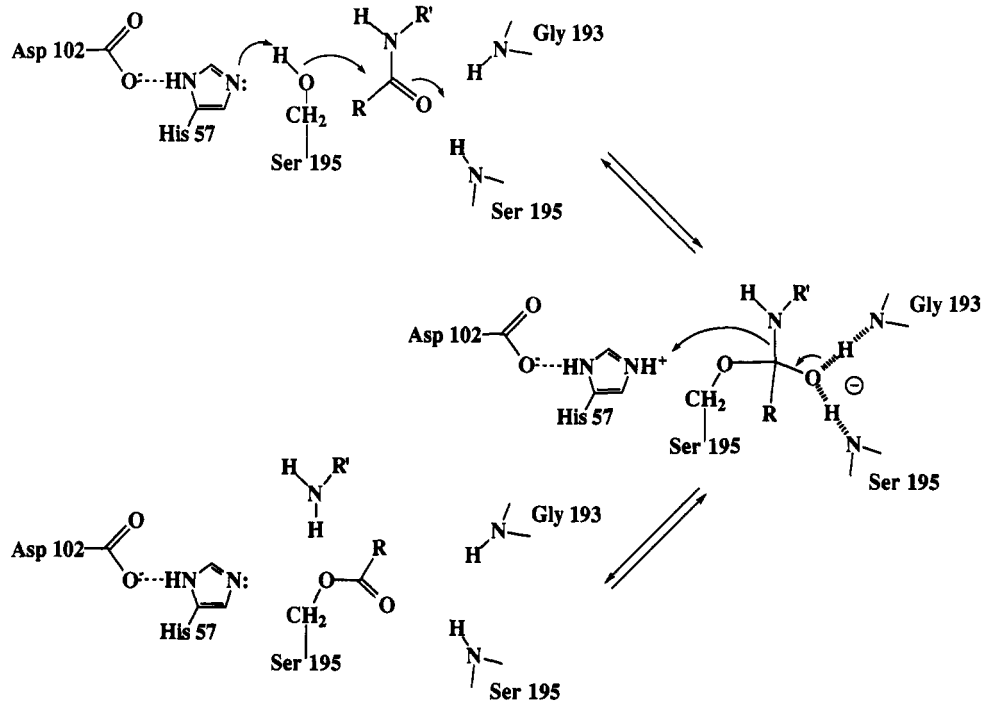
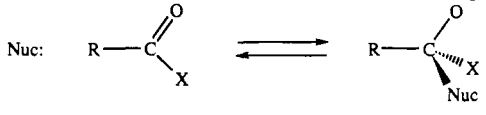


FIGURE 6: Mechanism of the reaction catalyzed by trypsin with concerted general acid-general base catalyzed formation of a tetrahedral intermediate stabilized by two short, strong hydrogen bonds (hash marks) with the peptidic NH groups of Gly 193 and Ser 195. The negative charge (⊖) is dispersed in the hydrogen bonds and not localized on the heteroatoms or the bridging protons.

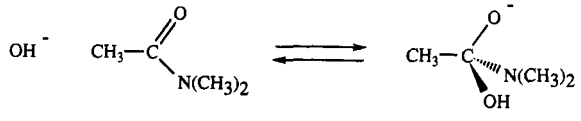
Acyl-Transfer Reactions: The Difficulties in Forming Tetrahedral Intermediates

We now apply our proposals to acyl-transfer reactions (e.g., peptide bond hydrolysis) in which a tetrahedral intermediate (**2**) is formed:



2

The $\Delta G°$ for the addition of hydroxide ion to *N,N*-dimethylacetamide (**8**) to form the anionic tetrahedral intermediate (**9**) is 18.8 kcal/mol (Guthrie, 1974). The $\Delta G^{\ddagger}_{int}$,



8 **9**

~12 kcal/mol, can be calculated from $\Delta G°$ and the observed $\Delta G^{\ddagger}$, ~24.6 kcal/mol (Guthrie, 1974). Guthrie (1983) has noted that the hydration of a variety of carbonyl compounds, including amides, to form tetrahedral intermediates can be described by a single value for $\Delta G^{\ddagger}_{int}$, 12.6 kcal/mol. These reactions, like those generating enolic intermediates, involve the development of negative charge on the carbonyl oxygen, so they also are intrinsically slow in aqueous solution.

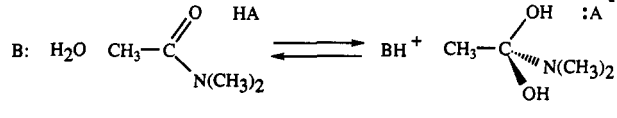
Acyl-Transfer Reactions: The Strategy for Reducing $\Delta G^{\ddagger}$

The active site of trypsin complexed with bovine pancreatic trypsin inhibitor (BPTI) is shown in Figure 5 (Marquart et al., 1983). The nucleophile, Ser 195, is juxtaposed to the carbonyl carbon of the scissile peptide bond (Lys 14 in BPTI). His 57, held in place by Asp 102, is the general base catalyst that assists the attack of Ser 195 on the substrate.

The carbonyl oxygen of the scissile peptide bond is hydrogen bonded to the peptidic NH groups of both Gly 193 and Ser 195. These interactions, often called the "oxyanion hole", have been used *qualitatively* to describe stabilization of the tetrahedral intermediate and, therefore, the transition state for its formation (Robertus et al., 1972).

However, we propose that the NH groups are general acid catalysts and that the reaction does not occur simply by general base catalyzed attack of the OH group of Ser 195 on the scissile amide bond but by *concerted* general acid-general base catalysis. In our mechanism partial transfer of protons from the NH groups toward the carbonyl oxygen occurs as the attack of the nucleophile is assisted by His 57. These proton transfers are thermodynamically feasible since a peptidic NH group is estimated to have a $pK_a \approx 15$ (Bordwell, 1988) and a neutral tetrahedral intermediate is estimated to have a $pK_a = 13.4$ (Guthrie, 1974).

Assuming concerted general acid-general base catalysis for formation of a neutral tetrahedral intermediate (**10**) in the active site of trypsin, the $\Delta G°$ can be calculated from that for



8 **10**

addition of hydroxide ion to **8** to form **9**, 18.8 kcal/mol, and the pK_as of **10** (13.4), H_2O, BH^+, and HA:

$$\Delta G° = 18.8 \text{ kcal/mol} = (1.4 \text{ kcal/mol})\{[pK_a(H_2O) - pK_a(BH^+)] + [(pK_a(HA) - pK_a(\mathbf{10})]\}$$

where BH^+ is the conjugate acid of His 57 ($pK_a \approx 7$) and HA

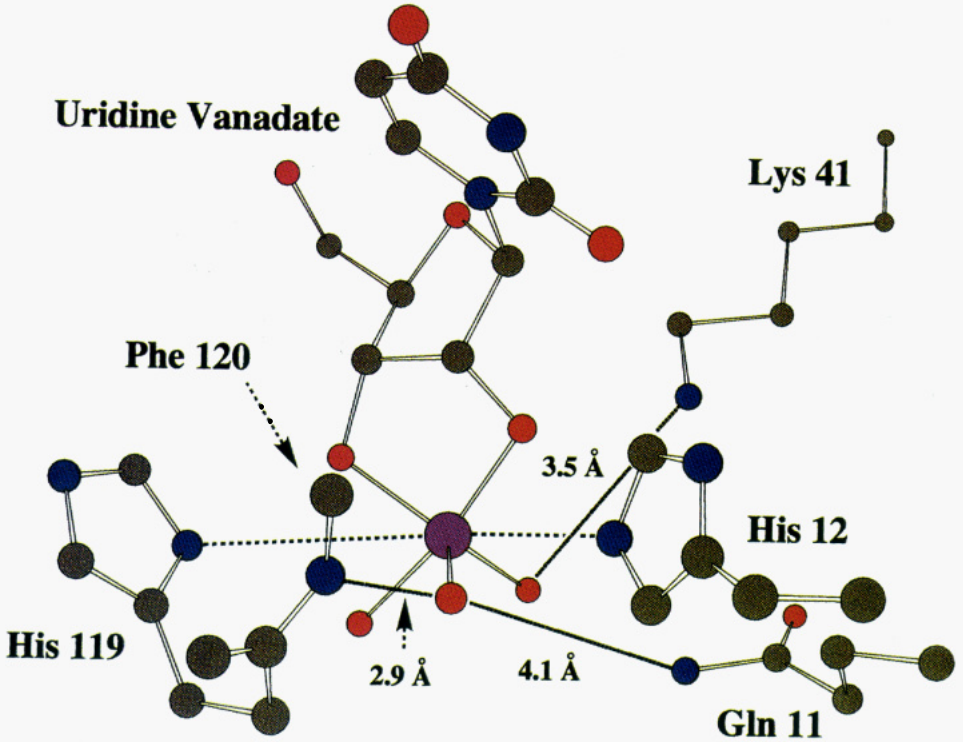


FIGURE 7: Active site of ribonuclease A with uridine vanadate bound in the active site (Borah et al., 1985). The interactions among His 12, His 119, and the vanadium oxygen of the transition state analog are shown by (- - -); the hydrogen bonds between Lys 41, the carboxamide of Gln 11, and the peptidic NH of Phe 120 and the vanadyl oxygens are shown by (—).

is a peptidic NH ($pK_a \approx 15$). With these values, $\Delta G° \approx 30.8$ kcal/mol.[1]

This value for $\Delta G°$ must be corrected for the entropic effects associated with intramolecular catalysis (Jencks, 1975; Kirby, 1980). Since effective molarities are as large as 10^8 for the equilibrium constants for nucleophilic addition reactions to carbonyl groups (Kirby, 1980), $\Delta G°$ can be reduced (by ≤11.2 kcal/mol) to ≥19.6 kcal/mol.

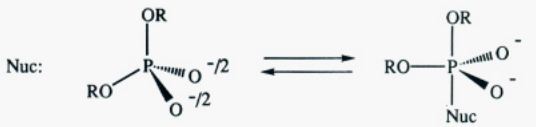
If $\Delta G° \geq 19.6$ kcal/mol and $\Delta G^{‡}_{int} = 12.6$ kcal/mol, $\Delta G^{‡} \geq 24.3$ kcal/mol for the formation of **10**. This value for $\Delta G^{‡}$ exceeds the value of 16 kcal/mol for the trypsin-catalyzed hydrolysis of an oligopeptide substrate ($k_{cat} = 40\ s^{-1}$; Corey et al., 1992).

In our proposed mechanism, the tetrahedral intermediate is stabilized by two short, strong hydrogen bonds in which the protons of the peptidic NH groups of Gly 193 and Ser 195 are *partially* transferred to the basic anionic tetrahedral intermediate (the pK_a of the neutral tetrahedral intermediate is similar to that of a peptidic NH group).[2] The positioning of the NH groups adjacent to the carbonyl oxygen also should reduce $\Delta G^{‡}_{int}$ (Jencks, 1975) and allow $\Delta G^{‡}$ to be approximated by $\Delta G°$. The amounts by which both $\Delta G°$ and $\Delta G^{‡}_{int}$ can be reduced are sufficient to account for the observed k_{cat}.

We propose that the mechanism of the reaction catalyzed by trypsin involves concerted general acid-general base catalyzed formation of a tetrahedral intermediate (Figure 6) that is facilitated by the formation of short, strong hydrogen bonds (represented by hash marks). The mechanisms and k_{cat}s of reactions catalyzed by other enzymes accomplishing acyl-transfer reactions can be similarly understood.

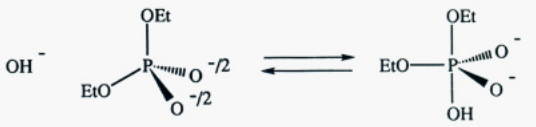
Displacement Reactions of Phosphodiesters: The Difficulties in Forming Phosphorane Intermediates

We finally apply our proposals to displacement reactions of phosphodiesters in which a phosphorane (**3**) can be formed:



3

The $\Delta G°$ for the addition of the hydroxide ion to the diethyl phosphate anion (**11**) to form the dianionic phosphorane (**12**) is 22.4 kcal/mol (Guthrie, 1977). The $\Delta G^{‡}_{int}$, ~21 kcal/mol, can be calculated from $\Delta G°$ and the observed $\Delta G^{‡}$, ~33 kcal/mol (Guthrie, 1977). Since similar $\Delta G^{‡}_{int}$s can be



11 **12**

calculated for the addition of water to diethyl phosphate anion, of hydroxide to triethyl phosphate, and of water to the triethyl phosphate (Guthrie, 1977), electrostatic repulsion between the nucleophile and the phosphate ester (Westheimer, 1987) apparently is not solely responsible for the low reactivities of phosphodiesters. Instead, these reactions also involve the development of negative charge on (two) phosphoryl oxygens, so they are intrinsically (very) slow in aqueous solution.

[1] The $\Delta G°$ for general base catalyzed formation of **9** is similar to this value since $pK_a(HA) \approx pK_a(\mathbf{10})$.

[2] Since the peptidic NH groups of both Gly 193 and Ser 195 are equivalently located for the formation of short, strong hydrogen bonds and are expected to have equivalent pK_as, we propose that two short, strong hydrogen bonds are formed. However, the necessary reduction in $\Delta G°$ could be accomplished by a single short, strong hydrogen bond.

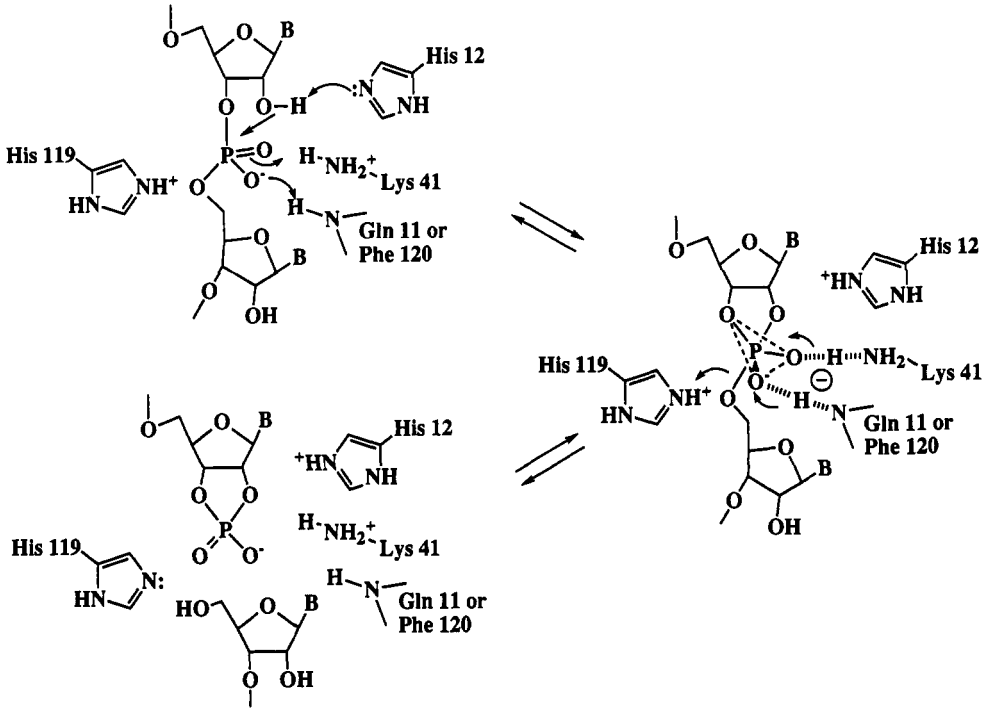


FIGURE 8: Mechanism of the reaction catalyzed by RNase A with concerted general acid-general base catalyzed formation of a phosphorane intermediate stabilized by two short, strong hydrogen bonds (hash marks) with Lys 41 and Gln 11 and/or the peptidic NH of Phe 120. The negative charge (⊖) is dispersed in the hydrogen bond and not localized on the heteroatoms or the bridging proton.

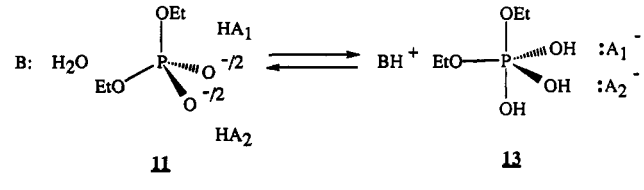
Displacement Reactions of Phosphodiesters: The Strategy for Reducing $\Delta G^{\ddagger}$

The active site of RNase A complexed with uridine vanadate is shown in Figure 7 (Borah et al., 1985). The general acid and base catalysts involved in proton transfer to and from leaving groups and nucleophiles, His 12 and His 119, are located on opposite sides of the transition-state analog.

Two vanadyl oxygens are close to Lys 41 *and* both the carboxamide group of Gln 11 and the peptidic NH of Phe 120. Lys 41 is often proposed as an electrostatic catalyst that stabilizes a pentacoordinate *transition state* in concerted displacement reactions.

However, we propose that Lys 41 and, perhaps, the carboxamide group of Gln 11 and/or the peptidic NH of Phe 120 function as general acid catalysts in concerted general acid-general base catalyzed formation of a phosphorane *intermediate*. These groups transfer protons toward the phosphoryl oxygens of the substrate as the attack of a nucleophile (the vicinal 2′-OH group or H_2O) is assisted by either His 12 or His 119. These proton transfers are thermodynamically feasible since the pK_a of Lys 41 is ~9 (Jentoft et al., 1981), those of the NH groups of Gln 11 and Phe 120 are estimated to be ~15 (Bordwell, 1988), and the first and second pK_as of a neutral phosphorane are estimated to be 7.2 and 12.0 (Guthrie, 1977).

Assuming concerted general acid-general base catalysis for formation of a neutral phosphorane (**13**) in the active site of RNase A, the ΔG° can be calculated from that for the addition



of hydroxide ion to **11** to form **12**, 22.4 kcal/mol, the first and second pK_as of **13**, and the pK_as of H_2O, BH^+, HA_1, and HA_2:

$$\Delta G^{\circ} = 22.4 \text{ kcal/mol} + (1.4 \text{ kcal/mol})\{[pK_a(H_2O) - pK_a(BH^+)] + [pK_a(HA_1) - pK_{a1}(\mathbf{13})] + [pK_a(HA_2) - pK_{a2}(\mathbf{13})]\}$$

where BH^+ is the conjugate acid of His 12 or His 119 ($pK_a \approx 7$), HA_1 is Lys 41 ($pK_a \approx 9$), and HA_2 is Gln 11 or Phe 120 ($pK_a \approx 15$). With these values, $\Delta G^{\circ} \approx 39.2$ kcal/mol.[3]

This value for ΔG° also must be corrected for the entropic effects associated with intramolecular catalysis (Jencks, 1975; Kirby, 1980). Since effective molarities are also as large as 10^8 for nucleophilic addition to phosphoryl groups (Kirby, 1980), ΔG° can be reduced (by ≤11.2 kcal/mol) to ≥28.0 kcal/mol.

If $\Delta G^{\circ} \geq 28$ kcal/mol and $\Delta G^{\ddagger}_{int} \approx 21$ kcal/mol, $\Delta G^{\ddagger} \geq 37.3$ kcal/mol for the formation of **13**. This value for $\Delta G^{\ddagger}$ exceeds the value of 14 kcal/mol for the RNase A catalyzed conversion of dinucleoside monophosphates to 2′,3′-cyclic nucleotides ($k_{cat} = 10^3$ s^{-1}; Richards & Wyckoff, 1971).

In our proposed mechanism, the phosphorane intermediate is stabilized by one or, perhaps, two short, strong hydrogen bonds that involve *partial* proton transfer to the basic anionic intermediate: the first involving Lys 41 and the second the NH group of Gln 11 or Phe 120.[4]

The available data suggest that a reduction in $\Delta G^{\ddagger}_{int}$ is possible only if the developing negative charge on *both* phosphoryl oxygens is stabilized by interaction with electrophilic catalysts (Guthrie, 1977). We further propose that the

[3] The ΔG° for general base catalyzed formation of **12** is similar to this value since $pK_a(HA_1) \approx pK_{a1}(\mathbf{13})$ and $pK_a(HA_2) \approx pK_{a2}(\mathbf{13})$.

[4] The pK_a of Lys 41 is nearly matched to that of the first pK_a of **13**. Although the pK_as of Gln 11 and Phe 120 are similar to the second pK_a of **13**, the matching may be less exact than those we described for the formation of enolic and tetrahedral intermediates. For this reason, the NH groups of Gln 11 and Phe 120 may be involved only in the reduction in $\Delta G^{\ddagger}_{int}$. The necessary reduction in ΔG° could be accomplished by a single short, strong hydrogen bond involving Lys 41.

positioning of the functional groups of Lys 41 *and* Gln 11 and/or Phe 120 adjacent to *both* phosphoryl oxygens is necessary and can reduce $\Delta G^{\ddagger}_{int}$ for formation of the phosphorane. The $\Delta G^{\ddagger}_{int}$ for the nonenzymatic reaction is very large, ~21 kcal/mol, so a reduction in its value can substantially decrease $\Delta G^{\ddagger}$ and may allow $\Delta G^{\ddagger}$ to be approximated by ΔG°. Taken together, the strength of at least one short, strong hydrogen bond between the phosphorane and the functional groups of Lys 41, Gln 11, and Phe 120 and a reduction in $\Delta G^{\ddagger}_{int}$ by the positioning of these groups adjacent to both phosphoryl oxygens are sufficient to account for the observed k_{cat}.

We propose that the mechanism of the reaction catalyzed by RNase A involves concerted general acid-general base catalyzed formation of a phosphorane (Figure 8) that is facilitated by the formation of short, strong hydrogen bonds (represented by hash marks). The mechanisms and k_{cat}s of reactions catalyzed by other enzymes accomplishing displacement reactions of phosphodiesters can be similarly understood.

Summary

We propose that the preferential binding of the transition states for formation of unstable intermediates in the active sites of enzymes that catalyze abstraction of the α-protons from carbon acids, acyl-transfer reactions, and displacement reactions of phosphodiesters can be understood. Using Marcus formalism, the energy of the transition state for formation of the intermediate from bound substrate, $\Delta G^{\ddagger}$, is determined by the energy of the intermediate relative to that of the substrate, ΔG°, and by an additional energy that describes the energy of the transition state relative to that of the intermediate and is associated with $\Delta G^{\ddagger}_{int}$. We propose that the positioning of a general acid group adjacent to the carbonyl or phosphoryl oxygens of the substrate is primarily responsible for reductions in both components of $\Delta G^{\ddagger}$: the reduction in ΔG° is accomplished by the formation of a short, strong hydrogen bond to the intermediate (the pK_as of the protonated intermediate and the general acid catalyst are matched), and the reduction in $\Delta G^{\ddagger}_{int}$ is accomplished by stabilization of the developing negative charge on the carbonyl or phosphoryl oxygens (without significant functional group or solvent reorganization). The magnitudes of the possible reductions in ΔG° and $\Delta G^{\ddagger}_{int}$ are sufficient to understand the observed k_{cat}s of these enzyme-catalyzed reactions.

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