Comparison of the Kinetics of the Papain-Catalyzed Hydrolysis of Glycine- and Alanine-Based Esters and Thiono Esters†

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ABSTRACT: The kinetic constants for the papain-catalyzed hydrolysis of a series of substrates with glycine or alanine in the P_1 position are discussed. The substrates have *N*-benzoyl, *N*-(*p*-nitrobenzoyl), *N*-(β-phenylpropionyl), or *N*-(methyloxycarbonyl)phenylalanine attached to the P_1 moiety, and kinetic constants are obtained for both esters and thiono esters. The results for the hydrolysis of esters can be readily interpreted in terms of the known specificity of papain. For any glycine ester the change in k_{cat}/K_m upon substituting C=S for C=O or upon substituting an α-CH_3 group is minimal. However, upon making both these substitutions, i.e., going from a glycine ester to an alanine thiono ester substrate, larger changes are seen for this ratio. Data for *N*-benzoyl- and *N*-(β-phenylpropionyl)glycine and -alanine methyl thiono esters show that k_2 is the parameter most affected by the double C=S and α-CH_3 substitution. A further conclusion is that the deacylation rate constants for any pair of glycine and alanine dithioacyl papains are similar; e.g., for the intermediates based on the "good" substrates PheAla and PheGly k_3 differs by only 20%. This is a surprising finding in light of the very different conformations and interactions of the bound acyl groups revealed by resonance Raman spectroscopy and raises the possibility that specific stereochemical effects, such as the oxyanion hole and general base catalysis, are not operating in the hydrolysis of dithioacyl papains.

A major goal in molecular enzymology is to relate the structural and dynamical properties of enzyme–substrate complexes to the rate constants for individual steps in the reaction. An approach we have taken is to obtain resonance Raman spectroscopic data for transient dithioacyl papains, to elicit structural information from the spectroscopic data by setting up a library of structure–spectra correlations on model compounds (Carey & Storer, 1984, 1985), and to obtain stopped-flow and steady-state kinetic data for the corresponding enzyme reactions (Storer & Carey, 1985). This approach has worked well for dithioacyl papains with glycine in the P_1 position; quite precise information has been obtained for some of the torsional angles of the enzyme-bound acyl group, and structure–reactivity relationships have emerged (Carey et al., 1984; Angus et al., 1986). An objective of the present series of papers is to extend our studies to include dithioacyl papains with alanine in the P_1 position.

The two preceding and accompanying publications show that the conformational preferences of the model compounds, glycine dithio esters and alanine dithio esters, are very similar (Lee et al., 1988) but that major conformational differences occur between dithioglycine and dithioalanine papains (Angus et al., 1988). In this paper we seek to relate these conformational differences to differential kinetic behavior. A surprising finding is that pairs of dithioacyl papains with either glycine or alanine in the P_1 site deacylate at similar rates, in spite of the different conformations and interactions found in the active site. This suggests that precise stereoelectronic effects do not bring about a major part of the observed rate acceleration and raises the possibility that the less specific physicochemical properties of proteins, such as their ability to function as supersolvents (Warshel, 1978), may have to be invoked to explain catalytic enhancement.

Experimental Procedures

Materials. Papain was prepared, activated, and assayed as previously described (Carey et al., 1984).

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Table I: Stoichiometries and Percent Acylation

acyl group	methyl thiono esters: max fractional acylation (f_m)	methyl thiono esters: stoichiometries[a]
N-benzoylglycine	0.96 ± 0.01	99 ± 1
N-benzoyl-DL-alanine	0.41 ± 0.14	(50)
N-(β-phenylpropionyl)glycine	0.92 ± 0.03	99 ± 1
N-(β-phenylpropionyl)-DL-alanine	0.47 ± 0.06	(50)
N-(β-phenylpropionyl)-DL-phenylalanine[b]		(50)
N-(methyloxycarbonyl)phenylalanylglycine	0.94 ± 0.01	104 ± 2
N-(methyloxycarbonyl)phenylalanyl-DL-alanine	1.2 ± 0.1	55 ± 1
N-(methyloxycarbonyl)phenylalanyl-L-alanine	1.2 ± 0.1	95 ± 3
N-(methyloxycarbonyl)phenylalanyl-L-phenylalanine[b]	1.1 ± 0.1	99 ± 1

[a] Values in parentheses are assumed since hydrolysis rates were so slow. [b] Determined at 30% acetonitrile (v/v).

The synthesis of the *N*-benzoyl- and *N*-(β-phenylpropionyl)-DL-alanine methyl thiono esters is described in the preceding paper (Angus et al., 1988). The *N*-benzoyl-, *N*-(p-nitrobenzoyl)-, and *N*-(β-phenylpropionyl)alanine and phenylalanine methyl esters were synthesized from their respective acid chlorides and amino acid methyl ester hydrochlorides. The (methyloxycarbonyl)-L-phenylalanine-L-alanine and L-phenylalanine methyl thiono esters were synthesized from the respective nitriles as previously described (Carey et al., 1984). However, the nitriles were synthesized from their respective methyl esters as described in Campbell et al. (1983).

Methods. The kinetic measurements were made by using a pH-stat (Carey et al., 1984; Angus et al., 1986). Maximum fractional acylation (f_m) was measured (Table I) at 20 °C with a Cary 219 spectrophotometer. The fractional acylation (f) was measured for several substrate concentrations by monitoring reaction mixtures at 315 nm; an extinction coefficient of 10^4 ($L \cdot mol^{-1}$) cm^{-1} (Storer et al., 1982) was used. The maximum fractional acylation (f_m) was obtained by a linear

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Table II: Kinetic Constants Obtained for the Papain-Catalyzed Hydrolysis of Methyl Esters and Methyl Thiono Esters at pH 6.0 and 20 °C[a]

	methyl ester			methyl thiono ester		
acyl group	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	[S] (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	[S] (mM)
N-benzoylglycine[b]	3.5 ± 0.2	150 ± 10	0–40	0.064 ± 0.002	215 ± 10	0–2.0
N-benzoyl-L-alanine	10.5 ± 3.0	190 ± 20 (3)	0–100			
N-benzoyl-DL-alanine[d]	8.2 ± 3.2	170 ± 60 (3)	0–100	0.096 ± 0.02	36.6 ± 7.3 (5)	0–10
N-(*p*-nitrobenzoyl)glycine[b]	11.0 ± 3.0	200 ± 10	0–10	0.180 ± 0.02	380 ± 40	0–10
N-(*p*-nitrobenzoyl)-L-alanine	10.5 ± 2.0	270 ± 50 (3)	0–15			
N-(*p*-nitrobenzoyl)-DL-alanine[d]	11.0 ± 2.0	260 ± 30 (2)	0–15	0.10 ± 0.17	47.0 ± 15.0 (4)	0–1.5
N-(β-phenylpropionyl)glycine[b]	4.7 ± 0.2	710 ± 30	0–20	0.17 ± 0.01	2600 ± 100	0–1.0
N-(β-phenylpropionyl)-L-alanine	11.0 ± 2.0	820 ± 40 (2)	0–70			
N-(β-phenylpropionyl)-DL-alanine[d]	11.0 ± 2.0	910 ± 10 (2)	0–90	0.290 ± 0.16	160 ± 50 (3)	0–10
N-(β-phenylpropionyl)-L-phenylalanine	1.1 ± 0.1	225 ± 40 (3)	0–8.0			
N-(β-phenylpropionyl)-DL-phenylalanine[c,d]				0.130 ± 0.07	80.0 ± 16 (3)	0–1.4
N-(methyloxycarbonyl)-L-phenylalanylglycine	6.2 ± 0.7	36000 ± 3000 (3)	0–1.5	0.53 ± 0.03	44000 ± 10000 (3)	0–0.15
N-(methyloxycarbonyl)-L-phenylalanyl-L-alanine	11.0 ± 1.0	34000 ± 4000 (3)	0–5.0	0.62 ± 0.02	6600 ± 1600 (3)	0–1.0
N-(methyloxycarbonyl)-L-phenylalanyl-DL-alanine[d]	10.5 ± 1.0	32000 ± 1000 (3)	0–10.0	0.67 ± 0.08	6800 ± 1200 (5)	0–2.0
N-(methyloxycarbonyl)-L-phenylalanyl-L-alanine[c]	9.0 ± 1.3	17000 ± 2000 (2)	0–6.0	0.45 ± 0.03	3700 ± 400 (2)	0–2.0
N-(methyloxycarbonyl)-L-phenylalanyl-L-phenylalanine[c]	2.8 ± 0.5	6800 ± 400 (3)	0–5.0	0.32 ± 0.01	1500 ± 200 (3)	0–1.8

[a] Values in parentheses indicate number of repeats. [b] Storer and Carey (1985). [c] 30% CH_3CN (v/v). [d] Results calculated by assuming only the L form is a substrate (see Table I).

least-squares analysis of a plot of $[S]/f$ vs [S] (see eq 4).

Results and Discussion

The kinetic model for the papain-catalyzed hydrolysis of *N*-acyl-L-amino acid esters via the well-known acyl-enzyme mechanism (Lowe, 1976; Polgar, 1977; Brocklehurst et al., 1981) is represented by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where ES is a Michaelis complex and ES′ is an acyl-enzyme intermediate. The Michaelis–Menten parameters k_{cat} and K_m obtained from steady-state reaction rate measurements are related to the rate constants in eq 1 by

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (2)$$

$$k_{cat}/K_m = k_1k_2/(k_{-1} + k_2) \qquad (3)$$

If the reasonable assumption is made that $k_{-1} \gg k_2$, then the first step in eq 1, the binding of substrate to the enzyme, can be said to be in quasi-equilibrium. Equation 3 then simplifies to $k_{cat}/K_m = k_2/K_s$, where K_s is the dissociation constant given by k_{-1}/k_{+1}. If deacylation is rate limiting, i.e., $k_3 \ll k_2$, then eq 2 becomes $k_{cat} = k_3$; however, if acylation is rate limiting, i.e., $k_2 \ll k_3$, then $k_{cat} = k_2$. During the steady-state phase of the reaction it can be shown that the fractional acylation (f) is given by

$$\frac{[ES']}{[E_{total}]} = f = \frac{k_2[S]}{k_3K_s + (k_2 + k_3)[S]} \qquad (4)$$

As $[S] \rightarrow \infty$ this equation simplifies to an expression for the maximum fractional acylation of

$$f_m = \frac{k_2}{k_2 + k_3} \qquad (5)$$

Therefore, a combination of eq 2 and 5 produces

$$k_{cat} = f_mk_3 \qquad (6)$$

Thus, measurement of k_{cat}, k_{cat}/K_m, and f_m for a given substrate can provide values for k_2/K_s and k_3 (if $k_2 \gg k_3$) or k_2 (if $k_2 \ll k_3$) or k_2 and k_3 (if $0 < f_m < 1$, i.e., $k_2 \simeq k_3$).

Esters. The kinetic constants obtained for a range of ester substrates with papain are given in Table II. If we consider the k_{cat}/K_m values, i.e., the specificity constants, for these substrates, they are consistent with the known specificity of the enzyme; that is, for the P_2 position of the substrate the k_{cat}/K_m values follow the order Phe > β-phenylpropionyl > *N*-benzoyl. For the P_1 position Gly ≃ Ala > Phe, and this indicates that for oxygen esters there appears to be no significant role in the recognition process, i.e., specificity, for the interaction between the methyl side chain of alanine and the S_1 site of the enzyme. On the other hand, the bulky Phe side chain does present a problem for the enzyme's S_1 subsite.

From the results in Table II it is clear that, for the oxygen esters, interactions between the substrate's P_2 residue and the enzyme's S_2 subsite play no significant role in defining k_{cat}. However, this constant is dependent upon interactions between the substrate and the enzyme in the S_1 subsite; i.e., k_{cat} values vary in the order Ala > Gly > Phe for the P_1 residue. Clearly, as shown by the k_{cat}/K_m values, the Phe side chain is not favored in the P_1 position of the substrate. For k_{cat}, unlike k_{cat}/K_m, the enzyme displays an approximately 2-fold preference for alanine over glycine at P_1. The consistency of this result for widely varying substrates indicates that this difference is not due to nonproductive binding but reflects a minor role of the alanine side chain in the deacylation process. Within experimental error identical kinetic constants are obtained when L- or DL-alanine-based substrates are used (in the case of DL, only the L form is hydrolyzed). This strongly indicates that the D form of the substrate does not bind significantly to the enzyme under the experimental conditions used for this study, a finding consistent with the results of de Jersey (1970).

Thiono Esters. The k_{cat}/K_m and k_{cat} values for various thiono ester substrates are compared with those of the corresponding oxygen ester substrates in Table II. On the basis of the above observation for the oxygen esters and on the results in Table II for *N*-(methyloxycarbonyl)phenylalanyl L- and DL-alanine thiono esters, we conclude that the D form of the alanine thiono esters does not bind to the enzyme under the conditions employed. For oxygen ester substrates it is generally assumed that acylation is faster than deacylation, i.e., $k_2 \gg k_3$, and therefore that $k_{cat} = k_3$. This has also been found to be the case for the majority of thiono esters, which have a maximum fractional acylation of 1.0 (Table I). However, two notable exceptions are *N*-benzoylalanine and *N*-(β-phenylpropionyl)alanine methyl thiono esters. As can be seen from Table I, $f_m < 1.0$, and using the values of f_m it has been possible to determine individual values for k_2, k_3, and

Table III: Comparison of Kinetic Constants for the Papain-Catalyzed Hydrolysis of Glycine and Alanine Methyl Thiono Esters

acyl group	K_s (mM)	k_2 (s^{-1})	k_2/K_s (M^{-1} s^{-1})	k_3 (s^{-1})
N-benzoylglycine[a]	≫5	≫2.35	214	0.064
N-benzoyl-DL-alanine[b]	4.4	0.162	36.6	0.235
ratio Gly/Ala	≫1.25	≫14.5	5.8	0.27
N-(β-phenylpropionyl)glycine[a]	≫5	≫12.8	259.0	0.165
N-(β-phenylpropionyl)-DL-alanine[b]	3.5	0.547	156.1	0.615
ratio Gly/Ala	≫1.4	≫23.4	16.6	0.27

[a] For the glycine methyl thiono esters the results are taken from Storer and Carey (1985). [b] For the alanine methyl thiono esters the constants were calculated by using the data in Tables I and II and eq 3, 5, and 6.

Table IV: Relative k_{cat}/K_m Values[a] for the Four Substrate Types, i.e., *N*-Acylglycine or *N*-Acylalanine Methyl or Methyl Thiono Esters

	methyl	methyl thiono
Gly	1	2
Ala	1.1	0.2

[a] Average values for the series of three *N*-benzoyl-, *N*-(β-phenylpropionyl)-, and *N*-(methyloxycarbonyl)-L-phenylalanine.

K_s for these two substrates. Table III compares the values for k_2, k_3, and K_s for *N*-benzoylalanine and *N*-(β-phenylpropionyl)alanine methyl thiono esters with the limiting values for these constants for the corresponding glycine-based substrates, obtained in a stopped-flow study (Storer & Carey, 1985). From the data in Tables II and III it can be deduced that substitution of alanine for glycine in the thiono ester substrates at the P_1 position has little influence on the deacylation process (as indicated by the k_3 or k_{cat} values) over and above the same increase observed for the oxygen esters. On the other hand, the k_{cat}/K_m (or k_2/K_s) values for the alanine thiono esters are significantly lower than those for the glycine thiono esters, i.e., 6–17-fold. This contrasts sharply with the approximate equality of k_{cat}/K_m values for the glycine and alanine oxygen esters. From Table III it can be seen that this effect of the methyl side chain on the k_{cat}/K_m (k_2/K_s) values for the alanine thiono esters is mainly reflected in a lowering of k_2, the rate constant for acylation, which is only partially offset by a smaller lowering of K_s; i.e., alanine thiono esters bind tighter to papain than glycine thiono esters but acylate more slowly.

When glycine oxygen esters and thiono esters are compared, the thiono esters have the larger k_{cat}/K_m values (i.e., 1.2–3.6-fold); the same comparison for alanine shows that the thiono esters have the lower k_{cat}/K_m values (i.e., 4.8–5.8-fold). For phenylalanine the thiono esters also have lower k_{cat}/K_m values (i.e., 3–4.4-fold). The similarity in magnitudes of the lowering of the k_{cat}/K_m values for alanine and phenylalanine on going from oxygen to thiono esters indicates that the effect of the side chain is not related to its bulk, suggesting that the β-CH_2 group is responsible for these effects, e.g., the lowering of k_2. The effect of the S for O substitution on k_{cat} for the alanine esters is similar to that previously reported and discussed for glycine esters (Storer & Carey, 1985).

Sensitivity of Kinetic Parameters to C=S plus α-Substitution. The effect of C=S substitution plus the presence of an α-side chain is best illustrated by comparing glycine and alanine esters and thiono esters. The results for k_{cat}/K_m have been discussed above and are summarized in Table IV. The key conclusion from Table IV is that C=S substitution in the glycine case (methyl → methyl thiono) or CH_3 substitution

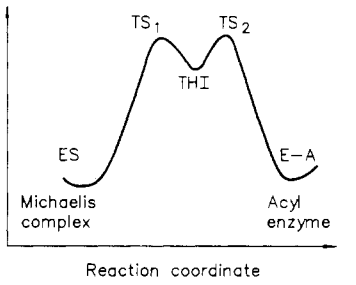

FIGURE 1: Energy profile for acylation.

for oxygen esters (Gly → Ala) produces a modest increase in k_{cat}/K_m. However, the presence of the α-CH_3 *and* C=S together bring about a substantial *decrease* (5-fold) in k_{cat}/K_m. Moreover, the data in Table III point to the k_2 parameter as being changed to a greater extent than K_s by the combined C=S and α-CH_3 substitutions.

If we view the potential energy curve for acylation in the following manner, there are a number of molecular factors that can change k_2. Thus, for the energy profile in Figure 1, tighter binding in the Michaelis complex with no effect on the curve near TS_1 (transition state 1), THI, and TS_2 will decrease k_2. A second but not necessarily independent way of altering k_2 is enzyme–substrate interactions, which change the shape of the potential curve near the top of the barriers. When thiono ester substrates of alanine and glycine are compared, there is resonance Raman (RR) spectroscopic evidence, in the preceding and accompanying publication (Angus et al., 1987), that the conformational space for alanine-based dithioacyl enzymes is quite different from that for glycine dithioacyl papains. RR data for glycine dithioacyl enzymes indicate that the acyl group takes up a relaxed nonperturbed conformation in which any enzyme–substrate contacts do not change the acyl group conformation from that found for a model glycine dithio ester in a B-conformer state in solution. In contrast, for alanine dithioacyl papains the acyl group conformation is highly perturbed from that for known "relaxed" standard states, demonstrating that there are energetically significant enzyme–substrate contacts which do not exist in the glycine case. The contacts in alanine dithioacyl papains may raise the energy of the EA and, since TS_2 has some EA character, raise the energy of TS_2, too. If raising TS_2 raises the highest point on the potential energy profile for acylation, then a smaller k_2 for alanine dithio esters will result.

The fact that the presence of *both* C=S and the α-CH_3 groups has a marked effect on k_{cat}/K_m values raises the question as to whether the highly perturbed conformational states seen for dithioacyl alanine papains are due to the dual presence of the C=S and α-CH_3 groups. In other words, do natural thiolacyl alanine papains, containing C=O and α-CH_3 groups instead of C=S and α-CH_3, have the same conformation as their dithioacyl counterparts in the active site? Since the effects on the kinetic parameters are not very large (approximately 10-fold in k_{cat}/K_m) and since there is more than one way of interpreting the kinetic differences, this must remain an open question. However it is worth reiterating that the "unusual" dithioacyl alanine RR spectra, and hence conformations, are a property of the alanine dithioacyl group *and* enzyme since the conformational preferences of model alanine dithio esters in solution or in the crystalline state appear to be quite similar to those of glycine dithio esters.

Different Dithioacyl Papain Structures Deacylate at Similar Rates. Separate concepts have to be invoked to explain the acylation and deacylation kinetic behavior of dithioacyl glycine and alanine papains. For acylation k_{cat}/K_m (Gly) is

Scheme I

$$\text{RCOAlaC(S)OCH}_3 + \text{HS–enzyme} \overset{K_s}{\rightleftharpoons}$$

$$\text{RCOAlaC(S)OCH}_3\text{HS–enzyme} \xrightarrow[\text{acylation}]{k_2}$$

$$\text{RCOAlaC(S)S–enzyme} \quad (\text{X}) \rightleftharpoons$$

$$\text{RCOAlaC(S)S–enzyme} \quad (\text{Y})$$

about 10 times larger than k_{cat}/K_m (Ala), and much of this difference resides in a faster acylation rate constant, k_2, for glycine. A plausible mechanism that encompasses this result, and the fact that the two dithioacyl papains have quite different acyl group conformations (Lee et al., 1988), introduces two acyl enzymes in equilibrium (Scheme I). It is postulated that for alanine-based substrates the initial intermediate X is formed in a B-type conformer (Lee et al., 1988) that has unfavorable enzyme–substrate contacts. The B-type conformer quickly relaxes to a non-B population Y, which is detected in the resonance Raman experiments. For glycine-based intermediates the initial acyl enzyme, X, is also a B-type conformer, but for glycine, unlike alanine, there are no unfavorable contacts with the enzyme. This has two consequences: k_2 for glycine will be higher than k_2 for alanine (as explained above) and, for glycine, the acyl-enzyme population X will remain predominantly as an X, i.e., B-type, population (seen in the RR spectrum) and not go into a Y population.

This explanation retains a single acylation catalytic mechanism for glycine and alanine thiono ester substrates, and in both cases $k_{cat}/K_m = k_2/K_s$. It is difficult to invoke a similar rationale for deacylation involving a single reaction pathway from a pair of equilibrating acyl enzymes. The problem is that the acyl groups exist in quite different conformations in glycine or alanine dithioacyl papains, and yet the rate constants for deacylation are similar (Tables II and III). The similarity is particularly striking for the "good" substrates based on PheAla and PheGly, which meet papains preference for Phe in the S_2 site. The k_3's for these substrates are 0.62 and 0.53 s^{-1}, respectively, but the RR data for the acyl enzymes demonstrate very different acyl group conformations and interactions in the active site (Lee et al., 1988).

An alternative, and favored, explanation for the deacylation results is that the rate of deacylation of dithioacyl papains is independent of the conformation of the amino acid residue in the P_1 site of the substrate. One possibility is that binding of the C=S group to the putative oxyanion hole (Drenth et al., 1976) is not used in the deacylation step of dithioacyl papains. Releasing this steric constraint means that the general base catalyzed hydrolysis step could proceed with equal facility for substrates in two quite different orientations in the active site. The role of the oxyanion hole is controversial in papain's mechanism, and Asboth et al. (1985) have suggested that it is not used in the acylation step.

Another explanation for the finding that $k_3^{Gly} \approx k_3^{Ala}$ involves the possibility that neither the oxyanion hole nor general base catalysis is operative in deacylation of dithioacyl papains. Recently we demonstrated that the solvent isotope effect for the deacylation of glycine dithioacyl papains is in the range compatible with general base catalysis but questioned that this mechanism was actually occurring in view of the fact that k_3 for thiono esters was pH independent (Storer & Carey, 1985). The small solvent isotope effects on k_{cat} for alanine [*N*-(methyloxycarbonyl)phenylalanyl-L-alanine methyl thiono ester $k_{cat}(H_2O)/k_{cat}(D_2O)$ = 1.76 (data not shown)] or glycine [*N*-β-phenylpropionylglycine methyl thiono ester $k_{cat}(H_2O)/k_{cat}(D_2O)$ = 1.67 (Storer & Carey, 1985)] thiono esters could equally well be attributed to a rate-limiting step involving an event other than general base catalyzed proton transfer. Thus, the kinetic, solvent isotope, and RR evidence taken together raise the possibility that the rate acceleration observed for the deacylation of dithioacyl papains (when compared to model systems) is solely a result of the proteinaceous environment making up the enzyme's active site. In other words, for the reactions we are considering here, we may be observing base-line catalysis—the rate acceleration left after specific, carefully controlled steric effects have been stripped away. The latter may, however, be a factor for the natural thiol ester intermediates and account for the additional rate of 10–100 in k_{cat} for thiol over dithio intermediates.

In recent years several novel theories have been brought forward to explain enzyme-based rate acceleration that go beyond standard chemical descriptions and have the underlying theme that enzymes have properties not commonly found in other forms of matter. For example, according to Warshel (1978) enzymes can bring about rate acceleration by acting as supersolvents, and Dewar and Storch (1985) have emphasized the importance of the elimination of water between interactive groups on the enzyme and substrate. Although the present findings from resonance Raman spectroscopy and enzyme kinetics cannot be construed as strong evidence for either of these theories of rate acceleration, or for other "unconventional" views (Careri et al., 1979; Frölich, 1975; Gavish, 1986; Welch et al., 1982), they do raise the possibility that effects unique to a proteinaceous environment are operating to promote deacylation.

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Relationship between Domain Closure and Binding, Catalysis, and Regulation in *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: Previous evidence, from both crystallographic and biochemical studies, has indicated that profound tertiary and quaternary changes in the structure of *Escherichia coli* aspartate transcarbamylase occur upon the binding of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA). In particular, within a single catalytic polypeptide chain, the aspartate binding domain relocates closer to the carbamyl phosphate binding domain, thereby resulting in a major reorganization of the interface between the two domains. Among the new interactions, salt bridges between Glu-50 and both Arg-167 and Arg-234 are formed. In the present study, site-directed mutagenesis is used to replace Glu-50 by glutamine in the catalytic chain. The Michaelis constant for aspartate of the mutant catalytic subunit is about 10-fold higher and the turnover number 10-fold lower than their respective couterparts in the wild-type catalytic subunit, whereas the dissociation constant for carbamyl phosphate is almost unchanged. For the holoenzyme, this substitution results in an 8-fold decrease in the specific activity, a 20-fold increase in the aspartate concentration that gives half of the maximal velocity, and a loss of cooperativity for both substrates. However, the mutant enzyme is not "frozen" in a low-affinity–low-activity conformation since PALA stimulates the activity severalfold and induces an increase in the sulfhydryl reactivity analogous to that of the wild-type enzyme. Together these results indicate that the stabilization of the aspartate binding domain near the carbamyl phosphate binding domain, through specific interdomain bridging interactions, is necessary for the high-affinity–high-activity configuration of the active site. The importance of these salt bridges for the homotropic and heterotropic interactions in aspartate transcarbamylase is discussed.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the formation of *N*-carbamyl-L-aspartate from carbamyl phosphate and L-aspartate. The enzyme, used as a model system to study the molecular mechanisms of allosteric regulation [see reviews by Gerhart (1970), Jacobson and Stark (1973), Schachman (1974), and Kantrowitz et al. (1980a,b)], exhibits positive cooperativity for both substrates (Gerhart & Pardee, 1962; Bethell et al., 1968), and its activity is inhibited by CTP and activated by ATP, the end products of the pyrimidine and purine pathways, respectively. The enzyme is composed of three regulatory dimers (i.e., regulatory subunits) and two catalytic trimers (i.e., catalytic subunits). The regulatory subunit binds CTP and ATP but is devoid of catalytic activity while the isolated catalytic subunit exhibits no homotropic cooperativity and is insensitive to the allosteric effectors. The active sites, three per catalytic subunit, are shared between adjacent catalytic chains within the trimer (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1985, Wente & Schachman, 1987). The amino acid sequences of the catalytic and regulatory polypeptide chains have been determined (Weber, 1968; Konigsberg & Henderson, 1983; Hoover et al., 1983; Schachman et al., 1984), and information about the three-dimensional structure of the unliganded enzyme (Honzatko et al., 1979, 1982; Ke et al., 1984) and the enzyme liganded with the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA)[1] (Ladner et al., 1982; Krause et al., 1985, 1987) is available at atomic resolution.

The kinetic and physicochemical properties of the enzyme can be rationalized in terms of a concerted transition between two alternate conformational states in equilibrium (Gerhart & Schachman, 1968; Hammes & Wu, 1971; Griffin et al., 1973; Kirshner & Schachman, 1973; Gibbons et al., 1976; Howlett & Schachman, 1977; Johnson & Schachman, 1980; Foote & Schachman, 1985). However, the two-state model (Monod et al., 1965) does not appear to satisfactorily account for all of the experimental data. In particular, ample evidence indicates that the homotropic and heterotropic interactions proceed by different mechanisms (Kerbiriou & Hervé, 1972, 1973; Kantrowitz et al., 1977; Kerbiriou et al., 1977; Kan-

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[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; T and R states, tight and relaxed states of the enzyme having low and high affinity, respectively, for the substrate; pHMB, *p*-(hydroxymercuri)-benzoate; $[S]_{0.5}$, substrate concentration at half the maximal observed specific activity; Tris, tris(hydroxymethyl)aminomethane; Gln-50 enzyme, mutant enzyme with glutamine substituted in place of glutamic acid at position 50 in the catalytic chain of aspartate transcarbamylase; holoenzyme, entire aspartate transcarbamylase molecule composed of two catalytic subunits and three regulatory subunits.

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