Mechanism of the Reaction Catalyzed by the Catalytic Subunit of Aspartate Transcarbamylase. Kinetic Studies with Acetyl Phosphate as Substrate†

E. Heyde and J. F. Morrison*

ABSTRACT: In an endeavor to obtain additional evidence for the mechanism of the reaction catalyzed by the catalytic subunit of aspartate transcarbamylase, kinetic studies have been made by using acetyl phosphate in place of carbamyl phosphate. The results of initial velocity, product inhibition, and dead-end inhibition studies are consistent with the reaction occurring by a rapid equilibrium, random mechanism involving the formation of the dead-end complexes: enzyme–aspartate–phosphate, enzyme–aspartate–acetyl aspartate, enzyme–acetyl phosphate–acetyl aspartate, and enzyme–(acetyl aspartate)$_2$. Thus the reaction mechanism appears to be consistent with that which has been considered to apply with carbamyl phosphate as a substrate. Comparisons of the values for the kinetic constants which are common to both reactions have shown that they are not always of a comparable magnitude. Possible explanations for the differences have been discussed.

The previous paper (Heyde *et al.*, 1973) has reported the results of kinetic studies which were directed toward the elucidation of the mechanism of the reaction catalyzed by the catalytic subunit of aspartate transcarbamylase with carbamyl phosphate and aspartate as substrates. It appeared that the kinetic data could be best explained in terms of the reaction having a rapid equilibrium, random mechanism with three dead-end complexes. However, a number of difficulties were encountered. These related largely to the low K_m value for carbamyl phosphate which precluded accurate determination of the values for certain kinetic parameters.

Since the conclusion reached about the reaction mechanism could not be considered as being completely definitive and because Porter *et al.* (1969) have postulated that the reaction proceeds *via* an ordered sequence, it appeared that there would be merit in undertaking further kinetic investigations with a substrate having a K_m value higher than that of carbamyl phosphate. The substrate chosen was acetyl phosphate and the results indicate that this reaction also can best be described as conforming to a rapid equilibrium random mechanism involving the formation of a number of dead-end complexes. The dead-end inhibition patterns obtained using succinate as an analog of aspartate provide strong evidence in favor of the random and against the previously proposed ordered mechanism (Porter *et al.*, 1969).

Experimental Section

Materials. *N*-Acetyl-L-aspartic acid was obtained from Cyclo Chemicals, while acetohydroxamic acid was synthesized by Dr. D. Magrath according to the general procedure described by Wise and Brandt (1955). Other reagents and the mercurial catalytic subunit of aspartate transcarbamylase were obtained or prepared as reported previously (Heyde *et al.*, 1973).

Methods. MEASUREMENT OF ENZYMIC ACTIVITY. Enzymic activity was determined at 28° in the presence of 0.05 M TES[1] buffer (pH 8.0). Reactions were initiated by the addition of enzyme (20 μg) and run for two time periods to ensure that initial velocities were being measured. At lower aspartate concentrations, the rate of formation of *N*-acetyl-[^{14}C]aspartate from [^{14}C]aspartate was followed using the procedure of Porter *et al.* (1969). Samples (100μl) were removed from reaction mixtures (0.5 ml) and passed through small columns of Dowex 50 to remove unreacted aspartate. At higher aspartate concentrations the volume of reaction mixtures was increased to 1.0 ml and the rate of acetyl phosphate disappearance was determined. For this purpose a slight modification of the hydroxylamine assay for acyl phosphates (Pechère and Capony, 1968) was used. The modification was introduced to increase the sensitivity of the method and consisted of the addition of the required amounts of color-producing reagents in half the volumes given by Pechère and Capony (1968). Absorbance measurements were made with a Gilford 240 spectrophotometer equipped with a digital readout and acetohydroxamic acid was used as the standard.

ANALYSIS OF DATA. The present results have been interpreted in terms of the same basic reaction mechanism as given by Heyde *et al.* (1973). Therefore, the methods used for analyzing the data and calculating values for the various kinetic parameters are the same as described in that paper. In addition, data conforming to parabolic competitive inhibition and to slope-parabolic, intercept-linear noncompetitive inhibition have been fitted to eq 1 and 2, respectively.

$$v = \frac{V[A]}{K_a\left(1 + \frac{[I]}{K_{i1}} + \frac{[I]^2}{K_{i2}}\right) + [A]} \quad (1)$$

$$v = \frac{V[A]}{K_a\left(1 + \frac{[I]}{K_{i1}} + \frac{[I]^2}{K_{i2}}\right) + [A]\left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, Australia 2601. *Received July 17, 1973.*

[1] Abbreviation used is: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

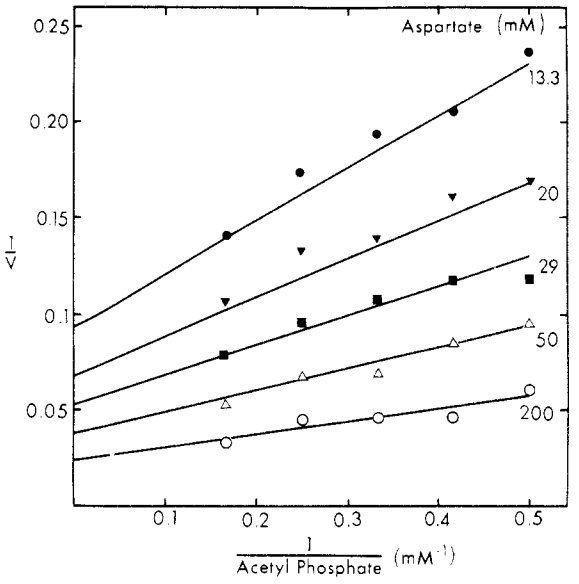

FIGURE 1: Effect of the concentrations of acetyl phosphate and aspartate on the initial velocity of the reaction. The data were fitted to eq 7 of Cleland (1963). Velocities are expressed as nanomoles of acetyl phosphate converted per microgram of enzyme per minute.

TABLE I: Kinetic Constants Determined from Initial Velocity Studies.

Kinetic Constant	Value (mM)
K_a	2.9 ± 0.4
K_{ia}	0.77 ± 0.14
K_b	48 ± 7
$K_{ia}K_b/K_a$[a]	22 ± 6[b]
K	13 ± 4[c]
V_m	55 ± 3 nmol/min per μg of enzyme

[a] This expression equals K_{ib} for a rapid equilibrium random mechanism. [b] Weighted mean of individual values. [c] Calculated from the values for K_a, K_{ia}, and K_b. Values are the weighted means of values obtained from five experiments including that illustrated in Figure 1. K_a and K_{ia} represent dissociation constants for the combination of acetyl phosphate (A) with the enzyme–aspartate complex and free enzyme, respectively; K_b and K_{ib} represent dissociation constants for the combination of aspartate (B) with the enzyme–acetyl phosphate complex and free enzyme, respectively.

Results

In the first instance, attempts were made to determine the initial velocity pattern for the reaction by use of the radioactive assay of Porter *et al.* (1969) which proved to be comparatively satisfactory with carbamyl phosphate as a substrate. However, it was not practical to use this method with acetyl phosphate as a substrate because the K_m value for aspartate in the presence of this compound is much higher than when carbamyl phosphate is present. The rate of P_i formation could not be determined on account of the instability of acetyl phosphate in the presence of acid molybdate and thus it has been necessary to carry out the initial velocity studies by measuring the rate of acetyl phosphate disappearance (Pechère and Capony, 1968). Typical results are illustrated in Figure 1 and these demonstrate that the reaction conforms to a sequential mechanism. The values obtained for the kinetic parameters by computer analysis of the data are listed in Table I.

Product Inhibition Studies. The patterns for the product inhibition by phosphate were similar to those obtained with carbamyl phosphate as a substrate (Figures 2a and 2b of Heyde *et al.*, 1973). Thus phosphate functions as a linear competitive inhibitor with respect to acetyl phosphate and as a linear noncompetitive inhibitor in relation to aspartate. On the other hand, the product inhibition patterns given by *N*-acetylaspartate are more complex (Figure 2). Thus, the inhibition with respect to acetyl phosphate is slope-parabolic, intercept-linear noncompetitive and with respect to aspartate seems to vary with the experimental conditions. At a lower fixed concentration of acetyl phosphate and with aspartate varying over a range of lower concentrations, the inhibition appears to be slope-parabolic competitive (Figure 2b) while at higher concentrations of both substrates (Figure 2c), the inhibition is best described as being of the linear noncompetitive type. However, it will be noted that the intercepts of Figure 2c vary to only a small extent. The product inhibition constants obtained from analysis of the data are recorded in Table II.

Dead-End Inhibition. Succinate functioned as an inhibitory analog of aspartate, giving rise to linear competitive inhibition and linear noncompetitive inhibition with respect to aspartate and acetyl phosphate, respectively. The variation of the slopes of the noncompetitive inhibition plot was much more marked than that of the vertical intercepts and thus the result differs from the noncompetitive pattern observed when the inhibition by succinate was studied with carbamyl phosphate as the variable substrate (Heyde *et al.*, 1973). Monomethyl phosphate acted as an inhibitory analog of acetyl phosphate causing linear competitive inhibition with respect to this substrate and linear noncompetitive inhibition in relation to aspartate. The kinetic constants associated with the inhibition of the reaction by the dead-end inhibitors are listed in Table III.

Maximum Velocity of the Reaction in the Presence of Carbamyl Phosphate and Acetyl Phosphate. A direct comparison was made of the maximum velocities of the reactions with carbamyl phosphate and acetyl phosphate as substrates, by varying the appropriate substrate pairs in constant ratio. The results indicated that the maximum velocity with acetyl phosphate was about 10% of that with carbamyl phosphate.

Discussion

For kinetic investigation of the reaction catalyzed by the catalytic subunit of aspartate transcarbamylase, acetyl phosphate has proved, in many respects, to be a more suitable substrate than carbamyl phosphate. In particular, its higher K_m value has allowed determination of a definitive sequential initial velocity pattern (Figure 1). However, acetyl phosphate is not an ideal substrate since there is no specific method for estimating *N*-acetylaspartate and inorganic phosphate cannot be determined because of the lability of acetyl phosphate in acid molybdate. Consequently, it has been necessary to resort to the measurement of the rate of acetyl phosphate disappearance as a method of determining initial velocities under conditions which do not permit the use of the more precise assay involving [^{14}C]aspartate. The kinetic investigations have also been hampered by the apparent irreversibility of the reaction. Nevertheless, the results do appear to indicate

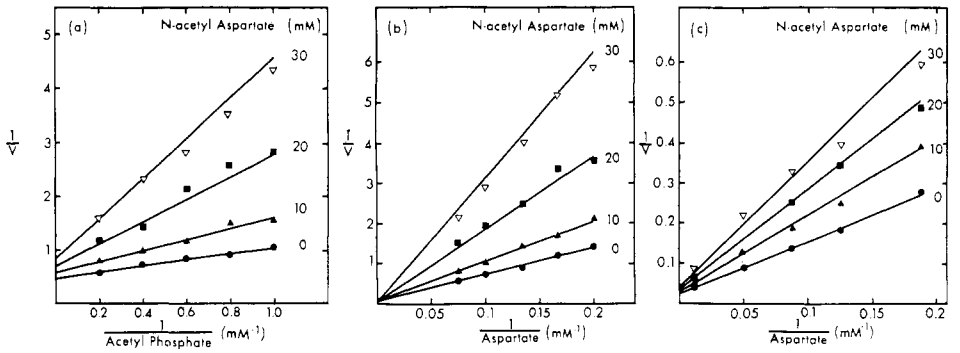

FIGURE 2: Product inhibition of the reaction by *N*-acetylaspartate: (a) acetyl phosphate varied at a fixed aspartate concentration of 5.0 mM; (b) aspartate varied at a fixed acetyl phosphate concentration of 1.0 mM; (c) aspartate varied at a fixed acetyl phosphate concentration of 4.0 mM. The data of a and b were fitted to eq 2 and 1, respectively, while the data of c were fitted to eq 10 of Cleland (1963). Velocities are expressed as nanomoles of *N*-acetylaspartate formed (a, b) or of acetyl phosphate converted (c), per microgram of enzyme per minute.

TABLE II: Apparent and True Constants Associated with the Product Inhibition of Aspartate Transcarbamylase.

Product Inhibitor	Varied Substrate	Fixed Substrate Concn (mM)	Apparent K_i (mM) Slope	Apparent K_i (mM) Intercept	True K_i[a] (mM)
Phosphate (Q)	Acetyl phosphate (A)	5.0	0.63 ± 0.04		
	Aspartate (B)	1.0	4.0 ± 1.0		1.7 ± 0.6 (K_{iq})
				0.72 ± 0.38	0.54 ± 0.30 (K_{IbQ})
N-Acetylaspartate (P)	Acetyl phosphate (A)	5.0	53 ± 41 (K_{i1})		
			190 ± 27 (K_{i2})	40 ± 9	36 ± 11 (K_{IaP})
	Aspartate (B)	1.0	84 ± 40 (K_{i1})		
			280 ± 30 (K_{i2})		
	Aspartate (B)	4.0	21 ± 3	46 ± 12	19 ± 6 (K_{IbP})

[a] Calculated using relationships given in Table VI of Heyde *et al.* (1973) and the fixed substrate concentrations. The apparent K_i values are the weighted means of values from two experiments and calculations were made on the basis that the reaction conforms to a rapid equilibrium random mechanism with three dead-end complexes. The values used for K_a, K_{ia}, and K_b were taken from Table I.

TABLE III: Kinetic Constants for Inhibition of Reaction by Succinate and Monomethyl Phosphate.

Inhibitor	Varied Substrate	Fixed Substrate Concn (mM)	Apparent K_i (mM) Slope	Apparent K_i (mM) Intercept	True K_i (mM)
Succinate	Acetyl phosphate	5.0	7.3 ± 0.7		6.0 ± 2.2 (K_i)
				47 ± 8	43 ± 11 (K_I)
	Aspartate	4.0	20 ± 1		
			(20 ± 5)[a]		
Monomethyl phosphate	Acetyl phosphate	5.0	0.76 ± 0.05		
			(0.28 ± 0.11)[a]		
	Aspartate	4.0	1.4 ± 0.2		0.23 ± 0.05 (K_i)
				6.1 ± 1.3	2.6 ± 0.7 (K_I)

[a] Calculated by substitution of the values for K_i and K_I into the relationships between true and apparent constants for the competitive inhibitions. The apparent K_i values are the weighted means of values from two experiments. The true values for the inhibition constants were calculated by assuming that the rate equation for the reaction in the presence of succinate is represented by the equation: $v = V[A][B]/(K_{ia}K_b + K_a[B] + K_b[A] + [A][B])$ with the $K_{ia}K_b$ and $K_b[A]$ terms multiplied by the factors $(1 + [I]/K_i)$ and $(1 + [I]/K_I)$, respectively. For the reaction in the presence of monomethyl phosphate values were determined by using the same equation in which the $K_{ia}K_b$ and $K_a[B]$ terms were multiplied by the factors $(1 + [I]/K_i)$ and $(1 + [I]/K_I)$, respectively. The value for K_{ib} was taken to be 22 ± 6 mM; the values for other constants used in the calculations are given in Table I.

that the reaction involving acetyl phosphate and aspartate proceeds *via* a rapid equilibrium random mechanism, and the arguments which lead to such a conclusion are developed below.

The results of the initial velocity studies (Figure 1) and the product inhibition by phosphate are in accord with the reaction having either (a) an ordered mechanism in which acetyl phosphate adds to the enzyme before aspartate and *N*-acetylaspartate is released before phosphate or (b) a random mechanism involving the formation of a dead-end enzyme–aspartate–phosphate complex. The product inhibition by *N*-acetylaspartate does not yield results which simply distinguish between these two mechanisms. If the reaction mechanism were ordered, the data of Figure 2 could be explained as being due to *N*-acetylaspartate acting both as a product inhibitor and as a dead-end inhibitor that combines with free enzyme. However, from studies of the reaction with carbamyl phosphate as a substrate, Heyde *et al.* (1973) have argued that, if the reaction were to conform to an ordered mechanism, carbamyl aspartate must act as a dead-end rather than as a regular product inhibitor. Thus it seems reasonable to assume that if the same mechanism were to apply to the reaction involving acetyl phosphate, the inhibition by *N*-acetylaspartate would be due also to its ability to act only as a dead-end inhibitor. Under these conditions, the data of Figure 2a would be obtained only if *N*-acetyiaspartate combined twice with free enzyme as well as with the enzyme–acetyl phosphate complex. It follows from these postulates that acetylaspartate would give rise to slope-parabolic, intercept-parabolic noncompetitive inhibition with respect to aspartate at fixed concentrations of acetyl phosphate in the region of the K_a and K_{ia} values (Table I). But the data of Figures 2b and 2c do not provide evidence in support of this prediction. On the other hand, all the data of Figure 3 are in accord with the reaction occurring by means of a rapid equilibrium random mechanism which allows for the formation of the dead-end complexes, enzyme–acetyl phosphate–acetyl aspartate and enzyme–aspartate–acetyl aspartate, as well as the combination of two molecules of *N*-acetylaspartate with the free enzyme. The initial rate equation for such a mechanism with aspartate (B) as the variable substrate may be expressed as

$$\frac{1}{v} = \frac{K_b}{V}\left[\frac{K_{ia}}{[A]}\left(1 + \frac{[P]}{K_{ip}} + \frac{[P]}{K_{Ip}} + \frac{[P]^2}{K_{Ip}K_{ipp}}\right) + 1 + \frac{[P]}{K_{iap}}\right]\frac{1}{[B]} + \frac{1}{V}\left[\frac{K_a}{[A]}\left(1 + \frac{[P]}{K_{ibp}}\right) + 1\right] \quad (3)$$

where K_{ip} and K_{iap} represent dissociation constants for the reaction of *N*-acetylaspartate (P) at the aspartate binding site of the free enzyme and the enzyme–acetyl phosphate complex, respectively; K_{Ip} and K_{ibp} represent dissociation constants for the reaction of P at the acetyl phosphate binding site of the free enzyme and the enzyme–aspartate complex, respectively; K_{ipp} represents the dissociation constant for the reaction of P at the acetyl phosphate binding site when P is already bound at the aspartate binding site. From eq 3 it is apparent that as the fixed concentration of acetyl phosphate (A) becomes large compared with K_{ia} (Table I), the variation of the slopes will change from a parabolic to a linear function of P (Figures 2b and 2c). Equation 3 also predicts that at concentrations of A comparable to the value of K_a (Table I), the intercepts should vary as a linear function of [P]. However, intercept variation is not readily observed (Figures 2b and 2c). This result indicates that the value of K_{ibp} is relatively high so that *N*-acetylaspartate reacts poorly with the enzyme–aspartate complex.

The aforementioned conclusion about the reaction mechanism is supported by the results of the dead-end inhibition by succinate which clearly indicate that the substrates must add to the enzyme in a random, and not ordered, manner. While the results of the dead-end inhibition by monomethyl phosphate are as expected for a random mechanism, it must be recognized that identical findings would be observed with the ordered mechanism.

On the assumption that the reaction has a rapid equilibrium random mechanism, the values for K_{ia}, K_a, and K_b (Table I) may be used to calculate a value for K_{ib} which represents the dissociation constant for the combination of aspartate with free enzyme. According to the method of calculation, two values have been determined (Table I). Since these are of a magnitude similar to the value of approximately 15 mM determined from binding experiments (Heyde *et al.*, 1973), the result is as expected for a reaction having a rapid equilibrium random mechanism. It is also of interest to note that both acetyl phosphate and aspartate combine more strongly with the free enzyme than with the appropriate enzyme–substrate complex.

The values of the kinetic parameters determined from the product inhibition data (Table II) cannot be used as a quantitative check on the correctness of the conclusion about the reaction mechanism. The nature of the rate equation for a rapid equilibrium random mechanism with all the proposed dead-end complexes is such that only a single value can be obtained for any particular inhibition constant. Additional supporting evidence in favor of the random combination of substrates with the enzyme comes from quantitative analysis of the dead-end inhibition data obtained with succinate and monomethyl phosphate. Thus, there is reasonable agreement between the experimental and calculated values for the apparent inhibition constants associated with the competitive inhibition of the reaction by these compounds (Table III).

It is of interest that significantly different maximum velocities have been observed for the reactions involving carbamyl phosphate and acetyl phosphate. This indicates that they cannot have a common rate-limiting step and excludes the possibility that both reactions occur by means of ordered mechanisms in which the release of phosphate is the slowest step. There are two other points arising from comparisons of the kinetic constants which are worthy of note. Firstly, the binding of *N*-acetylaspartate to the enzyme–aspartate complex is much weaker than that of carbamyl aspartate (Table II and Heyde *et al.*, 1973). Presumably these combinations occur through the acetyl or carbamyl moieties of the product molecules and their relative strengths reflect the fact that carbamyl phosphate has a greater affinity than acetyl phosphate for both the free enzyme and the enzyme–aspartate complex. Secondly, the combination of aspartate with the enzyme is facilitated by the presence of phosphate and *vice versa* (Table IV).

If it is true that the reaction catalyzed by the catalytic subunit of aspartate transcarbamylase conforms to a rapid equilibrium random mechanism, irrespective of whether carbamyl phosphate or acetyl phosphate is used as a substrate, then it follows that comparable values for certain kinetic constants should be obtained from studies on each of the reactions. For comparative purposes, the values calculated from the present and previous work (Heyde *et al.*, 1973) have been listed in Table IV. It is apparent that for the interaction of free enzyme with phosphate, monomethyl phosphate, and

TABLE IV: Comparison of the Kinetic Constants Determined by Using Carbamyl Phosphate and Acetyl Phosphate as Substrates.

Reaction	Apparent Dissociation Constant (mM)		True Dissociation Constant (mM)	
	Carbamyl Phosphate	Acetyl Phosphate	Carbamyl Phosphate	Acetyl Phosphate
E + phosphate	67 ± 20	4.0 ± 1.0	0.6 ± 0.2	1.7 ± 0.6
E + monomethyl phosphate	27 ± 2	1.4 ± 0.2	0.19 ± 0.03	0.23 ± 0.05
E + acetyl phosphate	38 ± 4	0.77 ± 0.14	0.28 ± 0.04	0.77 ± 0.14
E + succinate	0.26 ± 0.06	7.3 ± 0.7	0.26 ± 0.06	6.0 ± 2.2
E − aspartate + monomethyl phosphate	67 ± 10	6.1 ± 1.3	<0.4 [2.6][a]	2.6 ± 0.7
E − aspartate + acetyl phosphate	67 ± 16	2.9 ± 0.4	<0.18 [2.6][a]	2.9 ± 0.4
E − aspartate + phosphate	8.5 ± 1.3	0.72 ± 0.38	<0.05 [0.33][a]	0.54 ± 0.30
E − phosphate + aspartate			3.5 ± 1.3	11.9 ± 6.0

[a] Values obtained by assuming a K_a value of 40 μM.

acetyl phosphate (acting either as a substrate or inhibitor), there is reasonable agreement between the two sets of values, especially when the factors involved in the calculation of true dissociation constants are taken into account. However, there are marked discrepancies in the two values determined for the reaction of succinate with free enzyme and for the reaction of monomethyl phosphate, acetyl phosphate, and phosphate with the enzyme–aspartate complex. These differences could be significant, but it must be pointed out that, except in the case of succinate, they could arise as a consequence of the large corrections associated with the determination of true constants and/or because of the use of an incorrect K_a value for carbamyl phosphate. In this connection it is of interest that the discrepancies between each of the two calculated dissociation constants for the reaction of monomethyl phosphate, acetyl phosphate, or phosphate with the enzyme–aspartate complex disappear if the Michaelis constant for carbamyl phosphate is taken as 40 μM, rather than as <6 μM (Heyde *et al.*, 1973). However, if the value for K_a were 40 μM and the values for K_{ia}, K_b, and K_{ib} were correct, then the relationship that characterizes a random mechanism which is truly rapid equilibrium, *viz.* $K_{ia}K_b = K_aK_{ib}$, would not hold for the reaction involving carbamyl phosphate (Heyde *et al.*, 1973). The possibility would then arise that the carbamyl phosphate reaction, the acetyl phosphate reaction, or both might be random, but not truly rapid equilibrium. If this were so, it may not be valid to use the value for K_{ib}, as determined from studies with acetyl phosphate, for calculations with the data for the carbamyl phosphate reaction. Despite the quantitative discrepancies the qualitative results can be taken to indicate that the mechanism for each reaction is adequately described as rapid equilibrium, random. Consideration has also been given to other possible reasons for the difficulties encountered in the studies on the catalytic subunit of aspartate transcarbamylase and an alternative explanation is discussed below.

Throughout this work, it has been tacitly assumed that the reaction obeys Michaelis–Menten kinetics, and in a qualitative sense, the data illustrated in the figures appear to support this premise. However, it has been observed that, in the presence of ITP, double reciprocal plots with carbamyl phosphate as the variable substrate are nonlinear (Heyde, 1973) and that substrate inhibition by aspartate occurs in the presence of carbamyl phosphate, but not acetyl phosphate. These results suggest that the catalytic subunit, composed of three polypeptide chains, may always exhibit some degree of cooperativity so that the reaction conforms only approximately to Michaelis–Menten kinetics. Thus, errors could be introduced into the calculation of values for the kinetic parameters. Further, the occurrence of such cooperativity could mean that when carbamyl phosphate is varied over a range of concentrations which are high compared with its K_m value, the dissociation constant for succinate, as determined from the slope replot of the noncompetitive inhibition (Figure 4, Heyde *et al.*, 1973), does not give a measure of its combination with free enzyme, but rather with enzyme–carbamyl phosphate or enzyme–(carbamyl phosphate)$_2$. On the other hand, with acetyl phosphate varied in the region of its K_m value, the dissociation constant calculated in the same manner from the data (Table IV) may well give a measure of the binding of succinate to free enzyme. The apparently stronger combination of succinate in the presence of carbamyl phosphate (Table IV) could be explained on this basis and the explanation is in accord with the known effects of carbamyl phosphate in enhancing the combination of succinate with the enzyme (Collins and Stark, 1969). Confirmation of the postulate that the catalytic subunit of aspartate transcarbamylase can exhibit cooperativity would have to come from studies which utilize a wider range of substrate concentrations, especially those for the phosphorylated substrates. But experiments of this type would require more sensitive methods for determining initial velocities than those that can currently be foreseen.

Acknowledgments

The authors are grateful to Dr. W. W. Cleland for his comments on the manuscript and to Mrs. M. Labutis for technical assistance.

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Carbon Nuclear Magnetic Resonance Studies of the Histidine Residue in α-Lytic Protease. Implications for the Catalytic Mechanism of Serine Proteases†

Michael W. Hunkapiller,‡ Stephen H. Smallcombe, Donald R. Whitaker,§ and John H. Richards*

ABSTRACT: Selective ^{13}C enrichment of C-2 of the single histidine residue of the serine protease α-lytic protease has allowed direct study of the Asp-His-Ser catalytic triad. Both the chemical shift of C-2 and the coupling between C-2 and its directly bonded hydrogen have been observed as a function of pH. We interpret the results to indicate that only below pH 4 does the imidazole ring of the histidine residue become protonated and only above pH 6.7 does the aspartic acid residue lose a proton to generate a carboxylate anion. Thus, over the pH range 4–6.7, the catalytic triad consists of a *neutral* aspartic acid and a *neutral* histidine residue—not the ionized forms hitherto assumed. These new assignments for the ionization characteristics of the aspartic acid and histidine residues of the catalytic triad lead to a proposed catalytic mechanism that avoids any requirement for unfavorable charge separation. In this view, the histidine residue plays two roles: (i) it provides insulation between water and the buried carboxylate anion of the aspartate, thus ensuring the carboxylate group a hydrophobic environment, and (ii) it provides a relay for net transfer of protons from the serine hydroxyl to the carboxylate anion. The aspartate anion acts as the ultimate base which holds a proton during catalysis. An anionic, rather than a neutral, base has advantages; it both avoids the necessity of charge separation and, by giving the catalytic locus an overall negative charge, assists preferential expulsion of product relative to substrate from the active site of the enzyme. Relaxation measurements (T_1, T_2, and nuclear Overhauser enhancement) indicate that, over the pH range of enzymic activity, the histidine residue is held rigidly within the protein.

From the first discovery (Matthews *et al.*, 1967; Blow *et al.*, 1969) of a buried carboxylate anion as part of a precisely arranged catalytic triad of residues (consisting of the carboxylate anion of aspartate, the imidazole ring of histidine, and the hydroxyl group of serine), the detailed nature of the charge relays and accompanying proton transfers that occur during catalysis by serine proteases has been the focus of considerable interest. This array of three residues occurs in many enzymes which show clear homologies and most likely constitute a case of divergent evolution of a single family of proteins. (Members of this family include, for example, chymotrypsin, trypsin, elastase, and α-lytic protease [Olson *et al.*, 1970].) Even in enzymes which possess no other apparent homology, and are therefore probably unrelated in terms of their ultimate origins (such as the chymotrypsin family on the one hand and the subtilisin family on the other), the striking presence of this identical catalytic triad provides evolutionary testimony of its unique catalytic efficacy. The question of the true microscopic ionization behavior of the participants of this triad has, however, hindered the realization of a completely satisfactory account of the catalytic act.

^{13}C Nmr for Study of Proteins. Nuclear magnetic resonance (nmr) allows study of the environment of individual nuclei of molecules in solution and has recently found increasingly fruitful application to problems of biological importance. Meadows *et al.* (1967, 1969) used proton magnetic resonance to study ionization of the histidine residues in ribonuclease and the effect of inhibitor binding on the two catalytic histidine groups of the enzyme. Wuthrich *et al.* (1968) and Ogawa and Shulman (1971) observed those proton resonances of hemoglobin which are shifted by the ring currents of the porphyrin ring and thereby studied the effects on protein conformation of ligand binding to the heme groups. However, the relatively narrow range of proton chemical shifts and the resultant lack of dispersion usually require exchange with deuterium oxide to remove amide protons from the backbone peptide bonds and thereby expose signals from other protons of interest.

Groups containing fluorine nuclei have been covalently attached to proteins. Subsequent study by ^{19}F spectroscopy has been reported, for example, for hemoglobin and ribonuclease by Raftery *et al.* (1972) and Huestis and Raftery (1972). Though, in the cases mentioned, appropriate control studies showed only insignificant change in protein function as a result of attachment of label, such techniques may potentially cause unknown alterations in protein conformations.

Carbon magnetic resonance offers great potential for studies

† Contribution No. 4699 from the Church Laboratories of Chemical Biology, California Institute of Technology, Pasadena, California 91109. *Received June 13, 1973.* We wish to acknowledge generous financial support of this research by grants from the U. S. Public Health Service, NIGMS-16424 and NIGMS-10218, and the Medical Research Council of Canada, MA-4409.

‡ Recipient of a National Institutes of Health Traineeship.
§ Present address: Department of Biochemistry, University of Ottawa, Ottawa, Canada.