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Studies with Aspartate Transcarbamylase*

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ABSTRACT: Aspartate transcarbamylase (ATCase) is shown to have more than two active sites and therefore at least four and probably six by an alalysis of activation of the enzyme at low concentration of L-aspartate by an aspartate analog, maleate. Two groups of dicarboxylate dianions are shown to differ in their effect on the kinetic behavior of ATCase. At low L-aspartate concentration, one group activates ATCase at low concentrations and inhibits at high concentrations whereas the other group only inhibits the enzyme. Inhibition of both groups is offset by L-aspartate; however, inhibition by the second group, but not the first, is also offset by carbamyl phosphate. The difference in behavior of the two groups can be a consequence of a positive cooperative effect in the kinetic binding of L-aspartate and carbamyl phosphate. Bromosuccinate inactivates ATCase by alkylation; enzyme activity is protected by ureidosuccinate, maleate, succinate, and carbamyl phosphate (but not L-aspartate). In the presence of maleate and carbamyl phosphate reaction of ATCase with bromosuccinate causes activation of the enzyme and loss of CTP inhibition and maleate activation. Activation by bromosuccinate does not involve dissociation of the enzyme into subunits although interactions among the subunits evidently are disrupted. Two reversible treatments which result in loss of interactions among subunits of ATCase, high pH and 1 M urea, are also shown not to dissociate the enzyme.

Aspartate transcarbamylase (ATCase),[1] the first enzyme unique to pyrimidine biosynthesis in *Escherichia coli*, is inhibited in a partially competitive fashion by the end product of the pathway, CTP (Gerhart and Pardee, 1962–1964; Bethell *et al.*, 1968). Inhibition by CTP is thought to play an important role in regulation of pyrimidine biosynthesis in *Escherichia coli* (Yates and Pardee, 1956b; Gerhart and Pardee, 1962–1964). Although CTP inhibits competitively with both substrates, the CTP binding sites are distinct from the active sites (Gerhart and Pardee, 1962) and are located on different subunits (Gerhart and Schachman, 1965). The native enzyme is readily dissociated into subunits by heat treatment or reaction with PMB (Gerhart and Pardee, 1962–1964; Gerhart and Schachman, 1965) and two types of subunits can be separated (Gerhart and Schachman, 1965; Gerhart and Holoubek, 1967). The native enzyme has a molecular weight of 3.1×10^5 (Gerhart and Schachman, 1965) and consists of two active catalytic subunits, $\bar{M} = 1.0 \times 10^5$, and three regulatory (CTP binding) subunits, $\bar{M} = 3.4 \times 10^4$. Each catalytic subunit is composed of three polypeptide chains and each regulatory subunit of two polypeptide chains (Weber, 1968; Wiley and Lipscomb, 1968; Meighen *et al.*, 1970).

Gerhart and Pardee have inferred from kinetic studies that binding of one molecule of L-aspartate to ATCase enhances binding of successive L-aspartate molecules, and that CTP inhibits the enzyme at saturating concentrations of carbamyl phosphate by decreasing the affinity of the enzyme for L-aspartate (Gerhart and Pardee, 1963, 1964). This interpretation has been supported by binding sites with succinate and CTP (Changeux *et al.*, 1968). Changeux *et al.* have also shown that in the presence of a saturating concentration of carbamyl phosphate ATCase binds four molecules of succinate suggesting that the enzyme has four binding sites for L-aspartate.

In the first part of this paper we have examined effects of various dicarboxylate analogs of L-aspartate on the activity of ATCase at low concentrations of L-aspartate. In the second part of this paper we describe the effect of the reaction of bromosuccinate and ATCase on enzyme activity and the

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† NASA Fellowship, 1966–1968. This work forms part of the dissertation submitted to the Department of Biochemistry, Columbia University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] Abbreviations used are: ATCase, aspartate transcarbamylase; PMB, *p*-mercuribenzoate; BDEAE, benzyldiethylaminoethyl; CAP, carbamyl phosphate.

effect on the interactions among the subunits of ATCase. The effects of high pH and 1.0 M urea are also described.

Materials

Chemicals. All chemicals except ^{14}C-labeled bromosuccinic acid were obtained commercially in the purest grade available. Carbamyl phosphate was purified by ethanol precipitation and tested for purity (Gerhart and Pardee, 1962).

Bromosuccinic-1,4-^{14}C acid was synthesized from fumaric-1,4-^{14}C acid (International Chemical and Nuclear Corp.) (Fittig and Dorn, 1877): mp 160.5–161.5° (lit. mp 161°, Dictionary of Organic Compounds, 1965), yield 62%.

Methods

Enzyme Assay. ATCase activity was measured by the rate of formation of phosphate at 25° (Lowry and Lopez, 1946, as modified by Weitzman and Wilson, 1966). Generally 10–25 μl of enzyme was added to a reaction mixture containing buffer and substrates in 1.0 ml. Solutions of carbamyl phosphate were freshly prepared and were kept on ice. Reaction conditions were chosen to produce between 0.1 and 0.3 μmole of inorganic phosphate in a few minutes.

Protein Assay. Protein was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Enzyme Purification. ATCase was prepared from cells of *E. coli* strain HfrC-Ya-149, 4-21 kindly supplied by Dr. John C. Gerhart. The cells were grown by Drs. Woodruff and Stoudt of Merck & Co. in a salts–glycerol medium (Yates and Pardee, 1956a) supplemented with thiamine hydrochloride (1 mg/l.) and uracil (2 mg/l.) at 37° for 48 hr then harvested by centrifugation and frozen. Every step in the purification was carried out at about 4°. One unit of the enzyme activity was defined as the amount of enzyme which produced 1 μmole of phosphate/min in 0.050 M Tris–0.025 M L-aspartate–0.003 M carbamyl phosphate (pH 8.5). A portion of frozen cells was thawed and resuspended in 0.020 M Tris–0.008 M succinate–0.020 M magnesium acetate–0.010 M mercaptoethanol–0.0001 M EDTA (pH 7.5). The cells were disrupted by sonication and centrifuged. The enzyme was precipitated between 40 and 49% saturation of $(NH_4)_2SO_4$ and applied to a column of cellulose BDEAE-cellulose (2.3 × 29 cm) (Kremzner and Wilson, 1963) previously equilibrated with 0.005 M imidazole–0.001 M mercaptoethanol–0.0001 M EDTA–0.2 M KCl (pH 7.0). The enzyme was eluted from the column by 0.5 M KCl. Enzyme from this step was concentrated by absorbing it to a small BDEAE column (1.6 × 12 cm) and eluting it with a small volume of 0.7 M KCl. Concentrated enzyme was further purified with a Sephadex G-200 column (2.54 × 26.5 cm). The enzyme was slghtly retarded. The specific activity of eluted enzyme ranged from 80 to 140 units per mg of protein (2800–5000 units/mg using the unit defined by Shepherdson and Pardee, and Gerhart and Holouhek (Shepherdson and Pardee, 1960; Gerhard and Holouhek, 1967).

Heat Treatment of Enzyme. Catalytic subunits were prepared by changing the enzyme medium to 0.005 M phosphate–0.001 M mercaptoethanol–0.0001 M EDTA (pH 7.0) by passing the enzyme through a Sephadex G-25 column equilibrated with this buffer. The enzyme was then added to a prewarmed tube at 62°. After 10 min the enzyme was transferred to a precooled tube at 0° (Gerhart and Pardee, 1962; Weitzman and Wilson, 1966).

TABLE I: Per Cent Inhibition.

Reversible Inhibitor (M)		Aspartate 0.002	Aspartate 0.010	Aspartate 0.050		CAP 0.001	CAP 0.010
Pthalate (0.025)	*a*	48	32	0	*b*	43	26
U. S. (0.02)	*a*	42	26	5	*b*	27	5
Bromosuccinate (0.05)[e]	*a*	38	33	6	*c*	35	15
Glutarate (0.18)	*d*				*c*	45	30
Malonate (0.02)	*d*				*c*	42	46
Maleate							
(2×10^{-3})	*d*				*c*	30	32
(3×10^{-3})					*c*	47	50

[a] (CAP) = 0.001 M, pH 7.0, 25°. [b] (ASP) = 0.005 M. [c] (Asp) = 0.01 M. [d] Competitive with aspartate (Porter *et al.*, 1969). [e] DL compound 0.1 M but L assumed to be only active species. Per cent reversible inhibition of ATCase by various dicarboxylic acids at fixed CAP concentration and different aspartate concentration and also at fixed aspartate concentration and different CAP concentration.

Sucrose density gradient centrifugations were carried out according to the procedure of Martin and Ames (1961). Centrifugations were carried out in a Beckman Model L2-65B preparative ultracentrifuge using the SW-50L rotor at 35,000 rpm at 4°. Sucrose solutions (5 and 20%) were made up in 0.050 M Tris (pH 7.0 and 8.5), 0.050 M ethanolamine (pH 10.2), or 0.050 M Tris–1.0 M urea (pH 7.0). Gradient volumes were 5.0 ml; cold 0.20-ml samples were routinely applied to gradients.

Detection of Carbon-14. Carbon-14 in sucrose density gradient experiments was determined by dividing each gradient into 15 drop fractions then adding to each fraction 0.4 ml of 2 N NaOH in 85% ethanol, 2 ml of Colosolve CS-2 acid solubilizer, and 10 ml of scintillator solution (7 g of 2,5-diphenyloxazole and 0.42 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] in 1 l. of toluene). The samples were counted in a Beckman LS-250 scintillation counter.

Results

Effects of Dicarboxylic Acids on ATCase Activity at Low Concentrations of Aspartate. Two structural analogs of L-aspartate, maleate, and succinate, activate ATCase at low concentrations of aspartate (Gerhart and Pardee, 1963, 1964). We find that fumarate niether activates nor inhibits ATCase over a concentration range where the effect of maleate goes from a maximum activation of 60% to an inhibition of 60%. Phthalate does not activate ATCase at any concentration, but inhibits the enzyme competitively with L-aspartate (Table I). Ureidosuccinate, one of the products of the reaction catalyzed by ATCase, similar to phthalate, does not activate the enzyme but inhibits competitively with L-aspartate (Table I). Inhibition by both phthalate and ureidosuccinate is diminished by increasing the concentration of carbamyl phosphate. At concentrations of maleate which inhibit ATCase the inhibition is not diminished by increasing the concentration of carbamyl phosphate. Malonate activates the enzyme (Figure 1); glutarate does not activate but is a poor inhibitor. Inhibition by glutarate is diminished by an

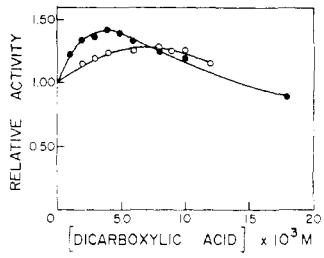

FIGURE 1: Activation of ATCase by malonate and L-malate at pH 7.0; 0.050 M Tris, 0.0015 M carbamyl phosphate, and either 0.002 M L-aspartate, malonate (●), or 0.001 M L-aspartate, L-malate (○), and 5.8 mg of ATCase per ml.

increase in carbamyl phosphate concentration while inhibition by a high concentration of malonate is not. Inhibition by those dicarboxylate dianions which reversibly activate ATCase at low concentrations, is independent of carbamyl phosphate concentration. Whereas, inhibition by dicarboxylate dianions which do not activate the enzyme is diminished by increasing the concentration of carbamyl phosphate.

Reichard and Hanshoff have shown that D-aspartate is not a substrate for ATCase (Reichard and Hanshoff, 1956). It is an extremely poor inhibitor and it does not activate the enzyme at low concentrations of L-aspartate.

Evidently D-aspartate is very poorly bound by ATCase. The proper positioning of the carboxyl groups is probably the gauche or the eclipsed configuration judging from the binding of maleate and the lack of binding of fumarate. It would appear that the proper positioning of the carboxyl groups places the amino group of D-aspartate in a position that renders the entire molecule unacceptable for binding. On this basis a small α substituent in the L configuration would be allowable and L-malate does bind and also activates the enzyme (Figure 1).

Number of Active Sites on ATCase. We have derived an equation in terms of the model of Monod *et al.* which describes the change in activity of ATCase with increasing concentrations of a substrate analog (Monod *et al.*, 1965). The fraction of active sites bound by L-aspartate in the presence of maleate is given by

$$r = \frac{\alpha[(1+\alpha)+\beta]^{n-1} + \alpha cL[(1+\alpha c)+\beta d]^{n-1}}{[(1+\alpha)+\beta]^{n} + L[(1+\alpha c)+\beta d]^{n}} \quad (1)$$

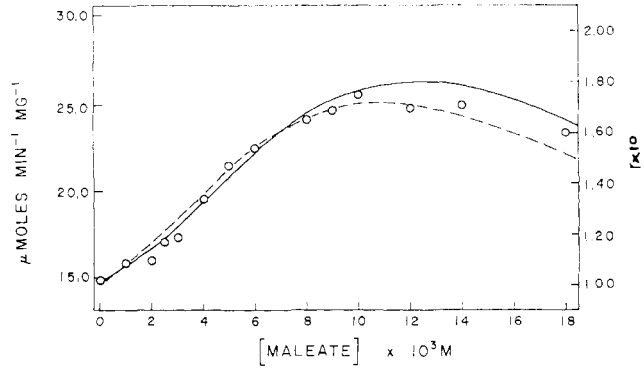

FIGURE 2: Experimental data for maleate activation of ATCase at pH 8.5 (0.005 M L-aspartate–0.001 M carbamyl phosphate) compared to theoretical curve calculated from eq 1 using the parameters in Table II (—), $n = 6$; - - -, $n = 4$).

TABLE II [a]

n	6	4
L	1100	1550
K_{Ra} (M)	5.0×10^{-3}	2.56×10^{-3}
α	1.0	1.95
K_{Rm} (M)	5.0×10^{-3}	2.2×10^{-3}
c	0.097	0.048
d	0.0455	0.010

[a] Parameters selected to fit eq 1 to experimental data for activation by maleate at pH 8.5. See Figure 2.

where α = [L-aspartate]/K_{Ra}, β = [maleate]/K_{Rm}, $c = K_{Ra}/K_{Ta}$, $d = K_{Rm}/K_{Tm}$, $L = T/R$, and n = number of active sites. K_{Ra} and K_{Ta} are dissociation constants for L-aspartate binding to R and T states, respectively. K_{Rm} and K_{Tm} are dissociation constants for maleate binding to R and T states respectively. Since the rate constants for all active sites in both R and T states are taken as identical (Monod *et al.*, 1965), enzyme activity is proportional to r. For an L-aspartate analog to activate ATCase c and d must be less than 1, L must be greater than 1, α must be small (<1), and n must be greater than 1.

If there are only two sites, $n = 2$, the curve of velocity *vs.* (maleate), the activation curve, cannot be sigmoid. The activation curve can be sigmoid if $n > 2$. This relationship gives us an additional means of learning something about the number of active sites and is of some interest since it is not certain that succinate binding corresponds to the number of functional aspartate binding sites.

As shown in Figure 2 the activation curve is distinctly sigmoid at pH 8.5. We have fitted this curve for $n = 4$ and $n = 6$ using the parameters given in Table II. Some of these parameters were obtained from the rate *vs.* (aspartate) curve using (Monod *et al.*, 1965)

$$r = \frac{\alpha(1+\alpha)^{n-1} + \alpha cL(1+\alpha c)^{n-1}}{(1+\alpha)^{n} + L(1+\alpha c)^{n}}$$

and the additional parameters from eq 1.

Since there are two catalytic units and the sigmoid activation curve requires $n > 2$, the minimum value for n is 4, *i.e.*, two active sites per catalytic unit. Since each catalytic unit consists of three polypeptide chains, the question naturally arises as to whether there may not be three active sites per catalytic unit which would give $n = 6$. The curves can be fitted reasonably well for $n = 4$ or $n = 6$ but the data are rather more sigmoid than can be obtained with $n = 4$ so that our results favor $n = 6$.

Reaction of ATCase with Bromosuccinate. Bromosuccinate was conceived as a possible trapping agent for that conformation of ATCase with enhanced affinity for L-aspartate. If bromosuccinate were to bind to sites on ATCase specific for L-aspartate and then, while held there, were to alkylate some reactive group, the resulting enzyme with a "succinate" molecule covalently bound to one active site might be trapped in the conformation which binds substrates well (R state). Trapping of the R state by reaction with bromosuccinate would progressively result in an increase of enzyme activity (measured at low concentrations of aspartate) as one or more

TABLE III [a]

Time (min)	% Inhibn by CTP (A)	% Activation by Maleate (B)
5	44	80
46		50
68	22	
90		30
150		4
185	15	
242	5	
265		−20
310		−23
370	0	−24

[a] The effect of the activation reaction of bromosuccinate (0.01 M DL-bromosuccinate and 0.050 M maleate (A)) or 0.050 M succinate (B), 0.005 M CAP, and 0.05 M Tris (pH 8.5), 25° on inhibition by CTP and activation by maleate. (A) Assay medium: 0.005 M L-aspartate, 0.0015 M CAP, 0.001 M CTP, and 0.05 M Tris, pH 8.5, 25°. (B) Assay medium: 0.005 M L-aspartate, 0.0015 M CAP, ±0.010 M maleate, and 0.05 M Tris, pH 8.5, 25°.

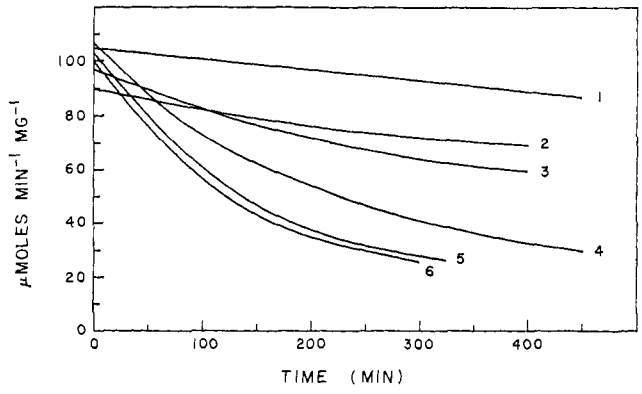

FIGURE 3: Effect of incubation with bromosuccinate on ATCase activity. Reaction mixtures consist of 0.050 M Tris, 0.010 M DL-bromosuccinate, and 0.05 mg/ml of ATCase plus (1) 0.005 M carbamyl phosphate, (2) 0.050 M DL-ureidosuccinate, (3) 0.050 M maleate, (4) 0.050 M succinate, (5) 0.050 M L-aspartate, and (6) no addition. pH 8.5 at 25°. Enzyme activity was determined after various times of incubation.

active sites became succinylated, followed by loss of activity as bromosuccinate reacted with the remaining active sites. This behavior was not observed when ATCase was treated with bromosuccinate.

At pH 8.5, 0.010 M bromosuccinate irreversibly inactivates ATCase with a half-time of about 90 min at 25° (Figure 3). The enzyme was not completely inactivated because of the instability of bromosuccinate under the conditions of these experiments. At pH 7.0, 0.010 M bromosuccinate inactivates ATCase very slowly with a half-time greater than 400 min; bromosuccinate is not stable enough at pH 10.2 to investigate its effect on ATCase at that pH. Heat-dissociated enzyme is inactivated by bromosuccinate at pH 8.5 at approximately the same rate as native enzyme.

If bromosuccinate inactivates ATCase by the mechanism of binding reversibly to L-aspartate sites followed by alkylation of nearby reactive groups on the enzyme, it ought to be possible to slow the rate of inactivation by reacting the enzyme with bromosuccinate in the presence of a high concentration of L-aspartate. In fact, 0.050 M L-aspartate has little effect on the rate of inactivation (Figure 3). However, compounds related to L-aspartate, ureidosuccinate, maleate, and succinate, do slow the rate of inactivation by bromosuccinate (Figure 3). At pH 7.0, where bromosuccinate irreversibly inactivates ATCase very slowly, the reversible inhibition by bromosuccinate is competitive with L-aspartate indicating that bromosuccinate does bind reversibly at the L-aspartate site on the enzyme. The fact that ureidosuccinate, maleate, and succinate slow the rate of inactivation by bromosuccinate and the fact that reversible inhibition by bromosuccinate at pH 7.0 is competitive with L-aspartate seems to indicate that inactivation involves reaction of bromosuccinate with some group(s) near the aspartate site. The failure of L-aspartate to slow inactivation and yet offset reversible inhibition is consistent with the conclusion that the binding of L-aspartate is very much poorer than the kinetic binding of L-aspartate. Published results indicate that L-aspartate binds poorly to ATCase (McClintock and Markus, 1968; Collins and Stark, 1969).

The other substrate for ATCase, carbamyl phosphate, readily protects against bromosuccinate inactivation (Figure 3). The reversible inhibition by bromosuccinate at pH 7.0 decreases when the concentration of carbamyl phosphate is increased, an effect which has been observed with other inhibiting dicarboxylic acids. Bromosuccinate does not produce reversible activation at low concentrations.

When ATCase is reacted with 0.010 M bromosuccinate in the presence of 0.050 M maleate and 0.005 M carbamyl phosphate at pH 8.5, its activity measured with 0.025 M L-aspartate at pH 8.5 increases slowly by about 50%. The slight activation (5% in 10 hr) observed in the presence of maleate and carbamyl phosphate alone could be the result of carbamylation of ATCase by cyanate (Collins and Stark, 1969; G. R. Stark, private communication), one of the breakdown products of carbamyl phosphate (Allen and Jones, 1964). The extent of activation by bromosuccinate is greater when enzyme activity is assayed at 0.005 M L-aspartate (pH 8.5) and amounts to a 3- to 4-fold increase. ATP (0.003 M) slows inactivation by bromosuccinate. Heat-dissociated enzyme is not activated by bromosuccinate but maleate and carbamyl phosphate do protect heated enzyme from inactivation. During activation of native enzyme the ability of the enzyme to be inhibited by CTP (Table III) and activated by maleate at low L-aspartate concentration (Table III) is progressively lost. Loss of CTP inhibition and maleate activation are consistent with dissociation of the enzyme into subunits (Gerhart and Pardee, 1962–1964; Gerhart and Schachman, 1965). However, enzyme activated by treatment with bromosuccinate in the presence of maleate and carbamyl phosphate sedimented in a sucrose density gradient at the same rate as native enzyme (Figure 4).

Bromosuccinate reacts with ATCase in two ways. In the presence of maleate (succinate) and carbamyl phosphate it reacts with the enzyme to cause activation which probably results from a loss of interactions among its subunits. Bromosuccinate alone, reacts with the enzyme to cause loss of activity; it also causes loss of activation by maleate. During inactivation by bromosuccinate the ratio of residual enzyme activity measured at 0.005 M L-aspartate in the presence and absence of 0.010 M maleate decreases (Figure 5). Since loss of maleate activation is also coincident with activation by

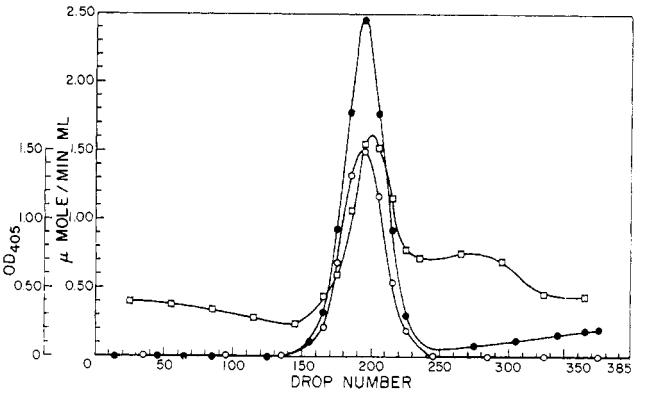

FIGURE 4: Sucrose density gradient centrifugation of ATCase activated by treatment with bromosuccinate in the presence of maleate and carbamyl phosphate. Sucrose gradients were 0.050 M Tris (pH 8.5). Catalase was determined by OD at 405 nm. (1) ATCase (0.055 mg/ml, specific activity 84 μmoles min^{-1} mg^{-1}) incubated in 0.050 M Tris, 0.050 M maleate, 0.005 M carbamyl phosphate, and 0.004 M DL-bromosuccinate (pH 8.5) at 25° for 680 min (●). Activation of ATCase was confirmed prior to centrifugation. (2) ATCase (0.55 mg/ml, specific activity 84 μmoles min^{-1} mg^{-1}) in 0.050 M Tris (pH 8.5) (○). (3) Catalase, 3 mg/ml, 405 nm (□).

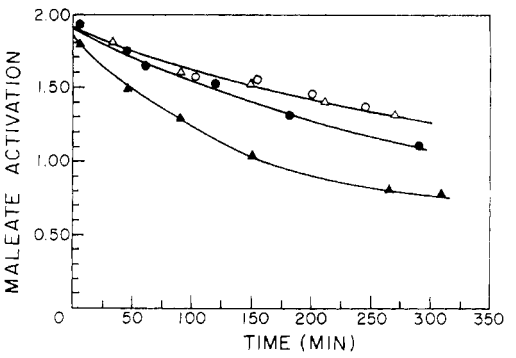

FIGURE 5: Effect of reaction of ATCase with bromosuccinate on maleate activation at low L-aspartate concentration. Incubation mixtures consist of 0.050 M Tris, 0.010 M DL-bromosuccinate (●) plus 0.050 M DL-ureidosuccinate (△), 0.005 M carbamyl phosphate (○), 0.050 M succinate, and 0.005 M carbamyl phosphate (▲) with 0.05 mg/ml of ATCase (specific activity 88 μmoles min^{-1} mg^{-1}) (pH 8.5) at 25°.

bromosuccinate, this result suggests that even in the absence of maleate (succinate) and carbamyl phosphate, bromosuccinate is reacting with some of the enzyme to cause activation but the net result of the two reactions is loss of enzyme activity. During protection of ATCase from bromosuccinate inactivation by ureidosuccinate or carbamyl phosphate, maleate activation of the enzyme decreases (Figure 5) indicating that protection by these compounds is actually the sum of their protecting the enzyme from reaction with bromosuccinate leading to inactivation and an increasee in activity due to reaction of the enzyme with bromosuccinate leading to activation. The results above suggest that the role of maleate (succinate) and carbamyl phosphate during activation of ATCase by bromosuccinate is to prevent reaction of bromosuccinate with groups near the active site which would inactivate the enzyme yet allowing the reaction of bromosuccinate with other groups that weaken the interaction among the subunits. However, the rate of decrease of the ratio of activities measured at 0.005 M aspartate in the presence and absence of 0.010 M maleate is faster when enzyme is reacted with bromosuccinate in the presence of succinate and carbamyl phosphate alone (Figure 5). This indicates that maleate (succinate) and carbamyl phosphate actually facilitate that reaction of bromosuccinate with ATCase which causes activation.

In an effort to understand better the differences between the two reactions of bromosuccinate with ATCase the enzyme was reacted with bromosuccinate-1,4-^{14}C and the degree of labeling on subunits of the enzyme examined by sucrose density gradient centrifugation. The enzyme was reacted with bromosuccinate-1,4-^{14}C, unbound ^{14}C compound was removed by passing the treated enzyme through a Sephadex G-100 column, labeled enzyme was sometimes dissociated by treatment with PMB, and centrifuged on sucrose density gradients. The results are shown in Figures 6 and 7. The fact that the major peak of radioactivity for enzyme not dissociated with PMB coincides with the position of untreated ATCase suggests that bromosuccinate-1,4-^{14}C reacts primarily with native enzyme and not a contaminant in the enzyme preparation. The smaller peaks of ^{14}C label appear at positions on the gradients corresponding to catalytic and regulatory subunits. The possibility of bromosuccinate-1,4-^{14}C reacting with a contaminant of the same molecular weight as ATCase is made highly unlikely by the fact that PMB dissociation of labeled enzyme moves all the ^{14}C label from a position on the sucrose gradient which corresponds to native enzyme to two positions which correspond to catalytic and regulatory subunits.

The number of molecules of succinate bound to each subunit of bromosuccinate-inactivated undissociated enzyme and activated undissociated enzyme was estimated from the distribution of ^{14}C on sucrose density gradient centrifugation patterns. Enzyme activated by treatment with bromosuccinate-1,4-^{14}C in the presence of maleate and carbamyl phosphate (see Figure 6 for details) contained 2.7 molecules of succinate on each catalytic subunit (0.9/catalytic polypeptide chain) and 3.7 molecules of succinate on each regulatory subunit (1.9/regulatory polypeptide chain). Enzyme inactivated by treatment with bromosuccinate-1,4-^{14}C (see Figure 7 for details) contained 2.4 molecules of succinate on each catalytic subunit (0.8/catalytic polypeptide chain) and 2.3 molecules of succinate on each regulatory subunit (1.1/regulatory polypeptide chain). During the activation reaction with bromosuccinate a small amount of active catalytic subunit is formed but the bulk of the enzyme has the native molecular weight. During the inactivating reaction there is considerable label on inactive subunits but most of the enzyme is undissociated.

Uncoupling of Subunit Interactions. In the presence of maleate (succinate) and carbamyl phosphate reaction of bromosuccinate with ATCase results in an "uncoupling" of interactions among subunits in ATCase without dissociation of the enzyme into subunits as is the case with heat (Gerhart and Pardee, 1962–1964; Weitzman and Wilson, 1966) and PMB treatment (Gerhart and Schachman, 1965, 1968). Two reversible treatments of ATCase employed by Weitzman and Wilson (Weitzman and Wilson, 1966) to disrupt interactions among subunits of ATCase were investigated by sucrose density gradient centrifugation to determine whether these treatments have caused dissociation of the enzyme. At pH 10.2 interactions among subunits of ATCase are reversibly lost; however, sucrose density gradient centrif-

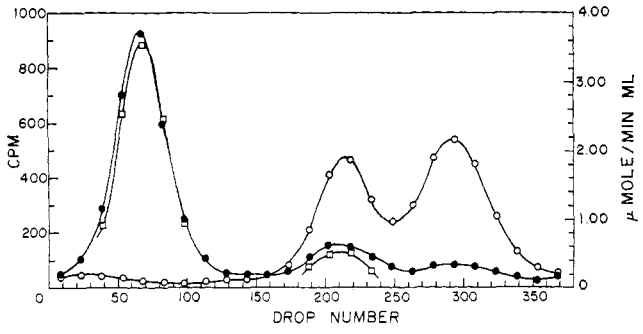

FIGURE 6: Sucrose gradient centrifugation of ATCase activated by incubation with bromosuccinate-1,4-^{14}C in the presence of maleate and carbamyl phosphate. ATCase (0.50 mg/ml, specific activity 80 μmoles min^{-1} mg^{-1}) was incubated with 0.050 M Tris, 0.010 M DL-bromosuccinate-1,4-^{14}C, 0.050 M maleate, and 0.005 M carbamyl phosphate (pH 8.5) at 25° for 400 min; the incubation mixture was then passed through a small Sephadex G-100 column to separate labeled enzyme from unreacted bromosuccinate-1,4-^{14}C. The labeled enzyme was divided into equal portions and layered on gradients (●, ^{14}C; □, enzyme activity) or dissociated by treatment with 0.89×10^{-3} M PMB for 30 min prior to layering on gradients (○, ^{14}C). Sucrose gradients were 0.050 M in Tris (pH 7.0); PMB was not present in any of the gradients.

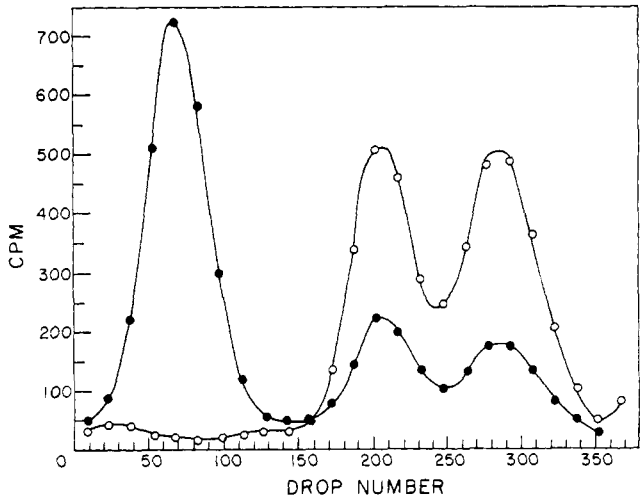

FIGURE 7: Sucrose density gradient centrifugation of ATCase inactivated by incubation with bromosuccinate-1,4-^{14}C. Enzyme (0.5 mg/ml, specific activity 80 μmoles min^{-1} mg^{-1}) was incubated with 0.050 M Tris and 0.010 M DL-bromosuccinate-1,4-^{14}C (pH 8.5) at 25° for 300 min. After incubation enzyme was treated as described in Figure 6. No treatment after inactivation (●); enzyme reacted with 0.89×10^{-3} M PMB after inactivation (○).

ugation of enzyme at pH 10.2 shows that the enzyme does not dissociate into subunits at this pH. Since disruption of interactions among subunits was recognized by an analysis of the enzyme's kinetic behavior, it is possible that at assay levels of enzyme concentration the enzyme may be dissociated at pH 10.2. Sucrose density gradient centrifugation of ATCase at pH 10.2 at protein concentrations comparable to those used in enzyme activity assays indicates that the enzyme has not dissociated at high pH even at low protein concentrations.

Moderate concentrations of urea (0.8–1.0 M) have also been shown to disrupt the interactions among subunits of ATCase (Gerhart and Pardee, 1962; Weitzman and Wilson, 1966). Like enzyme at pH 10.2 ATCase in 1 M urea has not dissociated into subunits but does sediment somewhat slower (10%) than native enzyme.

Discussion

Maleate and succinate at low concentrations have been shown previously to activate ATCase at low concentrations of L-aspartate (Gerhart and Pardee, 1963, 1964). Higher concentrations of maleate and succinate produce inhibition. In this investigation malonate and L-malate were also shown to activate ATCase at low concentrations of L-aspartate. Fumarate, phthalate, DL-ureidosuccinate, glutarate, and D-aspartate were shown not to activate the enzyme. All but the last are inhibitors. Thus some dicarboxylate dianions activate the enzyme at low concentrations and inhibit the enzyme at high concentrations. These compounds might be called activating analogs of L-aspartate, and the explanation for the behavior of these compounds has been given by Gerhart and Pardee. Other dicarboxylate dianions serve only as inhibitors; they are not activating analogs of L-aspartate. Inhibition of both groups is offset by L-aspartate but only the inhibition by the second group is offset by carbamyl phosphate. It appears that activating analogs of L-aspartate do not interfere with the binding of carbamyl phosphate and compounds of the second group do. However, this may not be a direct effect and another explanation is possible. Since the binding of succinate to native ATCase is enhanced by carbamyl phosphate (Changeux *et al.*, 1968; Fellenberg *et al.*, 1968; McClintock and Markus, 1969), we may reasonably assume that the binding of all activating analogs of aspartate is cooperative with carbamyl phosphate. Thus increasing (CAP) will tend to increase the binding of the activating analog and if the kinetic binding or K_m^{-1} of aspartate were not correspondingly increased, more inhibition would occur. Actually there is little change in inhibition, so we are lead to conclude that the kinetic binding of aspartate is also increased by CAP. There are no direct experiments bearing on this question. However, it is known that the kinetic binding of aspartate which of course is measured in the presence of CAP, is much greater than the binding of aspartate to the enzyme. The later quantity is in fact quite weak and difficult to evaluate. Moreover, it is thought that CAP must bind prior to aspartate (Porter *et al.*, 1969).

The other dicarboxylic acids are not cooperative with aspartate and we may assume that they are not cooperative with CAP. In this case increasing (CAP) increases the kinetic binding of aspartate without affecting the binding of the inhibiting dicarboxylic acid and therefore inhibition goes down. In this interpretation the L-aspartate site is the binding site for dicarboxylate inhibitors even though there is competition with CAP. If the effect of CAP is direct, an explanation would require that there is no mutual interaction between the activating analogs of aspartate and CAP but that there is mutual repulsion between the other dianions and CAP. It has been suggested (Porter *et al.*, 1969) that aspartate binds poorly because it gives up some of its binding energy in being compressed with CAP in the ternary complex. While this kind of explanation would be reasonable for some of the analogs, it is not apparent why CAP should interfere with the binding of glutarate and why CAP should not interfere with the binding of L-malate. The poor binding of aspartate might arise from a requirement for the eclipsed conformation.

It is of interest that Collins and Stark have shown that

carbamyl phosphate enhances binding of succinate and L-malate to catalytic subunit at pH 7.0 but not binding of L-aspartate (Collins and Stark, 1969). In nuclear magnetic resonance studies Schmidt *et al.* have shown that carbamyl phosphate induces broadening of the methylene proton signals of malonate and succinate in the presence of catalytic subunit but not that of glutarate (Schmidt *et al.*, 1969).

Although pertaining to the catalytic subunit these observations are similar to our indirect explanation of the effect of CAP on dicarboxylic acid inhibition. Some observations with the catalytic unit do not support a direct effect. Porter *et al.* have shown that inhibition of the catalytic subunit by ureidosuccinate or by glutarate is not competitive with carbamyl phosphate (Porter *et al.*, 1969). Collins and Stark have shown that inhibition of the catalytic subunit by ureidosuccinate is not competitive with L-aspartate (Collins and Stark, 1969). The difference between these observations with the catalytic unit and data presented in this paper for native enzyme could be a reflection of differences between the catalytic subunit and the native ATCase.

ATCase binds four molecules of succinate per molecule of native enzyme (Changeux *et al.*, 1968). Equilibrium dialysis was done in the presence of a saturating concentration of carbamyl phosphate because succinate does not bind well enough to ATCase in the absence of carbamyl phosphate. Since L-aspartate also appears to bind quite poorly to the enzyme (McClintock and Markus, 1968; Collins and Stark, 1969) direct measurement of the number of binding sites for L-aspartate is not possible at this time. Although it may be inferred from the number of succinates bound to the enzyme that there may be as many as four active sites and from studies which show that there are six catalytic polypeptide chains in each molecule of ATCase (Weber, 1968; Wiley and Lipscomb, 1968; Meighen *et al.*, 1970) that there may be as many as six sites, only two active sites are necessary for ATCase to show a sigmoid dependence of rate on L-aspartate concentration and to show activation by L-aspartate analogs at low concentrations of L-aspartate. The sigmoid dependence of enzymic rate on maleate concentration at pH 8.5 is not consistent with only two active sites per molecule of ATCase. Since there are two identical catalytic subunits in ATCase there must be an even number of active sites. Thus there must be at least four active sites and probably six since the enhancement curve is rather more sigmoid than can be accommodated by $n = 4$.

Bromosuccinate has been shown to inactivate ATCase by reacting with groups near the active sites. In the presence of maleate (succinate) and carbamyl phosphate reaction of enzyme with bromosuccinate leads to activation. Activation is accompanied by loss of CTP inhibition and loss of maleate activation at low concentrations of L-aspartate. This loss of interactions among subunits of ATCase by reaction with bromosuccinate in the presence of maleate and carbamyl phosphate does not involve dissociation of the enzyme into subunits. During inactivation by bromosuccinate the ability of the remaining active enzyme to be activated by maleate decreases indicating that the reaction which results in disruption of intersubunit interactions is also occurring in the absence of maleate (succinate) and carbamyl phosphate. When bromosuccinate is reacted with ATCase in the presence of maleate (succinate) and carbamyl phosphate, the enzyme is protected from the inactivating reaction and the results of the uncoupling reaction alone are observed. Succinate and carbamyl phosphate not only protect against the inactivating reaction but also enhance the rate of reaction of bromosuccinate with those groups on the enzyme which causes a loss of intersubunit interactions. These compounds similarly enhance the rate of reaction of PMB with the enzyme (Gerhart and Schachman, 1968; Fellenberg *et al.*, 1968).

During activation bromosuccinate reacts with a single site on each catalytic polypeptide and also with a single site during the inactivation reaction, but these sites are surely different for one produces inactivation and the other does not.

During the activation reaction bromosuccinate reacts with two sites in the regulatory polypeptide chain but only one site during inactivation. We do not know whether one of the two sites alkylated in the activation reaction is the same as the site alkylated during the inactivation reaction.

It is clear from the different groups that are alkylated that the conformation of the native enzyme is altered by the binding of carbamyl phosphate and maleate.

Because of the large number of sulfhydryl groups in this enzyme and because alteration of these groups by reaction with PMB, I_3^- (10^{-6} M, 10 sec, terminated with thiosulfate), or iodosobenzoate (10^{-3} M) results in dissociation (this work) it is reasonable to suppose that sulfhydryl groups are involved in the reaction with bromosuccinate especially on the regulatory subunits but of course other groups such as imidazole may be involved. In any event the introduction of two negative charges per site, 36 charges in all in the activated enzyme, could clearly alter the structure of the native enzyme.

It has been shown previously that heat treatment of ATCase or reaction of the enzyme with PMB destroys interactions among subunits of the enzyme The disruption of intersubunit interactions produced by these treatments has been demonstrated to be due to dissociation of the native enzyme into subunits (Gerhart and Pardee, 1962–1964; Gerhart and Schachman, 1965). In these studies various treatments of ATCase were shown to disrupt interactions among subunits of native enzyme without dissociating the enzyme into subunits. Reaction of enzyme with bromosuccinate in the presence of maleate and carbamyl phosphate, high pH (10.2) and moderate concentrations of urea all destroy the allosteric properties of the native enzyme (see Results and Gerhart and Pardee, 1962, and Weitzman and Wilson, 1966); however, none of these treatments results in dissociation of the enzyme. At pH 10.2 ATCase sediments slightly more slowly (5%) than at pH 7.0. It has been shown that in the presence of succinate and carbamyl phosphate ATCase sediments slower (3.6%) than in their absence (Gerhart and Schachman, 1968). Gerhart and Schachman have attributed this decrease in sedimentation rate to a change in conformation of ATCase mediated by binding of succinate and carbamyl phosphate and have correlated this swelling of the enzyme with enhanced binding of substrates. Both succinate and carbamyl phosphate are required for maximum effect on the sedimentation constant. It is possible then that at pH 10.2 the decreased sedimentation rate of the enzyme represents stabilization at this pH of the conformation of the enzyme with an enhanced affinity for aspartate. The change in properties of ATCase on increasing the pH to 10.2 could well be due to ionization of groups on side chains to give an enzyme molecule with increased negative charge.

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Conformation of Pyridine Dinucleotides in Solution*

John Jacobus

ABSTRACT: Evidence which has been previously presented for a helical conformation of pyridine dinucleotides is reinterpreted. Possible alternate conformations are discussed and it is concluded that the experimental data cannot be interpreted to define unambiguously the molecular geometry of pyridine dinucleotides.

Investigations directed toward the elucidation of the conformation of pyridine dinucleotides have yielded experimental data which has been rationalized on the basis of a helical model (Sarma *et al.*, 1968a,b, 1970; Sarma and Kaplan, 1969a,b, 1970a,b). A reinterpretation of the experimental data indicates that although a helical model may be correct, more prosaic alternatives are plausible, and the choice between a number of these alternate interpretations of the experimental data does not lead to a unique molecular geometry of dinucleotides in solution, *i.e.*, the data can be interpreted without consideration of any particular model of the molecules in question and need not be a consequence of the helicity of these molecules.

* Contribution from the Department of Chemistry, Clemson University, Clemson, South Carolina 29631. *Received September 10, 1970.* Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research.

Definitions

Nuclei or groups of nuclei in molecules can be classified on the basis of symmetry (Mislow and Raban, 1967). The ramifications of such classification as applied to nuclear magnetic resonance spectroscopy, as well as to chemical phenomena in general (Mislow, 1966), have been enunciated (Mislow and Raban, 1967). In order to facilitate the ensuing discussion, these classifications will be defined and employed. Numerous examples have been presented elsewhere (Mislow and Raban, 1967) and will not be reiterated.

Nuclei or groups of nuclei in molecules that can be interconverted by a rotational symmetry operation (Mislow, 1966) are defined as *equivalent*. Equivalent nuclei or groups reside in equivalent environments; such nuclei or groups are isochronous (chemical shift equivalent) in nuclear magnetic resonance spectroscopy.

Nuclei or groups of nuclei in molecules that can be interconverted by a reflectional, but not by a rotational, symmetry