heterogeneity has been proposed previously by Denburg and McElroy (1970). All attempts to separate two polypeptides from the apparently homogeneous enzyme have failed. However, knowledge that the two SH-I sulfhydryls do exist in the same polypeptide might serve as a basis for designing future separation schemes.

Registry No. pMB, 2979-65-9; MMTS, 2949-92-0; NEM, 128-53-0; IAAm, 144-48-9; DTNB, 69-78-3; ATP, 56-65-5; LH_2, 2591-17-5; L, 20115-09-7; LAMP, 24404-89-5; luciferase, 61970-00-1.

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Ligand Interactions at the Active Site of Aspartate Transcarbamoylase from *Escherichia coli*†

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ABSTRACT: The active site of aspartate transcarbamoylase from *Escherichia coli* was probed by studying the inhibitory effects of substrate analogues on the catalytic subunit of the enzyme. The inhibitors were chosen to satisfy the structural requirements for binding to either the phosphate or the dicarboxylate region. In addition, they also contained a side chain that would extend into the normal position occupied by the carbamoyl group. All the compounds tested showed competitive inhibition against carbamoyl phosphate. The ionic character of the side chain was found to be highly important in determining the affinity of the inhibitor. On the other hand, very little effect on binding was produced by changing the geometry of the functional group from trigonal to tetrahedral. Our findings suggest that the electrostatic stabilization of the negative charge that develops in the transition state may be a major factor in promoting catalysis. From the available X-ray diffraction data, we propose His-134 as the residue most likely to participate in this interaction. These results have significant implications on the design of reversible and irreversible inhibitors to this enzyme.

Aspartate transcarbamoylase (ATCase)[1] catalyzes the condensation of L-aspartate and carbamoyl phosphate to form carbamoyl-L-aspartate, which is the first intermediate in pyrimidine biosynthesis (Reichard & Hanshoff, 1956). The reaction is crucial for cell division since it provides the required precursors for the production of DNA. Accordingly, the mammalian enzyme is highly active in rapidly proliferating

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[1] Abbreviations: ATCase, aspartate transcarbamoylase (EC 2.1.3.2; aspartate carbamoyltransferase); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IR, infrared; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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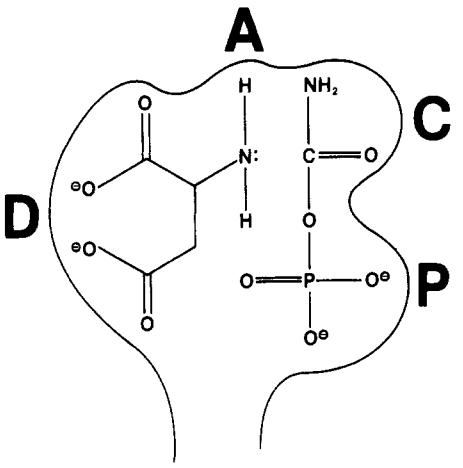


FIGURE 1: Diagrammatic representation of the active site of ATCase. The letters denote various regions referred to in the text: A, amino; C, carbamoyl; D, dicarboxylate; and P, phosphate.

tissues and is considered a suitable target for the action of antineoplastic agents.

The structure and function of the enzyme from *Escherichia coli* have been under intensive investigation [for a review see Kantrowitz et al. (1980a,b)]. The native enzyme is composed of distinct catalytic and regulatory subunits which can be separated (Gerhart & Schachman, 1965). Stark and coworkers have carried out penetrating studies on the isolated catalytic subunit and have clarified many aspects of the catalytic mechanism [see Jacobson & Stark (1973)]. This work has led to the discovery of the tight-binding inhibitor *N*-(phosphonoacetyl)-L-aspartate (PALA), which combines structural features of both substrates (Collins & Stark, 1971). PALA is also highly effective toward mammalian ATCase and inhibits tumor growth in experimental systems (Swyryd et al., 1974). These findings indicate that the *E. coli* enzyme may serve as a model for the further development of mechanism-based inhibitors of potential therapeutic value.

The object of the present study is to probe the active site of the catalytic subunit of *E. coli* ATCase by using a variety of synthetic as well as commercially available compounds. For the purpose of our discussion, this active site may be divided into four regions according to their interactions with substrate molecules (Figure 1). The extensive work of Stark and others has clearly delineated the dicarboxylate and phosphate regions (Porter et al., 1969; Davies et al., 1970). The former has a strong preference for two carboxyl groups that are in the cis conformation and are connected by two carbon atoms as exemplified by maleate (K_i = 4.3 mM). The latter region has a general requirement for a phosphate or phosphonate moiety but is otherwise nonspecific with regard to substituents. Even the terminal phosphate of CTP binds with an affinity (K_i = 0.32 mM) typical of simple phosphates. In addition, the amino region is known to exert a severe steric hindrance on substituents in this position. However, relatively little information is available concerning the carbamoyl region in spite of its obvious relevance to the catalytic mechanisms. Specifically, it seemed important to us to investigate whether geometrical or electrostatic destabilization of the substrates in their ground states might play a role in catalysis. In this paper we report results relevant to these aspects of the mechanism.

Materials and Methods

Source of Materials. ^{14}C-Labeled carbamoyl phosphate (10.4 mCi/mmol) was obtained from New England Nuclear and diluted with unlabeled dilithium carbamoyl phosphate in 200 mM sodium formate buffer, pH 3.8. The diluted sample (final concentration, 50 mM) was divided into small portions and stored at −70 °C. ACS scintillation fluid was supplied by Amersham Corp.

The synthetic reagents sulfur trioxide–pyridine complex, dibenzyl phosphite, *N*-chlorosuccinimide, 10% palladium/charcoal catalyst, and diethyl(bromoethyl)phosphonate and the inhibitors methylenediphosphonic acid, 1,2,3,4-butanetetracarboxylic acid, 1,2,4-benzenetricarboxylic acid, and 1,2,4,5-benzenetetracarboxylic acid were purchased from Aldrich. Other fine chemicals were products of Sigma Chemical Co.

N-Carbamoyl-L-aspartate was prepared by enzymatic synthesis (Porter et al., 1969). The column fractions containing the product were evaporated under vacuum. The residue was triturated with acetone and kept at −20 °C to give white crystals of the compound.

Aspartate transcarbamoylase was isolated by the method of Gerhart and Holoubek (1967). The subunits were separated by reaction with neohydrin, followed by DEAE-cellulose chromatography (Schachman, 1972). Preparations of the catalytic subunit were stored in 40 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Malate dehydrogenase and glutamate–oxaloacetate transaminase were both from pig heart and were supplied by Boehringer Mannheim in the form of ammonium sulfate suspensions. The transaminase was dialyzed against 0.1 M NaHEPES buffer, pH 7.5, shortly before use.

Analytical Procedures. Protein concentrations were generally determined from the absorbance at 280 nm. Values of absorbance for 1 mg/mL solutions (1-cm path length) were taken as 0.59 for aspartate transcarbamoylase, 0.72 for the catalytic subunit, and 0.32 for the zinc regulatory subunit (Nelbach et al., 1972).

Enzyme activity in the forward direction was measured by following incorporation of ^{14}C label from carbamoyl phosphate by using a modified version of the previous assay (Swyryd et al., 1974). The enzyme (1–4 ng of catalytic subunit/mL) was preincubated for 30 min at 25 °C with the inhibitor in NaHEPES buffer (100 mM, pH 7.5) containing sodium aspartate (15 mM) and bovine serum albumin (50 μg/mL). For convenience, the reaction was carried out directly in scintillation vials (Beckman, Mini Poly Q) with a final volume of 0.4 mL. The reaction was initiated by the addition of [^{14}C]carbamoyl phosphate (about 60 000 cpm per assay), then incubated for 15 min at 25 °C, and finally terminated by the addition of 50% trichloroacetic acid (0.1 mL). Subsequently, the vials were transferred to a boiling water bath for 5 min, and small amounts of crushed dry ice were added to complete the removal of $^{14}CO_2$. After the addition of 7 mL of ACS scintillation fluid, the radioactivity of the sample was measured in a Beckman LS-230 scintillation counter.

One unit of activity is defined in this work as the amount of enzyme catalyzing the formation of 1 μmol of carbamoyl phosphate per minute. Depending on the degree of inhibition, the amount of enzyme in the assay was varied to maintain approximately the same level of carbamoyl phosphate consumption (5–25%). Under these conditions, linearity and good signal-to-noise ratios were obtained.

Activity of the enzyme in the reverse direction was followed by coupling to NADH oxidation (Foote & Lipscomb, 1981). The assay mixture contained in a final volume of 0.8 mL of NaHEPES (80 μmol), α-ketoglutarate (1.6 μmol), NADH (0.12 μmol), bovine serum albumin (40 μg), glutamate–oxaloacetate transaminase (40 μg), malate dehydrogenase (1.9 μg), ATCase catalytic subunit (6.4 μg), and *N*-carbamoyl-L-

aspartate (1.33–4 μmol). The reaction was initiated by the addition of 20 μL of 0.2 M sodium arsenate and was monitored at 340 nm for 10 min at 25 °C. The reaction was linear with time over this period. Inhibitors were studied over a 4-fold range of concentrations in the neighborhood of the K_i value. *N*-Substituted derivatives of aspartate were checked for contamination by free aspartate by using the above coupled system. In addition, the rate of any spontaneous (nonenzymatic) breakdown was determined. Only *N*-phosphoryl-aspartate decomposed significantly (with a half-life of 11.6 h) under assay conditions. In this case, the enzymatic rate was obtained by correcting for the background value.

The pattern of inhibition was determined by analyzing the data with double-reciprocal plots (Lineweaver & Burk, 1934) while the inhibition constant was estimated according to Dixon (1953). At least two complete sets of duplicate assays (thirty-two in all) were conducted for each inhibitor with four different concentrations of both carbamoyl phosphate and inhibitor. The K_i values listed are average values of separate determinations.

Melting points were determined on a Fisher-Johns hot-stage apparatus and were uncorrected. Infrared measurements were carried out on a Perkin-Elmer grating spectrophotometer (PE-283). Proton nuclear magnetic resonance spectra were recorded on a Varian EM-390 spectrometer, and chemical shifts (δ) are reported as parts per million (ppm) downfield from tetramethylsilane. Synthetic intermediates and inhibitors were generally analyzed by thin-layer chromatography (TLC) on silica gel G (Analtech). The chromatograms were visualized in some cases by iodine vapor or ultraviolet light. Compounds containing thiol groups were detected by spraying with a 2% solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in aqueous methanol containing 10% water. The spots appear yellow after exposure to pyridine vapor. This method was also used for disulfides, which could be reduced by coating the chromatogram with a freshly prepared solution of $NaBH_4$ (1%) in methanol. The excess reagent was then removed by repeated spraying with 3 M ethanolic HCl before applying DTNB. Thiol ester derivatives were similarly identified by treating the TLC plate with ammonia vapor after spraying with the above DTNB reagent. Sulfonic acids were located by spraying with a 0.15% solution of sodium fluorescein in ethanol containing 15% water and 1.5% silver nitrate. Other acidic compounds were detected with a pH indicator consisting of a solution of 0.01% bromocresol green and 10% pyridine in acetone. Amines were identified by spraying with a freshly prepared 0.2% ninhydrin solution in 1-butanol containing 0.1% 2,4,6-collidine. Phosphonate derivatives were visualized by applying the reagent of Hanes and Isherwood (1949).

Free amino groups were determined by reaction with 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1966; Chan & Enns, 1981). Phosphate content was measured colorimetrically with molybdate/malachite green according to Chalvardjian and Rudnicki (1970) using Triton X-100 as a detergent (Duck-Chong, 1979).

N-(Methylsulfonyl)-L-aspartate. Methanesulfonyl chloride (153 mmol) was added slowly with stirring to a chilled solution of L-aspartic acid (76 mmol) in aqueous NaOH (2 M, 120 mL). The mixture was allowed to warm to room temperature and after 3 h was acidified with concentrated HCl (35 mL). Extraction with ethyl acetate, and subsequent drying and evaporation, gave *N*-(methylsulfonyl)-L-aspartic acid in 22% yield, mp 184–186 °C (from 1-propanol/petroleum ether): R_f = 0.88 (ninhydrin-negative) in $CH_2Cl_2/CH_3OH/HCO_2H$ (80:20:1); NMR (D_2O) δ 2.9 (d, 2 H, $-CH_2-$), 3.0 (s, 3 H, $-CH_3$), and 4.4 (t, 1 H, methine).

N-Sulfo-L-aspartate. L-Aspartic acid was sulfonated with sulfur trioxide–pyridine complex in potassium carbonate buffer according to the method of Baumgarten et al. (1932) and repeated to ensure complete derivatization. Owing to the previously known sensitivity of the derivative to the acid, it was isolated as the potassium salt. Following the original procedure, acetic acid was used for neutralization and the small contamination by acetate prevented characterization by elemental analysis. The product showed a single spot on TLC (R_f = 0.24) in 1-propanol/10 mM NaOH (7:3) as detected by iodine vapor and sulfonate spray. The chromatogram was also ninhydrin-negative unless pretreated with acid. IR (KBr) showed among other bands 1390- and 1200-cm^{-1} bands characteristic for sulfonyl groups. Quantitative analysis confirmed the presence of the predicted amount of free amino groups after acid treatment.

N-Phosphoryl-L-aspartate. Dibenzyl L-aspartate (22.3 mmol) was condensed with dibenzyl phosphochloridate (approximately 24 mmol) as prepared according to Atherton (1957) in $CHCl_3$ (110 mL) containing 7% Et_3N under conditions described by Li (1952). After washing and drying, the intermediate dibenzyl *N*-(dibenzylphosphoryl)aspartate was chromatographed on silica gel and eluted with CH_2Cl_2/CH_3OH (97:3) to give white crystals in 86% yield; mp 47–49 °C (from ether/petroleum ether, lit. mp 46–47 °C; Saias & Kornowski, 1968): NMR ($CDCl_3$) δ 2.9 (m, 2 H, $-CH_2-$), 3.9 (d, 1 H, NH), 4.2 (t, 1 H, methine), 5.0 (m, 8 H, aryl CH_2), and 7.3 (s, 20 H, arylH).

The benzyl groups were removed by hydrogenation in methanol containing 4 equiv of Et_3N with 10% Pd/C as catalyst according to Saias and Kornowski (1968). The trisodium salt of *N*-phosphoryl-L-aspartic acid was precipitated by the addition of 4 equiv of solid NaOH. The final product was extremely labile in acidic solutions. It was stabilized by dissolving in 10 mM NaOH and was used immediately to avoid decomposition. The actual concentration in the sample was analyzed by quantitative amino group analysis before and after acid hydrolysis and confirmed by phosphate analysis. The inhibition constants were determined with samples containing less than 10% contamination from hydrolysis.

DL-*(Sulfomethyl)succinate.* This compound was synthesized in three steps as follows. Itaconic acid (10 mmol) was mixed with thiolacetic acid (14 mmol) and heated with stirring at 80 °C for 3 h. Evaporation of excess thiolacetic acid under reduced pressure and trituration with benzene gave DL-[(acetylthio)methyl]succinic acid in 72% yield, mp 91–92 °C (from benzene, lit. mp 90.5–91.5 °C; Holmberg & Schjanberg, 1940): R_f = 0.75 in $CH_2Cl_2/CH_3OH/HCO_2H$ (90:10:1); IR (KBr) 1705 cm^{-1} (thiol ester); NMR (methanol-d_4) δ 2.25 (s, 3 H, methyl), 2.65 (d, 2 H, CH_2CO), and 2.8–3.4 (m, 3 H, CH_2–S and methine proton). Anal. Calcd for $C_7H_{10}O_5S$: C, 40.76; H, 4.89; S, 15.54. Found: C, 40.75; H, 4.78; S, 15.65.

The above thiol ester (1 mmol) was hydrolyzed with 2 N NaOH in 50% aqueous ethanol at 80 °C for 1 h. Acidification and extraction into ethyl acetate gave a mixture of the free thiol and its disulfide (0.15 g) with R_f = 0.74 and 0.46, respectively, in $CH_2Cl_2/CH_3OH/HCO_2H$ (90:10:1). This material was directly oxidized with performic acid (freshly prepared from 9.5 mL of formic acid and 0.5 mL of 30% H_2O_2) at room temperature for 12 h. After removal of solvent and reagent, the aqueous solution was neutralized with NaOH. The trisodium salt of DL-(sulfomethyl)succinic acid was precipitated with ethanol as a white amorphous solid in 58% yield, mp >300 °C: R_f = 0.39 in $CH_2Cl_2/CH_3OH/HCO_2H$

Table I: Inhibition of ATCase by Phosphate and Phosphonate Derivatives[a]

compd, R = $-PO_3^{2-}$	K_i (mM)
R–OH	0.36[b]
R–O–PO_3H^-	0.051[c]
R–CH_2–PO_3H^-	0.037
R–CH_2–CO_2^-	0.15[c]
R–CO_2^-	0.022
R–CH_2–NH_3^+	>40
R–CH_2–CH_2–NH_3^+	>40
R–O–CH_3	0.53[c]
R–CH_2–CH_2–SH	0.91

[a] Kinetic studies were performed at pH 7.5 and 25 °C with 15 mM aspartate and 10–50 μM carbamoyl phosphate. All compounds tested here show competitive inhibition against carbamoyl phosphate. [b] Literature values range from 0.6 (Heyde & Morrison, 1973) to 1.45 mM (Porter et al., 1969). [c] The constants reported by Porter et al. (1969) for these inhibitors are approximately twice the values given here.

(70:30:1); NMR (D_2O) δ 2.6–3.6 (m, 4 H, CH_2–CO and CH_2–S) and 3.8 (m, 1 H, methine proton). Anal. Calcd for $C_5H_5O_7SNa_3 \cdot H_2O$: C, 20.27; H, 2.38; S, 10.82. Found: C, 20.46; H, 2.21; S, 10.79.

(2-Mercaptoethyl)phosphonate. Diethyl (2-bromoethyl)-phosphonate (4 mmol) in 20 mL of absolute ethanol was added to a cooled solution of thiolacetic acid (5 mmol) and KOH (4.4 mmol) in 15 mL of absolute ethanol. After the mixture was stirred overnight at room temperature, it was filtered, evaporated, and dissolved in methylene chloride. Washing with saturated bicarbonate and removal of solvent gave diethyl [2-(acetylthio)ethyl]phosphonate as a yellow oil in 80% yield: R_f = 0.49 in CH_2Cl_2/CH_3OH (96:4); IR (film) 1695 cm^{-1} (thiol ester); NMR ($CDCl_3$) δ 1.3 (t, 6 H, CH_3–CH_2), 2.1 (m, 2 H, CH_2–P), 2.3 (s, 3 H, $SCOCH_3$), 3.0 (m, 2 H, CH_2–S), and 4.5 (m, 4 H, CH_3CH_2–).

The thiol ester (1.1 mmol) was hydrolyzed in 10 mL of 30% HBr in acetic acid at 80 °C for 3 h with the exclusion of moisture. After evaporation in vacuo, the residue was dissolved in 4 mL of methanol, and dicyclohexylamine (2 mmol) was added. Concentration and addition of acetone gave the dicyclohexylammonium salt of (2-mercaptoethyl)phosphonic acid as white crystals in 82% yield, mp 205–209 °C: R_f = 0.55 in 0.25 M LiCl on poly(ethylenimine)–cellulose plates; NMR (D_2O) δ 0.5–1.7 (m, 22 H, cyclohexyl protons) and 1.8–2.8 (m, 4 H, P–CH_2–CH_2–S). Anal. Calcd for $C_{14}H_{30}NO_3PS$: C, 51.97; H, 9.36; N, 4.33; S, 9.91. Found: C, 53.48; H, 8.88; N, 4.42; S, 9.87.

Results and Discussion

Although the carbamoyl region is the object of our study, it is too small to interact strongly and specifically with ligands by itself. Our approach, therefore, is to test the inhibitory effect of compounds that bind primarily to adjacent regions but have substituents capable of extending into the carbamoyl region. In this way, we can take advantage of the previous knowledge concerning the phosphate and dicarboxylate regions to align the ligand in the desired manner.

Phosphate and Phosphonate Derivatives. Our most clear-cut results were obtained with phosphate and phosphonate derivatives (Table I). Although many phosphorus compounds had been tested as inhibitors previously (Porter et al., 1969), no correlation had been made between binding affinity and the character of the side chain. In the present study, we found methylenediphosphonate and phosphonoformate to be potent inhibitors of ATCase. This observation, together with the already known affinity of the enzyme for pyrophosphate and phosphonoacetate, showed clearly the effect of negative charges proximal to the phosphate group. Since the inhibition was in all cases competitive toward carbamoyl phosphate, it is reasonable to assume the same mode of binding. The results therefore indicate the presence of an ionic interaction in the carbamoyl region. Although methylenediphosphonate was a much stronger inhibitor than phosphonoacetate, there is no definite preference for a tetrahedral configuration in this position. Thus the shorter homologue phosphonoformate had a remarkably high affinity in spite of its trigonal functional group.

Of particular significance was the observation that phosphonate derivatives containing positively charged amino groups were very poor inhibitors. The side chains of these compounds are not bulkier than the carbamoyl group. There is consequently no steric hindrance to exclude them from the carbamoyl region. Therefore, the extremely low affinity must be attributed to electrostatic repulsion at that site. The finding that neutral substituents have little effect on the strength of binding also supports the above interpretation.

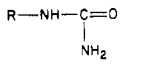
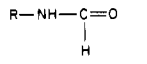
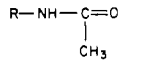
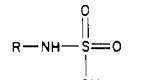
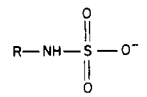
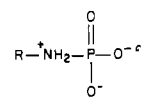
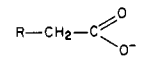
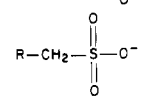
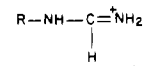
Aspartate and Methylsuccinate Derivatives. The ionic interaction in the carbamoyl region suggested by the above results was also probed from the direction of the dicarboxylate region. Among the various aspartate and methylsuccinate derivatives tested, the same preference by the enzyme for a negatively charged side chain was observed. For example, the change from the neutral *N*-(methylsulfonyl) substituent to the sterically similar but charged *N*-sulfo group increased the affinity 18-fold. The best inhibitor of this series was *N*-phosphorylaspartate, which binds approximately 100-fold tighter than the typical neutral derivative. As in the case of phosphonate derivatives, there is no clear preference for either the trigonal or tetrahedral configuration in these inhibitors. However, the interpretation of these results is subject to considerable uncertainty because of possible alternate modes of binding. The established kinetic mechanism (Porter et al., 1969; Collins & Stark, 1971) stipulates that the binding of carbamoyl phosphate must precede that of aspartate. Therefore, negatively charged aspartate derivatives might very well bind to the phosphate region. In fact, simple anions are known to be inhibitors at this site (Jacobson & Stark, 1975).[2] In our case, the lack of specificity could be shown by the fact that the dissociation constant of the D form of *N*-sulfoaspartate was only slightly higher than that of its L isomer (Table II).

In an attempt to resolve the above ambiguity, we tested the same inhibitors using the reverse reaction. Foote and Lipscomb (1981) had developed a coupled assay based on the transamination of aspartate and reduction of the oxaloacetate so generated. According to the sequential mechanism for this enzyme, phosphate would be the first substrate to bind in the reverse direction. The above series of inhibitors would then act as analogues of carbamoyl aspartate (the other substrate). In order to prevent undesirable binding to the phosphate region, excess phosphate could then be added. (In practice, arsenate would be used instead of phosphate since the spontaneous breakdown of carbamoyl arsenate would produce a linear assay.) However, we found that high arsenate (or phosphate) concentrations could not be used since they increased the K_m of carbamoyl aspartate[3] as well as the K_i of

[2] Although Jacobson and Stark (1975) described the binding of dicarboxylates and monoanions to the enzyme, it was not evident that any interactions with the carbamoyl region were involved.

[3] Foote and Lipscomb (1981) had also reported that the K_m of carbamoyl aspartate rose by more than 3-fold when the phosphate concentration was increased from 3 to 25 mM. These authors however did not discuss the possibility of binding to the carbamoyl region.

Table II: Inhibition of ATCase Catalytic Subunit by Dicarboxylate Derivatives[a]

compd.	K_i, forward reaction (mM)	K_i, reverse reaction (mM)
	0.95[b]	17.4 (K_m)
	7.4	16.9
	6.9	28.2
	9.1	44
	0.49 (L) 0.87 (D)	2.2 (L) 4.6 (D)
	0.05	0.48
	1.2 (DL)	5.1 (DL)
	2.9 (DL)	4.2 (DL)
	~40	>200

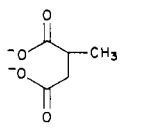
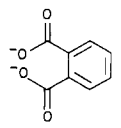
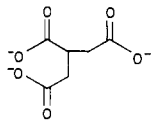
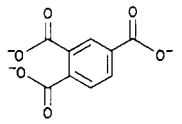
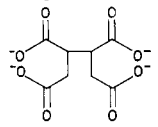
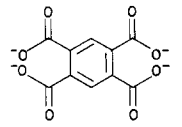
[a] The assay conditions for the forward reaction were the same as those given in Table I. In the reverse reaction the substrate concentrations were as follows: 5 mM arsenate and 1.65–5 mM carbamoyl aspartate. [b] The literature value for carbamoyl aspartate is 1.5 mM (Heyde et al., 1973). [c] This derivative is depicted as the zwitterion on the basis of previous work (Benkovic & Sampson, 1971).

the above inhibitors to experimentally unacceptable levels. (The most obvious interpretation of this behavior in view of our other results would be that phosphate might also bind in the carbamoyl region.) We are therefore forced to use a nonsaturating concentration of arsenate. At 5 mM arsenate (about $2K_m$) the pattern of K_i values of these inhibitors closely paralleled those measured in the forward reaction. In general, the affinities were lower by a factor of about 5. Unfortunately, again very little stereospecificity was shown toward the optical isomers of *N*-sulfoaspartate, suggesting that abnormal binding of these negatively charged side chains was occurring.

As in the case of the phosphonate derivatives, the effect of a positively charged side chain is highly relevant. Thus *N*-formimino-L-aspartate would not be expected to bind to the phosphate region because of its ionic character, and since it is smaller than carbamoyl aspartate it should not encounter steric hindrance at the carbamoyl region. Therefore, one must again invoke electrostatic repulsion as the reason for its extremely low affinity. If we use the K_m for carbamoyl aspartate as a measure of affinity in the normal binding mode, then the presence of a positive charge weakens the interaction by more than 10-fold.

Effect of Multiple Negative Charges. The dicarboxylate region is known to bind two spatially distinct negative charges, and the phosphate region represents an additional anion-binding pocket. If the above interpretation of our results is correct and the carbamoyl region has similar ionic properties, then there should be four separate sites that interact with negative charges. In such a situation, it is not surprising that the mode of binding of simple inhibitors may be uncertain.

Table III: Dependence of Inhibitor Affinity upon the Number of Carboxylate Groups[a]

inhibitor	K_i (mM)	inhibitor	K_i (mM)
	16		1.2
	1.2		0.16
	0.30		0.050

[a] For conditions see Table I. All compounds tested here show competitive inhibition against carbamoyl phosphate.

We have therefore used an alternative approach by examining the effect of multiple ionic substituents on the strength of binding to the enzyme. In both aliphatic and aromatic series, the affinity of the inhibitor is markedly enhanced as the number of carboxylate groups increases from two to four (Table III). Although the effect is greater for the addition of the third negative charge, there is little doubt that further contribution to binding is provided by the fourth carboxylate moiety. In these simple derivatives, it is most unlikely that the orientation of the substituents is optimal for interaction with the various protein side chains. Since the problem of achieving simultaneous interactions is magnified with an increasing number of functional groups, the relatively modest (3–4-fold) improvement in affinity for the fourth substituent is highly significant. The combined evidence therefore indicates the presence of a positively charged side chain in the carbamoyl region of the enzyme.

General Discussion. The location of a charged side chain in the critical carbamoyl region suggests that it might play an important role in catalysis. Several possible functions for this group may be considered. First, it may be the acid catalyst that directly donates a proton to the carbamoyl oxygen in the transition state. Stark and co-workers have presented evidence from ^{13}C NMR experiments (Roberts et a., 1976) that is consistent with such a process. In order to function as a proton donor, its pK_a should be near the pH of the reaction. Second, this group may interact with an ionizable moiety such as the hydroxyl of a tyrosine and lower the pK_a of the latter sufficiently for it to act as a proton donor. Finally, the function of this group may be to stabilize the negative charge that develops as the lone pair of electrons on the nitrogen attack to form a tetrahedral intermediate. In the case of lysozyme, a similar electrostatic stabilization of the transition state is considered to be the major factor in promoting catalysis (Warshel & Levitt, 1976).

The identity of the charged group implicated by our results remains a matter for conjecture. Chemical modification (Kantrowitz & Lipscomb, 1976) and X-ray crystallography (Honzatko & Lipscomb, 1982) both point to the presence of arginines in the active site. The location of Arg-54 and Arg-105 suggests that they might be involved in phosphate binding. However, Arg-167 is a possible candidate for interactions at the carbamoyl region. Several lysines have also been identified as essential residues (Greenwell et al., 1973; Lauritzen & Lipscomb, 1982). Although Lys-164 is in a position to interact with Tyr-165 and to facilitate proton

transfer, both residues are too far away to have any effect on the carbamoyl region. We believe that the group most likely involved in the binding of the above inhibitors is His-134. In the difference map obtained by X-ray diffraction of the enzyme–pyrophosphate complex (Honzatko & Lipscomb, 1982), the imidazole ring of this residue is in van der waals contact with the second phosphate group. Furthermore, the latest structure of the enzyme–PALA complex at 2.9 Å (Krause et al., 1985) showed this residue to be near the carbamoyl group of the inhibitor. Since the charge of a protonated histidine side chain may be delocalized between the two imidazole nitrogens, significant interactions may occur with inhibitor functional groups at different positions. This variation in the location of the charge may explain the unusual results concerning the tight binding of both pyrophosphate and phosphonoformate.

The nucleophilic attack of aspartate on carbamoyl phosphate is generally considered to proceed via a tetrahedral intermediate (Stark, 1971). An interesting question is whether the enzyme lowers the activation energy by geometrical destabilization of the substrate relative to the transition state. One possibility is for the active site to be complementary to the tetrahedral intermediate. If so, then substrate analogues with a tetrahedral configuration at the carbamoyl carbon should bind more tightly to aspartate transcarbamoylase. However, our results do not support this postulated stereochemical preference. It has also been reported previously that the tetrahedral alcohol obtained by the reduction of (4,5-dicarboxy-2-ketopentyl)phosphonate is a very poor inhibitor compared to its parent compound (Roberts et al., 1976). One possible explanation is that the geometry of the transition state being stabilized lies somewhere between the trigonal form of substrates (or products) and the tetrahedral form of the intermediate. In any case, electrostatic interactions may be generally more effective than physical distortion as a catalytic mechanism since it does not require the enzyme to be extremely rigid.

The anion-binding properties of the carbamoyl region suggest that certain charged compounds might be suitable reagents for the chemical modification of this site. Previously, the permanganate ion has been used as a probe of the phosphate region on account of its structural similarity to phosphate (Benisek, 1971). At low concentrations, it is known to react specifically with the single thiol group in the catalytic polypeptide. However, in view of the multiple ionic interactions at the active site, the observed specificity might be fortuitous. We have therefore tested similar compounds in an attempt to probe the carbamoyl region. Preliminary experiments show that the less reactive periodate ion appears to react selectively at this site. Inactivation of the enzyme occurs at low reagent concentrations and can be prevented by ligands capable of interacting at the carbamoyl region.[4]

Our results also imply that analogues of PALA containing a negative charge at a position equivalent to the acyl group might be extremely tight inhibitors of ATCase. The design and synthesis of this type of inhibitor are currently in progress in our laboratory.

Registry No. ATCase, 9012-49-1; $HOPO_3^{2-}$, 14066-19-4; $HO_3POPO_3^{2-}$, 42499-21-8; $H_2O_3PCH_2PO_3H_2$, 1984-15-2; $HO_2CCH_2PO_3H_2$, 4408-78-0; $HO_2CPO_3H_2$, 4428-95-9; $H_2NCH_2PO_3H_2$, 1066-51-9; $H_2N(CH_2)_2PO_3H_2$, 2041-14-7; $MeOPO_3H_2$, 812-00-0; $HS(CH_2)_2PO_3H_2$, 43064-23-9; L-$NH_2CONHCH(CH_2CO_2H)CO_2H$, 13184-27-5; L-$OHCNHCH(CH_2CO_2H)CO_2H$, 19427-28-2; L-$MeCONHCH(CH_2CO_2H)CO_2H$, 997-55-7; L-$MeSO_2NHCH(CH_2CO_2H)CO_2H$, 100466-75-9; L-$HO_3SNHCH(CH_2CO_2H)CO_2H$, 100466-76-0; D-$HO_3SNHCH(CH_2CO_2H)CO_2H$, 100466-77-1; L-$H_2O_3PNHCH(CH_2CO_2H)CO_2H$, 17497-10-8; (±)-$HO_2CCH_2CH(CH_2CO_2H)CO_2H$, 99-14-9; (±)-$HO_3SCH_2CH(CH_2CO_2H)CO_2H$, 100466-78-2; L-$NH{=}CHNHCH(CH_2CO_2H)CO_2H$, 2374-41-6; $HO_2CCH(Me)CH_2CO_2H$, 498-21-5; $HO_2CCH[CH(CO_2H)CH_2CO_2H]CH_2CO_2H$, 1703-58-8; *o*-$(HO_2C)_2C_6H_4$, 88-99-3; 1,2,4-$(HO_2C)_3C_6H_3$, 528-44-9; 1,2,4,5-$(HO_2C)_4C_6H_2$, 89-05-4; $(PhCH_2O)_2P(O)Cl$, 538-37-4; CH_3CSOH, 507-09-5; DL-$HO_2CCH(CH_2SAc)CH_2CO_2H$, 100466-79-3; DL-$NO_2CCH(CH_2SO_3Na)CH_2CO_2Na$, 100466-80-6; $(EtO)_2P(O)O(CH_2)_2Br$, 5324-30-1; $(EtO)_2P(O)O(CH_2)_2SAc$, 100466-81-7; L-His, 71-00-1; dicyclohexylammonium salt of (2-mercaptoethyl)phosphonic acid, 100466-82-8; L-aspartic acid, 56-84-8; dibenzyl L-aspartate, 2791-79-9; itaconic acid, 97-65-4.

[4] E. A. Jacobson and W. W.-C. Chan, unpublished results.

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DNA Polymerase α Associated Primase from Rat Liver: Physiological Variations†

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ABSTRACT: A primase activity associated to DNA polymerase α from rat liver is described. Both activities were absent in normal adult rat liver but were concomitantly induced after partial hepatectomy. As previously shown for polymerase α and DNA topoisomerase II, primase activity reached a maximum value 40–43 h after the partial removal of the liver. Primase activity was shown to catalyze dNMP incorporation on unprimed single-stranded DNA template (M13 DNA) in the presence of rNTP. The activity was not detectable on poly(dA) or poly(dG) but was efficient on poly(dT) or poly(dC). However, the reliability of the primase assay in the presence of poly(dC) was dependent upon the degree of purification of the enzyme. The ribo primers were about 10 nucleotides long, and the reaction was completely independent of α-amanitin, a strong inhibitor of RNA polymerases II and III. Primase and polymerase were found tightly associated. A cosedimentation on a 5–20% sucrose gradient was always obtained, independent of the ionic strength. There was also a close coincidence between α-polymerase and primase activities during phosphocellulose, hydroxylapatite, and single-stranded DNA Ultrogel chromatography. It has been previously demonstrated by us and others that primase and α-polymerase are on separated polypeptides. The association of two activities in the replication complex and the conditions allowing their separation are discussed.

The RNA priming of DNA chain synthesis has been demonstrated in polyoma virus (Eliasson & Reichard, 1978), simian virus (De Pamphillis et al., 1979), and mammalian cells (Waqar & Huberman, 1975; Tseng et al., 1979; Kitani et al., 1984). The RNA primers are oligoribodecanucleotides of variable composition, containing a purine ribonucleoside 5′-triphosphate. They are covalently attached to 5′ termini of newly synthesized DNA. In *Escherichia coli*, RNA priming is catalyzed by a specialized RNA polymerase called primase (Kornberg, 1982). Such a primase activity has been also described for bacteriophages T4 and T7 and the ColE1 plasmid (Kornberg, 1982).

More recently, a primase activity has been described in eukaryotic cells of different origins (Conaway & Lehman, 1982; Yagura et al., 1982; Méchali & Harland, 1982; Tseng & Ahlem, 1982; Kaufmann & Hoffman-Falk, 1982; Riedel et al., 1982; Shioda et al., 1982; Hübscher, 1983; Litvak et al., 1984; Philippe et al., 1984; Plevani et al., 1984; Singh & Dumas, 1984; Yamaguchi et al., 1985a). Detailed studies about the specificity of the RNA primer synthesis by the DNA polymerase–DNA primase complex from mammalian cells were recently reported (Hu et al., 1984; Yamaguchi et al., 1985b). The DNA primase was found associated with a subspecies of DNA polymerase α (Yagura et al., 1982; Riedel et al., 1982; Shioda et al., 1982) or with the α-polymerase (Conaway & Lehman, 1982a; Hübscher, 1983; Kaguni et al., 1983a; Wang et al., 1984; Gronostajski et al., 1984). In the latter case, determination of the subunit on which the primase activity resides has led to contradictory results. Hübscher has reported that the high molecular weight (125 000 daltons) subunit of calf thymus DNA polymerase α contained both primase and polymerase activities. On the other hand, primase activity was shown to reside in the 60 000-dalton and/or 50 000-dalton subunit of DNA polymerase from *Drosophila* embryos, whereas the polymerase activity is associated with the high molecular weight (182 000 daltons) subunit (Kaguni et al., 1983b).

It is well-known that there is a clear association between DNA polymerase α activity and mitotic activity, whereas β-polymerase levels did not change. Few data are available about primase activity, except that the levels of primase activity have been correlated positively with DNA synthesis, in spleen and cardiac muscle during postnatal development (Kozu et al., 1982). The finely programmed regeneration of rat liver after its partial removal gives us an attractive system to follow the variation of primase activity during DNA synthesis (Philippe et al., 1984). In this paper, we describe the variation

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