

Acid-base Catalysis in the Argininosuccinate Lyase Reaction*

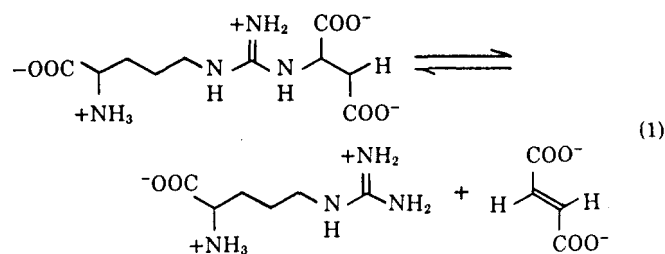
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The pH variation of the kinetic parameters, V_{\max} and V/K , was examined for the forward and reverse reaction of bovine liver argininosuccinate lyase. In the forward reaction the V_{\max} profile showed one group that must be unprotonated for activity over the pH range 5–10. The V/K profile for argininosuccinate showed one group that must be unprotonated and two groups that must be protonated for activity. The V_{\max} profile for the reverse reaction showed only one group that must be protonated for activity. These results support the proposal that catalysis is facilitated in the forward reaction by a general base that abstracts a proton from C-3 of argininosuccinate and a general acid that donates a proton to the guanidinium nitrogen during carbon-nitrogen bond cleavage. The enzyme is completely inactivated by diethyl pyrocarbonate or a water-soluble carbodiimide at pH 6. These experiments suggest that a histidine and a carboxyl group are at or near the active site and are essential for catalytic activity. The observed shifts of the pH profiles of the forward reaction with temperature and organic solvent (25% dioxane) were also consistent with a histidine and carboxylate group.

Argininosuccinate lyase (EC 4.3.2.1) catalyzes the following reaction (Equation 1).



This enzyme catalyzes a key step in the biosynthesis of urea and arginine in the liver of ureotelic species (1). The enzyme from beef liver has been extensively studied by Ratner and her colleagues over the last 30 years (2–6). However, there have been no attempts to identify those groups at the active site of this enzyme that facilitate in the binding of substrates and participate in the catalytic process. Unlike histidine ammonia lyase (7), phenylalanine ammonia lyase (8), and aspartase (9), this enzyme apparently does not contain a metal ion

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and/or a dehydroalanine residue that would participate in the bond breaking process.

Hansen and Havar (10) have pointed out that the cleavage of arginine from argininosuccinate will require at least two different acid-base groups at the active site for catalysis. A base is needed for the abstraction of a proton from C-3 and an acid is required for the donation of a proton to the guanidino nitrogen of arginine as it departs. Since the elimination is known to occur with *trans*-stereochemistry (4) it is highly unlikely that the group responsible for the abstraction of the proton from C-3 will be physically able to directly transfer the same proton to the nitrogen as arginine is cleaved. Thus, two separate acid-base groups are required and no net proton is released or taken up in this reaction over the pH range 5–10.

Recent inhibition studies with the nitro analog of argininosuccinate have indicated that the lyase reaction is initiated by the abstraction of a proton from C-3 of argininosuccinate to generate a carbanion intermediate (11). This intermediate then reacts to form enzyme-bound fumarate and arginine. Steady state and positional isotope exchange kinetic studies have shown the release of products from the ternary complex is random but that the release of fumarate is at least 10 times faster than the dissociation of arginine into the bulk solution (12, 13). It has also been shown that monofluorofumarate and acetylene dicarboxylate inhibit enzyme activity in a time-dependent process that is quite similar to the inactivation by mechanism-based inhibitors (14).

We have undertaken an extensive kinetic study of this reaction in an attempt to identify those amino acid residues that are essential for catalytic activity. In this report, use is made of the variation of the kinetic parameters V_{\max} and V/K with pH, temperature, and the addition of organic solvents to identify these groups (15). These studies have been complemented by the inactivation of argininosuccinate lyase with group-specific and affinity reagents.

MATERIALS AND METHODS

Argininosuccinate lyase was isolated from beef liver according to the procedure of Schulze *et al.* (6). All buffers used in the purification contained 0.1 mM EDTA and 1.0 mM dithiothreitol. Argininosuccinate was obtained from Sigma as its barium salt and was converted to the potassium salt and assayed as described by Havar *et al.* (16). 4-Bromocrotonic acid was synthesized according to the procedure of Pinza and Pifferi (17).

Enzyme Assays—Argininosuccinate lyase activity was assayed in the forward or reverse direction in 3.0 ml total volume in 1-cm cuvettes by monitoring the appearance or disappearance of fumarate at 240 or 255 nm with a Gilford 260 spectrophotometer and a 10-mV linear recorder. Full scale absorbance on the recorder was varied from 0.01 to 0.10 absorbance units. Temperature was maintained within 0.1 °C of the stated values with thermospacers and a circulating water bath. Reactions were initiated with the addition of enzyme with the aid of an adder mixer. pH values were measured with an Orion Research model 601A pH meter standardized at the given temperature to ± 0.01 pH unit. When the effect of dioxane on the pH

profile was being determined, the pH was measured before the addition of organic solvent. The cationic buffers, MES,¹ PIPES, HEPES, TAPS, CHES, and CAPS, were used at a concentration of 100 mM and within 1 pH unit of their pK values. The buffers were titrated to the desired pH by the addition of KOH. All reactions contained 100 mM KCl but no attempt was made to correct for small differences in the final ionic strength of individual reaction mixtures. All double reciprocal plots were linear over the concentration range of 0.015–10.0 mM. The time courses were linear throughout the incubation period which indicated that the enzyme was stable to the extremes of pH and solvent conditions.

Data Processing—Values of kinetic constants were determined by fitting initial velocity and concentration data in the appropriate rate equation by the least squares methods using the Fortran programs of Cleland (18). Substrate saturation curves were fitted to Equation 2.

$$v = VA/(K + A) \quad (2)$$

The pH profile data were fitted to one or more of the following equations:

$$\log y = \log(c/(1 + H/K_1)) \quad (3)$$

$$\log y = \log(c/(1 + K_1/H)) \quad (4)$$

$$\log y = \log(c/(1 + H/K_1 + K_2/H)) \quad (5)$$

$$\log y = \log(c/(1 + H/K_1 + K_2/H + K_3K_3/H^2)) \quad (6)$$

where $y = V$ or V/K , c = pH-independent value of y , K_1 , K_2 , and K_3 = dissociation constants of groups that ionize, H = hydrogen ion concentration, K = Michaelis constant, and V = maximal velocity. The enthalpies of ionization were determined from Equation 7.

$$pK = (\Delta H_{\text{ion}}/2.3RT) + b \quad (7)$$

The apparent pK values from fits of the data to Equations 5 and 6 are uncorrected (15).

Inactivation by Diethyl Pyrocarbonate—The inactivation of argininosuccinate lyase with diethyl pyrocarbonate was measured by incubating enzyme, 50 mM MES buffer, pH 6.0, 100 mM KCl, and various amounts of diethyl pyrocarbonate in a volume of 1.0 ml. At various times 0.1-ml aliquots were removed and added to a 0.20-ml solution of 100 mM imidazole, pH 7.0, to quench the reaction. Aliquots of the quenched reaction solution were then removed and assayed for argininosuccinate lyase activity. The rate constant for the spontaneous hydrolysis of diethyl pyrocarbonate was measured by incubating 10 mM diethyl pyrocarbonate, 50 mM MES, pH 6.0, 100 mM KCl, and enzyme. Aliquots were removed periodically and added to a solution of 100 mM imidazole, pH 7.0. The absorbance was measured at 230 nm (19).

The reactivation of diethyl pyrocarbonate-treated enzyme was attempted by adding an equal volume of a solution containing 100 mM hydroxylamine in 10 mM imidazole, pH 7.5, to a solution of argininosuccinate lyase that had been inactivated to less than 1% of its original activity by diethyl pyrocarbonate. Aliquots of 0.1 ml were removed periodically and assayed for the regain of argininosuccinate lyase activity.

The UV difference spectrum of the diethyl pyrocarbonate-treated enzyme was obtained by incubating argininosuccinate lyase (1 mg/ml) with 2.0 mM diethyl pyrocarbonate and monitoring the absorbance between 210 and 300 nm against a blank containing the same amount of enzyme but no diethyl pyrocarbonate. After the change in absorbance had ceased, hydroxylamine (100 mM) was added to both cuvettes and the UV spectrum monitored periodically.

Inactivation with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)—The inactivation of argininosuccinate lyase by EDC was measured by incubating enzyme with 50 mM MES, pH 6.0, 100 mM KCl, 0.5 M glycine methyl ester, and various levels of EDC. Aliquots (0.1 ml) were removed and assayed for remaining argininosuccinate lyase activity.

¹ The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonate; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; TAPS, 3-[tris(hydroxymethyl)methyl]amino-propanesulfonate; CHES, 2-(cyclohexylamino)ethanesulfonate; CAPS, 3-(cyclohexylamino)propanesulfonate; DEPC, diethyl pyrocarbonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

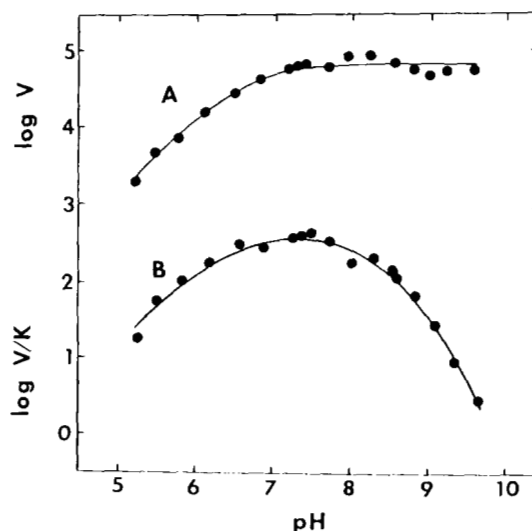


FIG. 1. pH profiles of the forward reaction of argininosuccinate lyase. A, V_{max} profile of the forward reaction of argininosuccinate lyase at 25 °C. The curve through the data points is a computer fit to Equation 3 with a pK of 6.7 ± 0.05 . B, V/K profile for argininosuccinate at 25 °C. The curve through the data points is a computer fit to Equation 6 with pK values of 6.4 ± 0.08 , 8.3 ± 0.16 , and 8.8 ± 0.4 . Additional details are given in the text.

RESULTS

Forward Reaction—The pH dependence of the kinetic parameters, V_{max} and V/K , for the cleavage of argininosuccinate to arginine and fumarate at 25 °C is shown in Fig. 1A. In the V_{max} versus pH profile a slope of 1 is seen below pH 6 and a plateau is observed above pH 7.5. The pK, as determined from a fit of the data to Equation 3, is 6.7 ± 0.05 . In contrast, the V/K versus pH profile (Fig. 1B) shows a slope of 1 below pH 6 and a slope of -1 and finally -2 above pH 8.5. The fit of the data for V/K versus pH to Equation 6 indicates pK values of 6.4 ± 0.08 , 8.3 ± 0.16 , and 8.8 ± 0.4 . Thus, the V/K profile indicates one group that must be unprotonated and two groups that must be protonated for activity.²

Reverse Reaction—In the reverse reaction the V_{max} versus pH profile (Fig. 2A) indicates one group on the enzyme that must be protonated for activity. A plateau is seen below pH 7.5 and a slope of -1 is observed above pH 8. From a fit of the data to Equation 4 a pK of 8.2 ± 0.1 is obtained. The profiles for V/K_{furn} and V/K_{Arg} (Fig. 2, B and C) are both bell-shaped from pH 5–9 indicating that one group must be protonated and the other unprotonated for activity. The fits of the data to Equation 5 indicate pK values of 6.5 ± 0.1 and 7.7 ± 0.1 for the V/K_{furn} data, and 6.5 ± 0.15 and 7.5 ± 0.1 for the V/K_{Arg} profile.³

Temperature Dependence—The temperature dependence of the pK values from the V_{max} and V/K profiles of the forward reaction was measured in an attempt to identify the groups that must be in a particular state of protonation for activity. A plot of pK versus $1/T$ at six temperatures between 20 and

² A fit of the argininosuccinate V/K data to Equation 5 indicated pK values of 6.6 ± 0.2 and 7.7 ± 0.2 . However, the square root of the residual least square (σ) was equal to 0.18 compared with a value of 0.098 from a fit of the same data to Equation 6.

³ A fit of the arginine V/K data to Equation 5 or 6 resulted in identical sigma values (0.13). However, the calculated pK value for the 3rd ionization in Equation 6 was 9.7 ± 0.9 . The large standard error for pK₃ does not appear to warrant a fit of the data to Equation 6. The calculated values for pK₁ and pK₂ remain the same in fits to either Equation 5 or 6.

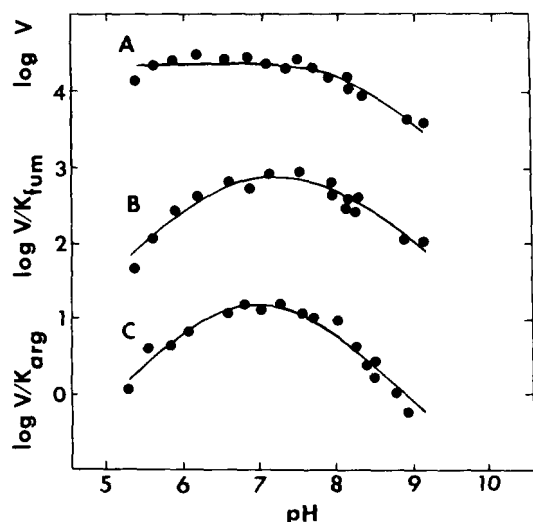


FIG. 2. pH profile of the reverse reaction of argininosuccinate lyase. A, V_{\max} profile of the reverse reaction of argininosuccinate lyase at 30 °C. Individual data points represent computer fits of velocities to Equation 2 at saturating arginine (30 mM) and varying fumarate. The curve through the data points is a computer fit to Equation 4 with pK of 8.2 ± 0.1 . B, V/K profile for fumarate at saturating arginine (30 mM). C, V/K profile for arginine at saturating fumarate (1.0 mM). The curves in B and C represent fits of the data to Equation 5. Additional details are given in the text. V_{\max} and V/K are in arbitrary units.

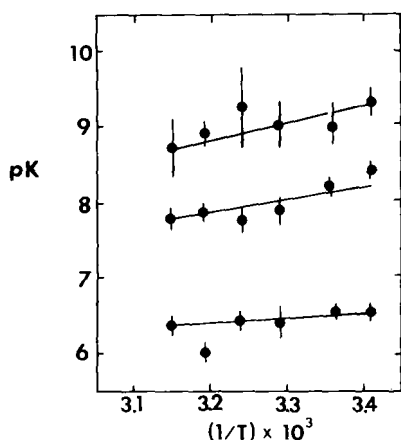


FIG. 3. Temperature dependence of the three ionizations observed in the V/K profiles of the forward reaction. The lines through the data points represent fits of the data to Equation 7. Additional details are given in the text.

45 °C for the three ionizations that appear in the V/K profile is shown in Fig. 3. Also shown is a fit of the data to Equation 7. The ΔH_{ion} for the three groups are 2.5 ± 1 kcal/mol, 6.9 ± 2 kcal/mol, and 9.0 ± 3 kcal/mol for K_1 , K_2 , and K_3 , respectively. A fit of the V_{\max} data (not shown) over this temperature range indicated a ΔH_{ion} of 1 ± 1 kcal/mol.

Effect of Organic Solvents—The effect of 25% (v/v) dioxane on the pH profiles of the forward reaction was measured in a series of cationic buffers in an attempt to determine whether the groups responsible for the ionizations observed in the pH profiles are due to cationic or neutral acid species. The V/K profile of the forward reaction in 25% dioxane could be fit to Equation 5. The pK values are 7.1 ± 0.1 , 8.2 ± 0.1 , and 9.7 ± 0.5 although the significance of the highest pK is doubtful. The V_{\max} profile was fit to Equation 3 with a pK of 6.9 ± 0.1 .

Inactivation by Diethyl Pyrocarbonate—Incubation of argi-

ninosuccinate lyase with diethyl pyrocarbonate at pH 6.0 results in the total loss of all enzymatic activity (>99%). The time course for the inactivation process is nonlinear when the logarithm of the residual activity is plotted *versus* time (data not shown). The data were subsequently corrected to take into account the decomposition of diethyl pyrocarbonate during the period of incubation. Gomi and Fujioka (20) have shown that the fraction of enzyme activity at time t may be expressed as

$$\ln(A/A_0) = -(k/k')I_0(1-e^{-k't}) \quad (8)$$

where I_0 is the initial reagent concentration, k is the bimolecular rate constant for reaction of the enzyme with diethyl pyrocarbonate, and k' is the first order rate constant for the hydrolysis of the reagent. Shown in Fig. 4 is a plot of the natural log of the residual activity *versus* $(1-e^{-k't})k'$ where k' is equal to 0.023 min^{-1} .

The time courses for the inactivation of argininosuccinate lyase by diethyl pyrocarbonate (1–4 mM) are biphasic. There is a rapid initial phase (<10 s) followed by a slower phase that is linear *versus* time to less than 10% of the remaining enzyme activity. A second order rate constant of $4.6 \text{ M}^{-1} \text{ s}^{-1}$ was calculated from the data of Fig. 4. The enzyme can be protected from inactivation by the inclusion of fumarate and/or arginine in the reaction mixture (data not shown). Either fumarate or arginine alone protects the enzyme from inactivation but greater protection is afforded by a ternary complex of enzyme, fumarate, and arginine.

The reaction of diethyl pyrocarbonate with argininosuccinate lyase produces an increase in absorbance at 236 nm. This result is consistent with the modification of a histidine residue (21). No change was noted at 280 nm where a decrease in absorbance would have been observed if a tyrosine had been modified during the time of incubation (22). When $19 \mu\text{M}$ argininosuccinate lyase is incubated at pH 6.0 with 2.0 mM diethyl pyrocarbonate, the absorbance at 235 nm increases by 0.12 absorbance units after 4 min. Assuming an extinction coefficient of $3200 \text{ M}^{-1} \text{ cm}^{-1}$ (21) for the appearance of *N*-carbethoxyhistidine, the concentration of modified histidines was found to be $37 \mu\text{M}$. At this point less than 10% of the original activity remained. The number of essential histidine residues is therefore ≤ 2 . The number of modified histidine residues after 55 min increases to ~ 4 .

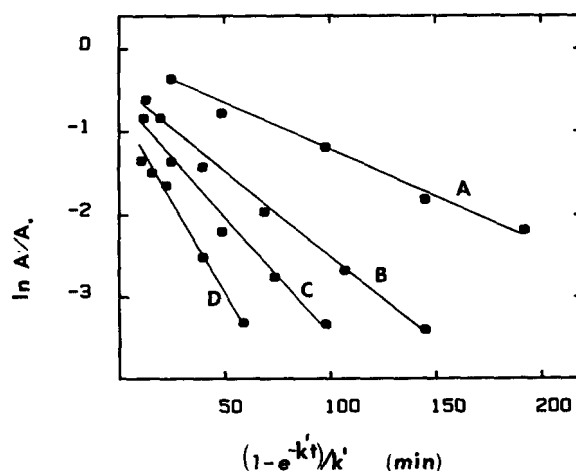


FIG. 4. Inactivation of argininosuccinate lyase by diethyl pyrocarbonate at pH 6.0. A, 1.0 mM; B, 2.0 mM; C, 3.0 mM; D, 4.0 mM. In this plot k' is the first order rate constant for the hydrolysis of diethyl pyrocarbonate and is equal to 0.023 min^{-1} . Additional details are given in the text.

Neutral hydroxylamine has been shown to remove carbethoxy groups from modified histidine residues as well as tyrosine and serine but not from cysteine, arginine, or lysine residues (19). When 100 mM hydroxylamine, pH 7.0, is added to a solution of argininosuccinate lyase that had been inactivated to less than 1% of its original activity by 1 mM diethyl pyrocarbonate greater than 80% of the original activity can be recovered after 5 h. The time course for the regain of activity is illustrated in Fig. 5. The pseudo first order rate constant is 0.39 h^{-1} . The absorbance maximum at 236 nm totally disappeared after incubation with hydroxylamine.

Inactivation by 1-Ethyl-3-(3-diethylaminopropyl)carbodiimide—The requirement for an essential carboxyl side chain in argininosuccinate lyase was probed by amidation with glycine methyl ester after activation by a water-soluble carbodiimide (23). When argininosuccinate lyase is incubated with EDC (10 mM) in the presence of glycine methyl ester (0.5 M) all of the enzymatic activity is lost after 4 h of incubation. No activity is lost if EDC is omitted from the reaction mixture. The time courses for the rate of inactivation at various levels of EDC (2.5–10 mM) are shown in Fig. 6. The pseudo first order plots are linear to less than 10% remaining enzyme activity at pH 6.0. The second order rate constant obtained from a replot of the data in Fig. 6 is $33 \text{ M}^{-1} \text{ min}^{-1}$. In the absence of the glycine methyl ester the time

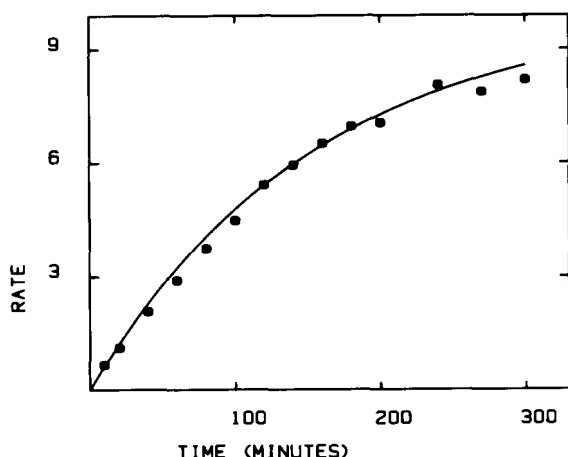


FIG. 5. Reactivation of argininosuccinate lyase by hydroxylamine after inactivation by 1.0 mM diethyl pyrocarbonate. The solid line represents a first order rate constant of 0.39 h^{-1} .

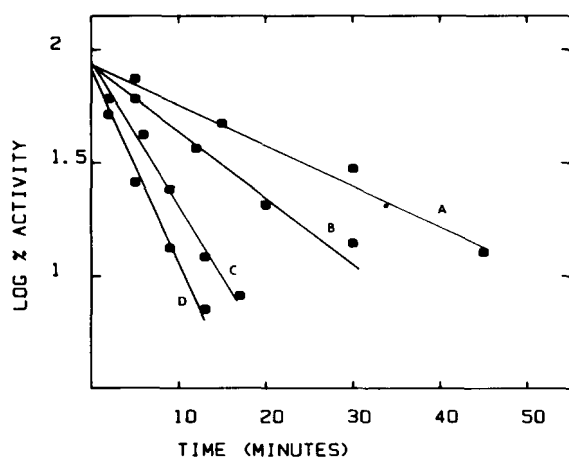


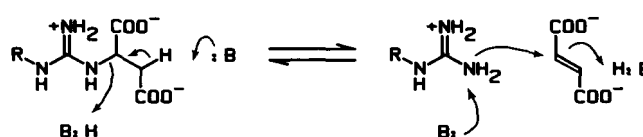
FIG. 6. Inactivation of argininosuccinate lyase by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at pH 6.0. A, 12.5 mM; B, 20 mM; C, 37.5 mM; D, 50 mM. Additional details are given in the text.

courses for the inactivation process were nonlinear and the rates became slower at longer periods of incubation.

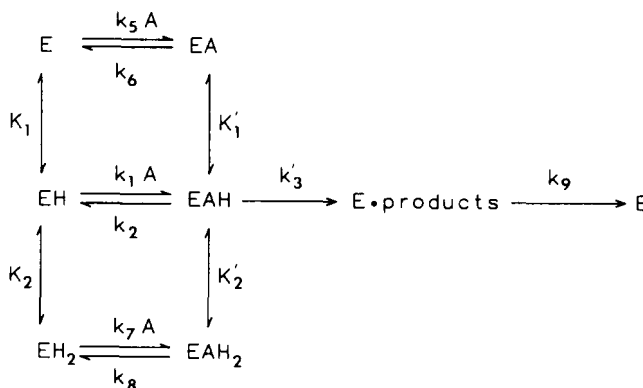
The inclusion of the substrates, arginine, and/or fumarate in the reaction mixture provided significant protection against inactivation by the carbodiimide. The rate of inactivation is retarded by either fumarate or arginine alone but even greater protection is observed with both the substrates present. However, in the absence of added glycine methyl ester (data not shown) the substrate protection results were different. The addition of 5 mM fumarate increased the rate of inactivation by 3-fold and 10 mM arginine had relatively no effect. A mixture of fumarate and arginine decreased the rate of inactivation by a factor of 2.

DISCUSSION

Variation of Kinetic Parameters with pH—A simplified representation of acid-base catalysis in the argininosuccinate lyase reaction is illustrated in Scheme 1. In this scheme an enzyme-base abstracts a proton from C-3 of argininosuccinate while an enzyme-acid donates a proton to the guanidino nitrogen during carbon-nitrogen bond cleavage (guanidino $\text{pK} = 12.5$). For the reverse reaction, the state of protonation of these groups is reversed. A proton is donated to fumarate and a hydrogen ion is abstracted from arginine. In this report we have attempted to provide evidence for the participation of acid-base catalysis in the cleavage of argininosuccinate to fumarate and arginine by argininosuccinate lyase. Our initial approach has been to measure the kinetic parameters with pH in order to determine the state of protonation of the ionizable functional groups that are required before binding and/or catalysis can occur. The identification of essential ionizable groups at the active sites of enzymes from changes in kinetic parameters with pH can only be accomplished if certain relationships among the various microscopic rate constants can be reasonably assumed (18, 24, 25). A minimal kinetic scheme for the argininosuccinate lyase reaction where one group must be ionized and one group must be protonated for enzyme activity is illustrated in Scheme 2. In such a mechanism activity will be lost at low and high pH as the two groups required for catalysis become both protonated or both deprotonated. It has been clearly shown that the pK values determined from a plot of $\log V/K$ versus pH represent the



SCHEME 1



SCHEME 2

actual dissociation constants of protons from free enzyme and unbound substrates only if the protonation steps represented by K_1 and K_2 are at equilibrium during the steady state (18, 24, 25). Furthermore, the rate constant for the release of the substrate from the *EA* complex (k_2) must be fast relative to V_{\max} in the forward reaction (18). If these conditions are not met then the pH profiles can become distorted by the appearance of "humps" and "hollows" in the usual bell-shaped profiles (18).

These two criteria appear to be satisfied in the reaction catalyzed by argininosuccinate lyase. The k_{cat} for the reaction is estimated to be $\sim 6 \text{ s}^{-1}$ at pH 7.5 and 25 °C. This is at least 3 orders of magnitude slower than any reasonable rate of dissociation of protons from the free enzyme (25). Therefore the proton exchange reactions of free enzyme will always be at thermodynamic equilibrium in the steady state. Furthermore the V_{\max} for the cleavage of argininosuccinate to fumarate and arginine is 40% slower than the turnover rate of the reverse reaction (12). Since the release of argininosuccinate from the *E*-argininosuccinate complex can be no slower than the slowest step of the reverse reaction the rate constant for the release of argininosuccinate (k_2) from *EHA* must be faster than the V_{\max} of the forward reaction. Therefore, the pK values determined from plots of $\log V/K$ versus pH will provide reasonably good estimates for the dissociation constant of groups that must be in the proper state of protonation in the free enzyme and unbound substrate.

The pH profile for V/K in the direction of argininosuccinate cleavage indicates one group that must be unprotonated and two groups that must be protonated for activity. The only ionizable group of argininosuccinate that can ionize in the pH range 5–10 is the α -amino group of the arginine moiety. All three carboxyls ionize below pH 5 while the guanidino group ionizes above 12. That leaves only the protonated group with a pK of ~ 8.8 as a possibility for an ionization originating from the substrate. The α -amino group of arginine has a measured pK of 9.1 (26). This same pK of 8.8 is also observed in the pH versus pK_i profile of the nitro analog of argininosuccinate (11). The remaining two groups should therefore have originated from amino acid side chains of the protein itself. This bell-shaped profile is as would be predicted based on the proposal as depicted in Scheme 1.

The enzyme group that must be unprotonated and the enzyme group that must be protonated for activity are probably the base and acid, respectively, that participate in the cleavage of argininosuccinate to fumarate and arginine. The V_{\max} profile for the forward reaction only shows a group that must be unprotonated for activity with a pK of 6.7. This is only slightly different than the pK of 6.4 that was observed in the V/K profile. The pK for the group that must be protonated has apparently been shifted beyond pH 9 since there is no indication that activity is lost to at least pH 9.5. The most likely explanation is that the pK for this group has been dramatically shifted because of hydrogen bonding to the guanidino group of argininosuccinate after the substrate has bound to the active site. Alternatively, the increase in pK could also be due to complete shielding of the enzyme group from bulk solvent by the addition of substrate to the active site.

According to the principle of microscopic reversibility the state of protonation for the reverse reaction must be opposite to what is required for the forward reaction. The pH profile for V_{\max} shows only one group that must be protonated for activity. This is as expected for the group that must donate a proton to fumarate during catalysis of the reverse direction. The other group, which is most likely hydrogen bonded to the

guanidino group of arginine, has had its pK shifted to less than 5.5 since no loss of activity is apparent on the low side of the pH profile.

It might be argued that the loss of activity at high and low pH could be due to ionization at sites remote from the active site or inactivation of the enzyme itself. Either of these possibilities is unlikely. The time courses for the rate measurements were fully linear throughout the assay period, thus indicating that the enzyme was stable long enough to determine the rate constant. Furthermore, full activity is observed for the forward reaction at high pH and full activity is observed for the reverse reaction at low pH. It is highly unlikely that a change in protein conformation due to some ionization away from the active site would differentially affect the forward and reverse reactions.

Inactivation by Group-specific Reagents—The inactivation of argininosuccinate lyase by diethyl pyrocarbonate and the water-soluble carbodiimide was attempted in order to provide additional experimental support for the essential amino acids at the active site of the enzyme that can participate in acid-base catalysis.⁴ Diethyl pyrocarbonate has been used primarily for the modification of histidine residues while the carbodiimide functions as a reagent for the derivatization of the carboxyl group of aspartate and glutamate. These reagents were chosen because imidazole and carboxyl groups are the two most likely candidates for participation in acid-base catalysis at the active sites of enzymes.

In this report we have been able to show that argininosuccinate lyase is rapidly and completely inactivated by diethyl pyrocarbonate at pH 6.0. The data appear to indicate that the inactivation is due to the modification of 1 or more histidine residues that are at or near the active site and are essential for catalytic activity. DEPC is, however, also known to react with other amino acid side chains such as cysteine, arginine, lysine, and tyrosine (19). Inactivation due to the modification of cysteine, arginine, or lysine is unlikely because modification of these groups is known not to be reversible with neutral hydroxylamine (19). Moreover, if a tyrosine had reacted with DEPC a change in absorbance would have been apparent in the UV spectrum at 278 nm but no difference was noted between the labeled and unlabeled enzyme (22). In addition, Lusty and Ratner (5) have reacted all available cysteine residues with 5,5'-dithiobis-(2-nitrobenzoate) with no loss of activity.

The properties of the modified enzyme are entirely consistent with the labeling of histidine residues. The modified enzyme shows an increase in absorbance near 240 nm and this absorbance increase is eliminated by reaction with neutral hydroxylamine and is concomitant with the regain of enzymatic activity. When over 90% of the initial activity is lost between 1 and 2 histidines have been labeled as indicated by the change in absorbance at 240 nm. The rate of inactivation is substantially reduced by the presence of arginine and fumarate in the reaction medium suggesting that the site of labeling is at or near the active site. Taken together, the results strongly support the conclusion that a histidine residue is essential for catalytic activity.

Argininosuccinate lyase is completely inactivated by the water-soluble carbodiimide in the presence of glycine methyl

⁴ The inactivation and labeling of the active site of argininosuccinate lyase has also been attempted with iodoacetamide, iodoacetic acid, 4-bromocrotonic acid, and the epoxide of fumaric acid. Of these compounds only 4-bromocrotonic acid inactivated the enzyme activity. The rate of inactivation at saturating bromocrotonic acid was 4.8/h and the K_i was 170 mM. The identity of the attacking nucleophile has not been identified (L. Garrard, unpublished observations).

ester at pH 6.0. Carbodiimides in acid pH are known to react almost exclusively with carboxylic acids, sulfhydryls, and to some extent tyrosine residues (27). Although carbodiimides can react with sulfhydryl groups to form isothioureas (27) the modification of sulfhydryl groups is very unlikely to result in the inactivation of argininosuccinate lyase because it has been previously shown that all of the available sulfhydryl groups under nondenaturing conditions can be reacted with 5,5'-dithiobis-(2-nitrobenzoate) and no inactivation is observed. It has also been observed that the enzyme is protected against inactivation by the substrates, fumarate and arginine. This would appear to suggest that these compounds can protect the enzyme activity by binding at the active site and shielding the essential residue from the reagent. This is supported by the observation that neither maleate (1 mM) nor glycine (10 mM) can protect the enzyme from inactivation by the carbodiimide.

Variation of pK with Temperature and Organic Solvent—The variation of the kinetic parameters with pH supports the proposal that the enzyme has at least two acid-base groups at the active site needed for catalytic activity. The group specific reagents, DEPC and EDC, have indicated that at least 1 histidine and 1 carboxyl are at or near the active site and essential for catalytic activity. An attempt to assign specific functional roles to these essential amino acids was made by trying to determine the pK values of these groups from the rates of inactivation with pH. These studies were unsuccessful. The identity of the ionizable functional groups appearing in the pH profiles was attempted by the variation of temperature and the addition of organic solvents to the reaction medium.

The identification of functional groups from a determination of the temperature dependence of the pK is based on the observation that the ΔH_{ion} for the ionizable groups of the amino acids are characteristic of the group that ionizes. For example, the ΔH_{ion} for carboxyl and imidazole groups are ± 1.5 and $6\text{--}7.5$ kcal/mol, respectively. The temperature dependence of the pK of the group that must be unprotonated for activity of the forward reaction shows a ΔH_{ion} of 2.5 and 1.0 kcal/mol for V/K and V_{max} , respectively. These values are consistent with the ionization of a carboxyl group. The other group that must be protonated for activity shows a ΔH_{ion} of 7 kcal/mol which is consistent with the ionization of either an imidazole, sulfhydryl (6.5–7 kcal/mol), or tyrosine (6 kcal/mol) residue. These assignments must be viewed with some caution however. Knowles (24) has forcefully pointed out that when the pK of some functional group is perturbed by its local environment there is no *a priori* reason to assume that the ΔH term remains constant.

The other method that has been used with some success in assigning specific residues to ionizations observed in pH rate profiles is the solvent perturbation technique (28). This technique relies on the observation that the pK for cationic acids is relatively unaffected by the addition of organic solvents to the medium while neutral acids generally increase in the pK. Therefore in a cationic buffer system the apparent pK for the ionization of neutral acids (carboxyl, sulfhydryl, and phenolic groups) will increase while the apparent pK for cationic acids (imidazole and amino groups) should remain about the same (28). Although this technique has successfully predicted the presence of 2 essential histidine residues in ribonuclease (28) and a carboxylate in hexokinase (29) it has recently been demonstrated by Grace and Dunaway-Mariano (30) that this

method must now be used with some caution since water-organic solvent mixtures can also influence the average conformational state of the protein with unpredictable results.

The ionizations that appear to be involved in acid-base catalysis of the forward reaction of argininosuccinate lyase are differentially perturbed by the addition of 25% dioxane to the reaction mixture. The group that must be unprotonated for activity in the V/K profile is shifted 0.7 units while the group that must be protonated for activity is relatively unaffected. The small ΔH_{ion} and the solvent perturbation characteristic of a neutral acid suggest that the unprotonated group with a pK of 6.4 may be a carboxyl of aspartate or glutamate. The protonated group with a pK of 8.3 is consistent with an imidazole of histidine since it appears to be a cationic acid with a ΔH_{ion} of ~ 7 kcal/mol. Similar results have been observed in the reaction catalyzed by fumarase (15, 31).

Conclusions—The pH rate profiles for the reaction catalyzed by argininosuccinate lyase indicate the requirement for an active site residue that must be unprotonated and at least one group that must be protonated for catalytic activity. It is suggested that these groups are involved in acid-base catalysis of the reaction by the abstraction of a proton from C-3 of argininosuccinate and the donation of a proton to the guanidino leaving group. The inactivation of all enzymatic activity by the group-specific reagents DEPC and EDC have indicated that at least 1 histidine and 1 carboxyl are at or near the active site that are essential for catalytic activity. The requirement for an essential histidine and carboxyl is also supported by the shifts in the pH rate profiles with temperature and organic solvents. However, these assignments must be viewed as tentative until the x-ray structure of this enzyme is solved and the active site is located.

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