Aspartokinase–Homoserine Dehydrogenase I from *Escherichia coli*: pH and Chemical Modification Studies of the Kinase Activity†

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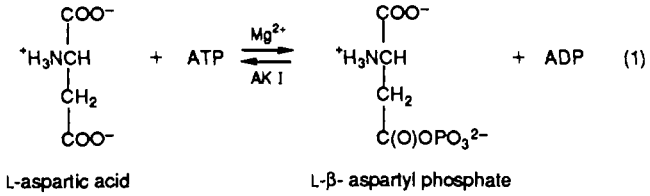
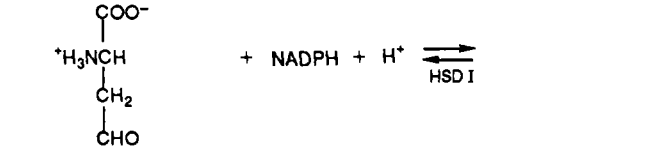
ABSTRACT: The pH variation of the kinetic parameters was examined for the kinase activity of the bifunctional enzyme aspartokinase–homoserine dehydrogenase I isolated from *Escherichia coli*. The V/K profile for L-aspartic acid indicates the loss of activity upon protonation of a cationic acid type group with a pK value near neutrality. Incubation of the enzyme with diethyl pyrocarbonate at pH 6.0 results in a loss of enzymic activity. The reversal of this reaction by neutral hydroxylamine, the appearance of a peak at 242 nm for the inactivated enzyme, and the observation of a pK value of 7.0 obtained from variation of the inactivation rate with pH all suggest that enzyme inactivation occurs by modification of histidine residues. The substrate L-aspartic acid protects one residue against inactivation, which implies that this histidine may participate in substrate binding or catalysis. Activity loss was also observed at high pH due to the ionization of a neutral acid group with a pK value of 9.8. The reactions of AK–HSD I with *N*-acetylimidazole and tetranitromethane have been investigated to obtain information about the functional role of tyrosyl residues in the enzyme. The acylation of tyrosines leads to inactivation of the enzyme, which can then be fully reversed by treatment with hydroxylamine. Incubation of the enzyme with tetranitromethane at pH 9.5 also leads to rapid inactivation, and the substrates of the kinase reaction provide substantial protection against inactivation. However, three tyrosines are protected by substrates, implying a structural role for these amino acids.

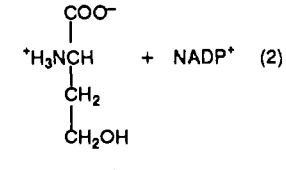
Aspartokinase–homoserine dehydrogenase I (AK–HSD I,[1] EC 2.7.2.4) from *Escherichia coli* catalyzes two nonconsecutive steps (reactions 1 and 2) in the biosynthetic pathway from L-aspartate to the amino acids threonine, isoleucine, and methionine. AK–HSD I is one of three isofunctional enzymes in *E. coli* that catalyze the phosphorylation of aspartic acid that is the commitment step in this biosynthetic pathway (Truffa-Bachi et al., 1974).



L-aspartic acid L-β- aspartyl phosphate



L-aspartic β-semialdehyde



L- homoserine

This bifunctional enzyme is a well-characterized allosteric protein, with the feedback inhibition by threonine of its two catalytic activities comprising a major feature of the complex regulation scheme of threonine biosynthesis (Cohen et al., 1967). The enzyme is composed of four identical subunits with an overall molecular weight of 360 000 (Falcoz-Kelly et al., 1972; Wampler, 1972), and the two catalytic activities reside on separate structural domains of each polypeptide chain (Veron et al., 1972). Thr A, a part of the thr operon in *E. coli*, codes for the protein. This gene has been cloned and sequenced, and the primary structure of AK–HSD I has been determined (Katinka et al., 1980). However, no X-ray crystallographic structure of the enzyme has been reported to date.

Chemical modification studies of the enzyme have indicated the involvement of sulfhydryl groups (Truffa-Bachi et al., 1966, 1968) and arginine residues (Tsien & Takahashi, 1978) in a structural rather than a catalytic role. In the present work, we have utilized the pH variation in enzyme kinetic parameters and in the UV/visible spectrum, and also chemical modification techniques, to aid in the identification of the groups involved in the catalytic function of the aspartokinase activity of this enzyme.

Experimental Procedures

Materials. *N*-Acetylimidazole, buffer salts, substrates, substrate analogues, and coupling enzymes were purchased

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[1] Abbreviations: Aces, 2-[(carbamoylmethyl)amino]ethanesulfonic acid; ABME, L-aspartate β-methyl ester; AK–HSD I, aspartokinase–homoserine dehydrogenase I; AMP-PNP, 5′-adenylyl imidodiphosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DEP, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NAI, *N*-acetylimidazole; PMB, *p*-(chloromercuri)benzoate; TNM, tetranitromethane.

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from Sigma. Diethyl pyrocarbonate, *p*-(chloromercuri)-benzoate, and tetranitromethane were supplied by Aldrich. All other reagents were of the highest purity commercially available. AK–HSD I was purified from *E. coli* by the method of Karsten et al. (1985), suspended in 100 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol, and then stored in liquid nitrogen.

Enzyme Assays. Aspartokinase activity was routinely assayed in the forward direction (reaction 1) by coupling the production of ADP with pyruvate kinase and lactate dehydrogenase. The 1.00-mL reaction mixture consisted of 100 mM Hepes, pH 8.0, 100 mM KCl, 3.6 mM magnesium acetate, 1.8 mM ATP, 0.7 mM phosphoenolpyruvate, 0.1 mM NADH, 40 units of lactate dehydrogenase, 20 units of pyruvate kinase, 15 mM L-aspartic acid, and 8–15 μg of AK–HSD I. Initial velocities were measured at 30 ± 1 °C by following the disappearance of NADH at 340 nm by using a Perkin-Elmer Lambda-1 spectrophotometer. In control experiments the levels of the coupling enzymes were varied to ensure that the aspartokinase reaction was completely rate limiting, and no lags were observed in the disappearance of NADH upon initiation of the reaction by the addition of AK–HSD I.

Homoserine dehydrogenase activity was monitored at 30 ± 1 °C in a 1.00-mL assay mixture containing 100 mM Ches, pH 9.0, 400 mM KCl, 12.5 mM L-homoserine, 0.3 mM $NADP^+$, and 0.1 mM DTT. Initial velocities were measured by following the appearance of NADPH at 340 nm as described above.

pH Studies. A constant ionic strength buffer (100 mM Aces/52 mM Tris/52 mM ethanolamine; $I = 0.1$ M) was used to cover the pH range from 6 to 11 (Morrison & Ellis, 1982). This buffer system was adjusted to the desired pH by using either tetraethylammonium hydroxide or hydrochloric acid. For the pH profiles run in neutral acid type buffers, a mixed 3,3-dimethylglutarate (30 mM)/*p*-phenolsulfonate (10 mM) buffer system was used with NaCl added to bring the total ionic strength to 0.1 M. These buffer components were examined separately and were found to have no effect on the rates of the enzyme-catalyzed reaction at the concentrations used across the pH range of interest.

The K_m values of the substrates were determined at the extremes of the pH range used to ensure saturation of the fixed substrate in the reaction mixture. For pH studies run in the presence of 20% dimethyl sulfoxide (Me_2SO) as the perturbing organic solvent, the pH was determined before the addition of the organic solvent (Cleland, 1977). Absorption spectra of AK–HSD I at different pH values were obtained by using a Hewlett-Packard 8452A diode array spectrophotometer. Addition of 10% 1,4-dioxane, which provided a larger window to monitor the spectral changes in these absorption studies, was used as the perturbing organic solvent instead of Me_2SO. Titrations were also performed on the model compound *N*-acetyl-L-tyrosine under similar conditions.

Data Analysis. All data were fitted to the BASIC versions of the computer programs of Cleland (1979), which weight the data by assuming equal variance for the velocities of the fitted parameter. Reciprocal plots at each pH, at saturating levels of the fixed substrate, were fitted to eq 3, where v is the experimentally determined velocity, V is the maximum velocity, A is the variable substrate concentration, and K is the Michaelis constant. All data to determine pK values were fitted to eq 4 or 5, where y is V/K or V_{max}, C is the pH-independent value of this parameter, and K_1 and K_2 are the dissociation constants of the groups on the acidic and basic side, respectively.

$$v = VA/(K + A) \tag{3}$$

$$\log y = \log\left(\frac{C}{1 + [H^+]/K_1}\right) \tag{4}$$

$$\log y = \left(\frac{C}{1 + [H^+]/K_1 + K_2/[H^+]}\right) \tag{5}$$

Treatment with Diethyl Pyrocarbonate. Chemical modification of AK–HSD I by diethyl pyrocarbonate (DEP) was carried out at 25 °C in a 1.00-mL reaction mixture containing 100 mM phosphate, pH 6.0, 100 mM KCl, 0.5 mM dithiothreitol (DTT), 10% glycerol, 0.1 mM EDTA, and 0.16 mg of enzyme. The reaction was initiated by the addition of 20 μL of stock solutions of different DEP concentrations dissolved in 100% acetonitrile, and 5-μL aliquots of the reaction mixture were assayed for remaining activity at the indicated time intervals. The concentration of stock solutions of diethyl pyrocarbonate was determined by monitoring the increase in absorbance at 230 nm on reaction with 10 mM imidazole in 0.1 M phosphate buffer, pH 6.0, by use of an ϵ value of 3.0×10^3 M^{-1} cm^{-1} (Melchior & Fahrney, 1970; Miles, 1977).

The pseudo-first-order rate constants for inactivation by DEP, k_{obs}, were calculated according to

$$\ln (V_t/V_0) = -k_{obs}t \tag{6}$$

where V_0 and V_t represent the enzyme activity at times 0 and t, respectively. For protection studies, the enzyme was preincubated with effectors at pH 6.0, and the modification reaction was initiated by the addition of the reagent. In the pH-dependent studies of the rate of inactivation, a mixed Mes (40 mM)/Hepes (60 mM) buffer system was used to cover the pH range from 5.5 to 8.5. NaCl was added to bring the total ionic strength to 0.1 M. The number of residues modified in the presence or absence of 50 mM L-aspartic acid was determined by correlating the inactivation of the enzyme (0.64 mg/mL) by 1.5 mM DEP with the increase in absorbance at 242 nm.

Reversal of the Diethyl Pyrocarbonate Inactivation. A solution containing 0.16 mg/mL of AK–HSD I was allowed to react at 25 °C with 1.6 mM DEP for 30 min. Enzyme assays at that time showed about 50% residual activity. At this point, hydroxylamine hydrochloride, pH 7.0, was added to the reaction mixture to a final concentration of 1 N. Aliquots were then removed and assayed for the increase in enzyme activity at the designated time intervals.

Treatment with N-Acetylimidazole. Modification of AK–HSD I by NAI was carried out at 25 °C in a 1.00-mL reaction mixture containing 100 mM Hepes, pH 7.5, 100 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 10% glycerol, and 0.10 mg of enzyme. A desired volume of a stock solution of NAI in benzene was placed in a vial, and nitrogen gas was passed over the solution to remove the benzene. The enzyme solution was added to the dry NAI, and 5-μL aliquots of the modified enzyme were assayed for remaining activity at the indicated time intervals. Initial rates were determined from the linear portion of the curves. The pseudo-first-order rate constants for inactivation, k_{obs}, were calculated by using eq 6. Procedures for the protection and reversal of inactivation were the same as those utilized with diethyl pyrocarbonate, except for the buffers used.

The number of residues modified was determined by incubating 0.64 mg/mL AK–HSD I with 0.1 M NAI for 30 min in the reaction mixture described. The modified enzyme was then dialyzed for 4 h at 4 °C against the same buffer system to remove excess NAI. The increase in absorbance at 278 nm

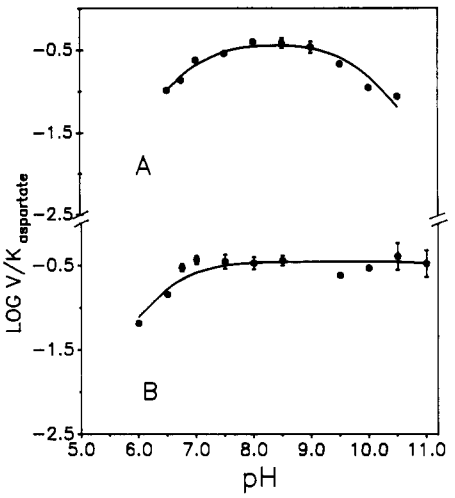

FIGURE 1: V/K profile for L-aspartic acid. Assays were run in the cationic acid buffer system (100 mM Aces/52 mM Tris/52 mM ethanolamine; I = 0.1 M) in (A) the absence and (B) the presence of 20% dimethyl sulfoxide. The line drawn through the experimental data points is derived from a fit to the appropriate equations for pH dependence as described under Experimental Procedures.

was monitored after treating the enzyme solution with hydroxylamine, pH 7.5, to a final concentration of 0.1 N.

Preparation of Sulfhydryl-Masked AK-HSD I. A stock solution of PMB (1 mM) was prepared by dissolving the sodium salt of the reagent in 100 mM Hepes and adjusting the pH to 7.0. The solution was then standardized on the basis of $\epsilon_{233} = 1.69 \times 10^4$ (Boyer, 1954). AK-HSD I was dialyzed at 4 °C against 100 mM Hepes, pH 8.0, containing 100 mM KCl, 0.1 mM EDTA, and 10% glycerol for 4 h prior to modification. The enzyme (0.22 mg/mL) was then incubated with 100 μM p-(chloromercuri)benzoate (PMB) at room temperature for 15 min. No enzyme activity remained after this treatment. The modified enzyme was fully reactivated by treatment with 50 mM DTT for 2 min.

Treatment with Tetranitromethane. Tetranitromethane was dissolved in absolute ethanol just before use. Modification experiments were performed at 25 °C by the addition of tetranitromethane in ethanol to the sulfhydryl-masked enzyme in a pH 8.0 buffer system containing 100 mM Hepes, 100 mM KCl, 0.1 mM EDTA, and 10% glycerol. The progress of the reaction was monitored by diluting aliquots at the indicated times into 50 mM DTT to unmask the sulfhydryl groups, and then assaying for aspartokinase activity. The modification procedures were repeated at pH 9.5 with a buffer system of 100 mM Ches, 100 mM KCl, 0.1 mM EDTA, and 10% glycerol. DTT (0.5 mM) was incorporated in the incubation mixture for the unmasked enzyme. Inactivation rate constants were calculated as above. Protection studies were carried out on both masked and unmasked enzyme at pH 8.0 and 9.5.

RESULTS

Effects of pH on AK-HSD I Properties. The $V/K_{aspartate}$ profile shows a maximum value in the pH range from 7.5 to 9 which decreases on either side of this maximum (Figure 1A). On the low-pH side a decrease is observed with a limiting slope of +1, and on the high-pH side of the maximum the $V/K_{aspartate}$ profile decreases to a limiting slope of −1. The data from the pH dependence of $V/K_{aspartate}$ were fitted to eq 5 (solid line in Figure 1A). From this fit a pK value of 6.9 ± 0.1 was obtained on the low-pH side of the profile, and a value of 9.8 ± 0.1 was determined on the high-pH side. The pK values of $V/K_{aspartate}$ profile were also examined in the presence of 20% Me_2SO in a cationic buffer system. These solvent perturbation studies were run to aid in the identification of the acid type (neutral or cationic) of the ionizable groups on the enzyme (Cleland, 1977). The ionization on the low side of the pH profile was relatively unchanged by the addition of Me_2SO (Figure 1B). However, the pK value on the high-pH side was shifted from 9.8 to >11 by the addition of Me_2SO. These data were fitted to eq 4 (solid line in Figure 1B). The effects of increasing the hydrophobicity of the solvent on the two pK values were also examined in the neutral acid buffer system. The results of this series of experiments are shown in Table I.

Table I: Effect of Me_2SO on pK Values of the $V/K_{aspartate}$ Profile

	acidic pK		basic pK	
buffer type[a]	−Me_2SO	+Me_2SO[b]	−Me_2SO	+Me_2SO
neutral	6.5 ± 0.1	**5.3 ± 0.3**	9.2 ± 0.1	9.0 ± 0.1
cationic	6.9 ± 0.1	6.6 ± 0.1	9.8 ± 0.1	**>11**

[a] A constant ionic strength buffer of the cationic acid type (100 mM Aces/52 mM Tris/52 mM ethanolamine; I = 0.1 M) was used to cover the range from pH 6 to pH 11. For the profiles run in neutral acid buffer, a mixed 30 mM 3,3-dimethylglutarate/10 mM p-phenolsulfonate system was utilized, with NaCl added to bring the total ionic strength to 0.1 M. [b] Studies were conducted in the presence of 20% dimethyl sulfoxide as the perturbing organic solvent.

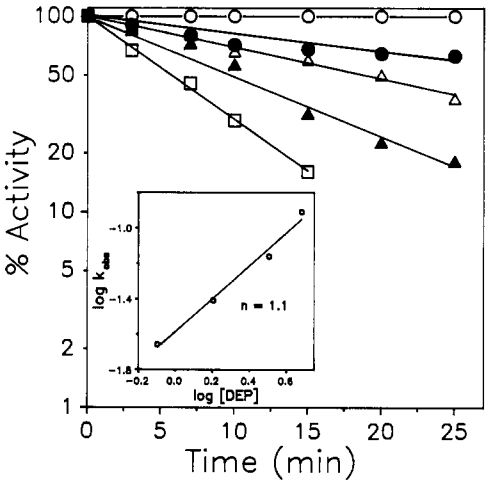

FIGURE 2: Inactivation of aspartokinase I by diethyl pyrocarbonate. Enzyme (0.16 mg/mL) was incubated at 25 °C with 0 mM (○), 0.80 mM (●), 1.6 mM (△), 3.2 mM (▲), and 4.8 mM DEP (□) in 100 mM phosphate buffer, pH 6.0. Aliquots were removed at various time intervals and assayed as described under Experimental Procedures. The inset shows a plot of the logarithmic dependence of the inactivation rate constant on the concentration of DEP.

The UV/visible spectrum of AK-HSD I exhibits a shift in λ_{max} from 234 nm to 244 nm when titrated from pH 6 to pH 11. This shift is consistent with the spectral characteristic of tyrosine residues (Dawson et al., 1986). Titration of the model compound N-acetyl-L-tyrosine resulted in a spectral shift that is similar to that observed with the enzyme, with a pK value of 10.2. This pK value for N-acetyl-L-tyrosine was observed to shift up to 12.2 in the presence of 10% dioxane (data not shown). In parallel with this trend seen for the ionization of the model compound N-acetyl-L-tyrosine, the spectral shifts in the UV/visible titration curves for AK-HSD I extrapolate to pK values of 10.5 and >12 in the absence and presence of 10% dioxane, respectively.

Inactivation of AK-HSD I by Diethyl Pyrocarbonate. Incubation of AK-HSD I with various concentrations of diethyl pyrocarbonate at pH 6.0 results in a time-dependent inactivation of the aspartokinase activity by a pseudo-first-order process, with the rate of inactivation dependent on DEP concentration (Figure 2). A plot of log k_{obs} vs log [DEP]

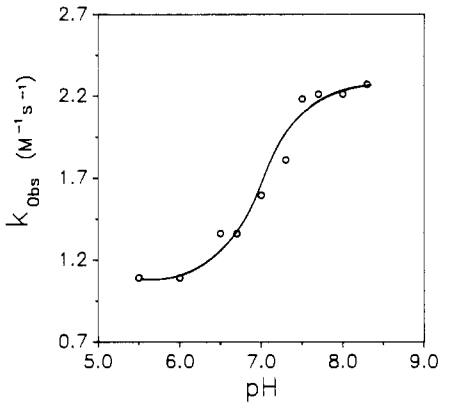

FIGURE 3: Effect of pH on the rate of inactivation of AK I by diethyl pyrocarbonate. Enzyme (0.16 mg/mL) was incubated at 25 °C with 1.2 mM DEP in a mixed Mes (40 mM)/Hepes (60 mM) buffer system of different pH values. NaCl was added to bring the total ionic strength to 0.1 M. Samples withdrawn at designated time intervals were assayed for enzyme activity, and the observed pseudo-first-order rate constants for inactivation, k_{obs}, were determined from these data.

(inset of Figure 2) gives a slope of 1.1, suggesting that the inactivation is also first order in DEP. A second-order rate constant for inactivation, 0.4 M^{-1} s^{-1}, was determined from a plot of k_{obs} vs [DEP]. DEP shows a selectivity for the modification of histidine residues in proteins, but it has also been reported to react with other types of residues (Lundblad & Noyes, 1984; Melchior & Fahrney, 1970; Miles, 1977). Of the residues that can potentially be modified by DEP, only histidine and tyrosine can be regenerated by treatment with hydroxylamine (Miles, 1977). Residues such as lysine, arginine, and cysteine which are acylated by DEP are more resistant to nucleophilic attack by hydroxylamine. AK–HSD I that had been 50% inactivated with DEP was treated with hydroxylamine to a final concentration of 1 N for 30 min. This treatment resulted in restoration of the enzymic activity to between 85 and 90%, as compared to the control that had not been treated with DEP.

Modification of histidine and tyrosine by diethyl pyrocarbonate can be distinguished spectrophotometrically. Formation of *N*-carbethoxyhistidine results in an increased absorbance at 242 nm, whereas acetylation of tyrosine causes a decrease in absorbance at 278 nm (Miles, 1977; Setlow & Mansour, 1970). The differential spectra between the native enzyme and the DEP-treated enzyme were recorded during the process of derivatization. Formation of a peak was observed in the 242-nm region, without a significant decrease at 278 nm, consistent with the modification of histidyl residues by DEP. On prolonged incubation an additional peak was observed at about 230 nm which is consistent with the formation of a disubstituted histidine (Miles, 1977). A study of the pH dependence of the rate of inactivation of aspartokinase I by DEP gave a p*K* value of 7.0 (Figure 3). Modification by DEP was carried out in the absence and presence of substrates and competitive inhibitors in order to determine if the inactivation is due to modification of a residue at the active site of the enzyme. Extensive protection against inactivation was afforded by saturating amounts of the abortive complex L-aspartate/5′-adenylyl imidodiphosphate (AMP-PNP), and also by L-aspartic acid and L-threonine examined separately (Table II). Complete inactivation of aspartokinase I correlates with the modification of 2–3 histidyl residues/subunit (Figure 4). However, the modification of 0.6 less histidines/subunit was observed after 30 min in the presence of saturating L-aspartic acid, resulting in 40% more residual enzyme activity compared to the control experiment.

Table II: Effect of Substrates and Substrate Analogues on Inactivation of the Aspartokinase Activity of AK–HSD I

ligand	concn (mM)	rate of inactivation k_{obs} (M^{-1} s^{-1})	% protection
(A) DEP Inactivation[a]			
control		1.9	
L-aspartic acid	30	0.51	73
ABME	15	0.41	78
N-formyl-L-aspartic acid	4	1.9	0
MgATP	14	1.2	37
MgdATP	13	1.1	42
L-aspartic acid/AMP-PNP	30/10	0.18	91
L-threonine	10	0.28	85
(B) TNM Inactivation[b]			
control		50	
L-aspartic acid	15	26	48
	50	0	100
ABME	15	19	62
	20	11	78
MgATP	7	15	70
	18	5	90
L-threonine	10	14	72
(C) NAI Inactivation[c]			
control		0.28	
L-aspartic acid	15	0.05	82
ABME	15	0.07	75
MgATP	4	0.07	75
L-threonine	10	0.14	50

[a] Conditions: 0.16 mg of enzyme was incubated at 25 °C with one of the ligands listed in a 1.00-mL buffer system consisting of 100 mM phosphate, pH 6.0, 100 mM KCl, 0.5 mM DTT, 10% glycerol, and 0.1 mM EDTA. The modification reaction was initiated by adding DEP to a final concentration of 1.6 mM. Five-microliter aliquots of the reaction mixture were assayed for remaining enzymatic activity at various time intervals. [b] Conditions: Modification of the enzyme by 10 μM TNM was performed at 25 °C in 100 mM Ches buffer, pH 9.5, containing 100 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.1 mM EDTA. The effect of the presence of each ligand in the incubation mixture was determined at the concentrations shown. Initial rates were calculated from the linear portion of the curves. [c] Conditions: Modification of AK–HSD I (0.10 mg/mL) by 15 mM NAI was carried out at 25 °C in a reaction mixture containing 100 mM Hepes, pH 7.5, 100 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 10% glycerol, and one of the ligands listed above. A desired volume of a stock solution of NAI in benzene was placed in a vial, and nitrogen gas was passed over the solution to remove the solvent. The enzyme solution was added to the dry NAI, and aliquots of the modified enzyme were assayed for remaining activity at the indicated time intervals. Initial rates were determined from the linear portion of the curves.

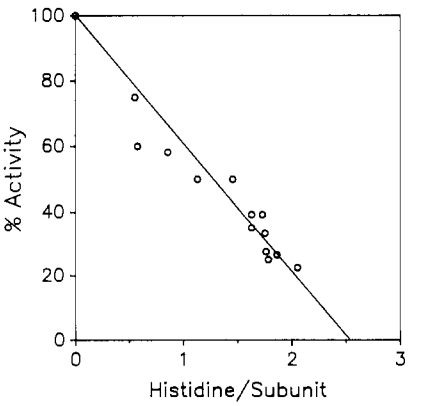

FIGURE 4: Correlation of the inactivation of aspartokinase I with the number of histidyl residues modified by diethyl pyrocarbonate. AK–HSD I (0.64 mg/mL) was incubated at 25 °C in a reaction mixture containing 100 mM phosphate, pH 6.0, 100 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, and 1.5 mM DEP. Aliquots were withdrawn at designated time intervals and assayed for remaining activity. The increase in absorbance of the enzyme solution at 242 nm vs time was monitored.

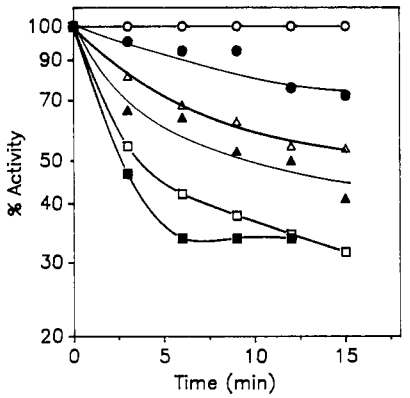

FIGURE 5: Effect of varying concentrations of *N*-acetylimidazole on aspartokinase I activity as a function of time. Enzyme (0.10 mg/mL) was incubated at 25 °C with 0 mM (○), 1.89 mM (●), 7.56 mM (△), 15 mM (▲), 26.5 mM (□), and 45.4 mM NAI (■) in 100 mM Hepes buffer, pH 7.5. Aliquots were removed and assayed for enzyme activity at various time intervals.

Loss of the homoserine dehydrogenase activity of this bifunctional enzyme also occurred during the modification of the enzyme by diethyl pyrocarbonate. A rate constant of 0.6 M^{-1} s^{-1} was observed for this process, which is not substantially different from the value of 0.4 M^{-1} s^{-1} determined for the aspartokinase activity. Saturating levels of the substrates of the kinase reaction, L-aspartic acid and MgATP, afforded almost 80% protection against the loss of homoserine dehydrogenase activity. Protection against inactivation of the HSD activity was also obtained by reactants that bind at that active site. However, while the abortive ternary complex formed by 3-amino-$NADP^+$ and L-homoserine protected against the loss of the dehydrogenase activity, this protection was not competitive. These results indicate that the DEP-modified group is not located at the HSD active site.

Inactivation of AK-HSD I by N-Acetylimidazole. *N*-Acetylimidazole is a relatively specific reagent for the determination of "free" tyrosyl residues in proteins. Deacetylation of the resulting *o*-acetyltyrosine is rapidly accomplished with hydroxylamine (Riordan et al., 1965). Treatment of AK-HSD I with *N*-acetylimidazole results in a rapid inactivation of the enzyme which then slowed down, presumably due to instability of the reagent in aqueous solution (Figure 5). Complete reactivation was observed when the derivatized enzyme was treated with neutral hydroxylamine (data not shown). Quantitation of the stoichiometry of inactivation was monitored at 278 nm upon hydroxylamine treatment, and a value of 6 tyrosyl residues/subunit was obtained. All the substrates, L-aspartic acid, ATP, and the alternate substrate ABME, afforded substantial protection against inactivation of aspartokinase I by *N*-acetylimidazole, whereas the allosteric inhibitor L-threonine showed little protection (Table II). In the presence of saturating L-aspartic acid the number of modified tyrosyl residues per subunit decreased from 6 to 3.

Table III: Effect of Substrates and Substrate Analogues on NAI Inactivation of the Homoserine Dehydrogenase Activity of AK-HSD I[a]

ligand	concn (mM)	rate of inactivation k_{obs} (M^{-1} s^{-1})	% protection
control		0.052	
L-aspartic acid	15	0.043	17
	30	0.021	60
MgATP	10	0.025	52
$NADP^+$	1.5	0.049	6
3-amino-$NADP^+$/ L-homoserine	1/62.5	0.050	4

[a] Conditions: AK-HSD I (0.20 mg/mL) was incubated at 25 °C with one of the ligands in a reaction mixture containing 100 mM Hepes, pH 7.5, 100 mM KCl, 0.5 mM DTT, 0.1 mM DTT, and 10% glycerol. NAI in benzene (16 mM) was transferred to a vial, and nitrogen gas was passed over the solution to remove the benzene. The enzyme solution was added to the dry NAI, and the change in activity with time was determined. The initial rates of inactivation were calculated from tangents drawn to the curves.

The other catalytic activity of the enzyme, the homoserine dehydrogenase activity, was also affected by treatment with NAI, with the rate of inactivation about a factor of 5 slower than the loss of kinase activity (Table III). However, the substrates of the dehydrogenase reaction did not offer any degree of protection. In contrast, the substrates of the kinase activity considerably decreased the rate of inactivation of homoserine dehydrogenase (Table III).

Inactivation of AK-HSD I by Tetranitromethane. Tetranitromethane (TNM) has been found to be a reasonably specific mild reagent for the nitration of tyrosyl residues of proteins at pH 8. At pH 6 cysteine, the only other amino acid affected, is oxidized without affecting tyrosine (Sokolovsky et al., 1966). Loss of the kinase activity and desensitization of the dehydrogenase activity of AK-HSD I have been observed with a variety of sulfhydryl reagents (Veron et al., 1972; Truffa-Bachi et al., 1966). In view of these results, it became necessary to protect the SH groups of AK-HSD I prior to carrying out the modification with TNM.

The reaction of the sulfhydryl-masked AK-HSD I with TNM caused an irreversible loss of the kinase activity (Figure

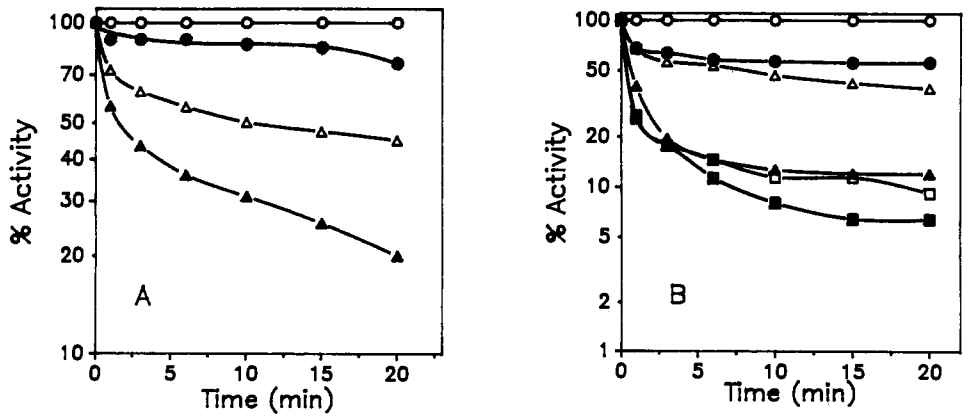

FIGURE 6: Inactivation of aspartokinase I by tetranitromethane. (A) Sulfhydryl-masked AK-HSD I was incubated at 25 °C with 0 mM (○), 2 mM (●), 4 mM (△), and 6 mM TNM (▲) in 100 mM Hepes, pH 8.0. Aliquots were withdrawn periodically and assayed after the sulfhydryl groups were recovered by treatment with DTT. (B) Unmasked enzyme was treated at 25 °C with 0 μM (○), 1 μM (●), 10 μM (△), 15 μM (▲), 20 μM (□), and 30 μM TNM (■) in 100 mM Ches, pH 9.5. Aliquots were withdrawn at designated time intervals and assayed.

6A). No protection was offered by any of the substrates (data not shown); however, this may be due to the loss of the binding capacity of the sulfhydryl-masked enzyme for its substrates. The nitration rate of tyrosine with TNM is markedly enhanced at high pH, accompanied by increased specificity (Means & Feeney, 1971). Rapid initial inactivation of AK–HSD I is observed at pH 9.5 at very low levels of TNM, with a subsequent decrease in the rate of inactivation due to the instability of the reagent (Figure 6B). Extensive protection was afforded by the substrates L-aspartate, MgATP, and L-aspartate β-methyl ester when added in saturating amounts (Table II). The presence of L-threonine in the reaction mixture likewise was effective in decreasing the inactivation rate. To determine if the protection observed with these compounds is due to competitive binding at the site of inactivation, the concentrations of the protectants L-threonine and ABME were varied against different levels of tetranitromethane in separate experiments. A competitive pattern was observed when the substrate analogue, ABME, was varied; however, L-threonine gave an uncompetitive protection pattern against TNM (data not shown). Logarithmic plots of the rate constants of inactivation at pH 8.0 and pH 9.5 vs TNM concentration both gave a slope of 0.8 ± 0.3, indicating that the inactivation process is first order with respect to modifier concentration.

Discussion

Kinetic and Spectral Studies. Chemical modification and pH dependence of the kinetic parameters are two widely used techniques for the identification of the functional groups of an enzyme. A pH-dependent decrease in the V/K or V_{max} profile gives information regarding groups on the enzyme, or on the substrates, that are involved in either binding or catalysis. The V/K profile reveals information concerning changes in the state of ionization of essential functional groups on the free enzyme (or the binary enzyme–substrate complex in the case of bireactant enzymes) and/or substrates that affect activity, while the V_{max} profile reflects changes in the state of ionization of groups on the central enzyme–substrate complexes (Cleland, 1982). Solvent perturbation studies of the apparent pK values are an aid in establishing the acid type of the group(s) involved in enzymic activity. Modification of the amino acid residues on the enzyme by group-specific and affinity reagents augments the information obtained through kinetic studies, and protection against inactivation by substrates and products of the reaction can give an indication of the location of these groups on the enzyme. The application of these approaches in concert may allow a definitive assignment of the functional groups involved in the catalytic mechanism of an enzyme.

In the bifunctional enzyme aspartokinase–homoserine dehydrogenase I, a change in the kinase activity near neutral pH is seen in the $V/K_{aspartate}$ profile run in cationic acid buffers. This pK, corresponding to a value of 6.9, is unaffected by the addition of organic solvents. These results indicate that the group that is being protonated to cause loss of enzymic activity is of the cationic acid type, since a neutral acid group would have the ionization suppressed (and therefore the pK elevated) by the addition of organic solvents. This assignment is supported by the pH profiles examined in neutral acid buffers. Ionization of a neutral acid buffer also leads to charge separation, which is suppressed by the addition of organic solvent. In the presence of an organic solvent little net change in the pK value would be seen if the group on the enzyme is of the neutral acid type, since both the enzymic group and the buffer would have suppressed ionization. The observed *decrease* in the pK on the low-pH side caused by the addition of 20% Me_2SO to the AK–HSD I reaction mixture in neutral acid buffers (Table I) confirms the assignment of this group as a cationic acid type. On the basis of the observed pK value and acid type, a histidine is the most likely candidate for this enzymic group.

A pK of 9.8 is observed on the basic side of the $V/K_{aspartate}$ profile, and the ionization of this group is shifted up by greater than 1.5 pH units in the presence of 20% dimethyl sulfoxide. Addition of Me_2SO to the reaction mixture in neutral acid buffers did not alter the pK value that was observed in the absence of organic solvent (Table I). An increase in pK with the addition of an organic solvent in cationic buffers is exhibited by residues of the neutral acid type such as the sulfhydryl group of cysteine and the phenolic hydroxyl group of tyrosine. The assignment of this group as tyrosine is supported by the pH-dependent spectral changes that are observed with AK–HSD I. The pK value of the UV/visible spectral shift of the enzyme is virtually the same as the pK value observed in the $V/K_{aspartate}$ pH profile. A similar spectral shift and pK value is observed for the model compound *N*-acetyl-L-tyrosine, and the spectral pK values of both the enzyme and the model compound are shifted up by several pH units in the presence of organic solvents.

Inactivation Studies. Inactivation of the kinase activity of AK–HSD I by diethyl pyrocarbonate at pH 6.0 is characterized by a pseudo-first-order kinetics with, however, a relatively low rate constant. The magnitude of the second-order rate constant can frequently serve as an indicator of the specificity of modification (Cousineau & Meighen, 1976); however, such is not always the case. For example, the inactivation of thermolysin at pH 6.2 by histidine modification proceeds with a low rate constant, 0.18 M^{-1} s^{-1} (Burstein et al., 1974), compared to a value of 30 M^{-1} s^{-1} for pig heart lactate dehydrogenase (Holbrook & Ingram, 1973). However, in each case a single histidine residue was selectively modified. Although a relatively low rate constant was observed for the reaction of AK–HSD I with DEP, spectral results obtained during the process of derivatization are consistent with the selective modification of a histidine residue. A prominent peak at 242 nm was observed with no concomitant decrease at 278 nm, eliminating the possibility that the inactivation might have been due to the modification of tyrosine residues. The inactivation was determined to be first order with respect to the histidine modifier concentration from a logarithmic plot of the apparent rate constants vs the concentration of DEP (inset of Figure 2).

The reactivation of the DEP-treated enzyme by neutral hydroxylamine provides evidence that the loss of enzymic activity is due to the modification of histidine, rather than either lysine or cysteine. In the event that disubstituted histidine is produced during prolonged incubations, treatment with hydroxylamine results in the scission of the imidazole ring (Miles, 1977). This reaction may account for the incomplete (85–90%) recovery of activity of the DEP-inactivated enzyme. The variation of the rate of inactivation with pH suggests that inactivation is dependent on the unprotonated form of an amino acid side chain with an apparent pK of 7.0. The above data, along with the observation that inactivation is reversed by hydroxylamine, strongly indicate that inactivation of aspartokinase I by DEP is due to the modification of histidine residue(s).

L-Aspartate, but not MgATP, effectively decreases the rate of inactivation by DEP at pH 6.0, indicating that the histidine(s) that are being modified may be located at or near the L-aspartate binding site of the enzyme. This conclusion is

further supported by the protection afforded by L-aspartate β-methyl ester, an alternate substrate in the kinase reaction (results not shown). Binding of the allosteric inhibitor L-threonine to the threonine sites results in a change in the conformation of the enzyme (Janin & Iwatsubo, 1969), which is also apparently sufficient to slow the rate of inactivation of the enzyme. Modification of 2–3 histidyl residues/subunit resulted in complete loss of the kinase activity (Figure 4). However, the protection offered by L-aspartic acid against derivatization of the enzyme by DEP suggests the presence of approximately 1 histidine/subunit in the substrate binding site.

Inactivation of the homoserine dehydrogenase of AK-HSD I by diethyl pyrocarbonate appears to be a consequence of the modification of a histidine residue at the kinase catalytic site. While the substrates of the homoserine dehydrogenase activity protect against the effect of the modifying agent to a fair extent, the mode of protection offered by 3-amino-$NADP^+$ and L-homoserine is not competitive against the inactivator, which is consistent with the derivatization occurring at the kinase active site.

Both *N*-acetylimidazole (Riordan et al., 1965) and tetranitromethane (Sokolovsky et al., 1966) possess a high reactivity and a high specificity toward tyrosyl residues. Acetylation of AK-HSD I resulted in a rapid initial loss of the kinase activity, which then slowed due to the instability of NAI in aqueous solution. Complete reversal of the modification was achieved in the presence of neutral hydroxylamine, which ruled out the possibility that inactivation was due to derivatization of the enzyme amino groups. Acetylation by NAI also resulted in the loss of homoserine dehydrogenase activity of the enzyme. Competitive protection against inactivation of the two catalytic activities was provided by the substrates of the kinase activity, L-aspartic acid and MgATP. Acetylation of six tyrosyl residues/subunit resulted in complete inactivation of the enzyme. Of the total of 18 tyrosine groups in AK-HSD I, 3 have been located in the aspartokinase domain (Katinka et al., 1980). Three of the six modified tyrosyl residues are protected in the presence of L-aspartic acid, suggesting that the loss of activity upon derivatization of tyrosine by NAI may be due to a structural change in the aspartokinase domain rather than being the result of the modification of a specific active site tyrosine residue.

Tetranitromethane, a reasonably specific reagent for the nitration of tyrosyl residues of proteins at pH 8, has been observed to oxidize cysteine at pH 6 (Sokolovsky et al., 1966). To eliminate nonspecific modification, the sulfhydryl groups of AK-HSD I were derivatized with PMB prior to nitration. Treatment with tetranitromethane resulted in an irreversible inactivation of the sulfhydryl-masked enzyme. However, a linear loss of activity with time at a certain level of TNM was not achieved as a consequence of the instability of the reagent in aqueous media. The lack of protection by the substrates against inactivation may be either due to modification of a tyrosine that is remote from the active site or, more likely, the result of a loss of the binding capacity of the SH-blocked enzyme. Improved reactivity of tetranitromethane toward tyrosine is achieved at high pH (Means & Feeney, 1971). At very low levels of TNM, rapid initial inactivation of AK-HSD I was observed, with a subsequent decrease in the rate of inactivation due to the instability of the reagent. L-Aspartic acid, MgATP, and ABME afforded extensive protection against modification of the enzyme by TNM. The noncompetitive protection offered by the allosteric inhibitor L-threonine is probably a result of a conformational change in the enzyme which renders the active site inaccessible to modification. These data provide further support to the conclusions drawn from the modification studies using NAI.

The results of both the chemical modification and pH variation studies suggest the involvement of histidine and tyrosine residues in the kinase active site of AK-HSD I. Additional studies will be required to establish the chemical mechanism of this enzyme and the particular roles played by these functional groups.

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