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Interaction of Substrates and Inhibitors with the Homoserine Dehydrogenase of Kinase-Inactivated Aspartokinase I†

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ABSTRACT: The aspartokinase activity of the aspartokinase-homoserine dehydrogenase complex of *Escherichia coli* was affinity labeled with substrates ATP, aspartate, and feedback inhibitor threonine. Exchange-inert ternary adducts of Co(III)-aspartokinase and either ATP, aspartate, or threonine were formed by oxidation of corresponding Co(II) ternary complexes with H_2O_2. The ternary enzyme-Co(III)-threonine adduct (I) had 3.8 threonine binding sites per tetramer, one-half that of the native enzyme. The binding of threonine to I was still cooperative as determined by equilibrium dialysis (n_H = 2.2) or by studying inhibition of residual dehydrogenase activity (n_H = 2.7). Threonine still protected the SH groups of I against 5,5′-dithiobis(2-nitrobenzoate) (DTNB) reaction but the number of SH groups reacting with thiol reagents (DTNB) was reduced by 1-2 per subunit in the absence of threonine. This suggests either that Co(III) is bound to the enzyme via sulfhydryl groups or that 1-2 SH groups are buried or rendered inaccessible in I. The binding of threonine to sites not blocked by the affinity labeling produced changes in the circular dichroism of the complex comparable to changes produced by threonine binding to native enzyme and also protected against proteolytic digestion. The major conformational changes produced by threonine are thus ascribable to binding at this one class of regulatory sites. The interactions of kinase substrates with various aspartokinase-Co(III) complexes containing ATP, aspartate, or threonine and a threonine-insensitive homoserine dehydrogenase produced by mild proteolysis were studied. The inhibition of homoserine dehydrogenase by kinase substrates is not due to binding of these inhibitors at the kinase active site but was shown to be due to binding to sites within the dehydrogenase domain of the enzyme. L-α-Aminobutyrate, a presumed threonine analogue, also inhibits the dehydrogenase by binding at the same or similar sites in the dehydrogenase domain and not at a threonine regulatory site.

The feedback inhibition of the aspartokinase I-homoserine dehydrogenase I complex by threonine is one regulatory mechanism controlling L-threonine biosynthesis in *E. coli*. The tetrameric enzyme carries two activities on each of its identical polypeptide chains (Falcoz-Kelly et al., 1972). The two reactions catalyzed are:

aspartate + Mg-ATP → aspartyl phosphate + Mg-ADP

and

aspartate-β-semialdehyde + NADPH → homoserine + $NADP^+$

In addition to four pairs of active centers, aspartokinase-homoserine dehydrogenase possesses eight threonine binding sites (Falcoz-Kelly et al., 1972; Véron et al., 1973). Nuclear relaxation studies with 1-[^{13}C]threonine revealed that at least one threonine site is adjacent to the kinase metal-ion cofactor site. The threonine C_1 to Mn(II) distance is 4.4 ± 0.3 Å (Tilak et al., 1976). This distance estimate was corroborated by the discovery that L-threonine could be specifically incorporated into the inner sphere of an exchange-inert Co(III)-aspartokinase complex (Wright et al., 1976a). Both substrates of the kinase reaction are stably incorporated into similar complexes

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by this affinity-labeling technique. Furthermore, the competition observed between L-threonine and L-aspartate for a Co(III) coordination site indicated that the binding sites for inhibitor and substrate overlap (Wright et al., 1976a). The location and function of the four remaining threonine sites have yet to be ascertained. L-Threonine is not the only inhibitor of the enzyme complex. The substrates of one enzymic activity inhibit the other (Patte et al., 1966).

Modified aspartokinases which possess homoserine dehydrogenase activity may be divided into two classes based on their kinetic properties. The first class contains those proteins which lack aspartokinase activity and possess threonine insensitive homoserine dehydrogenase activity. One such derivative is the dimeric homoserine dehydrogenase fragment produced by proteolytic digestion. This fragment has a molecular weight of 110 000 as compared with 360 000 for the native enzyme (Véron et al., 1972). Another derivative in this class is native enzyme which has been treated with a variety of thiol reagents (Véron et al., 1973).

Ryzewski and Takahashi (1975) reported a second class of derivative produced by affinity labeling aspartokinase with Co(III). This modified enzyme lacks aspartokinase activity but retains homoserine dehydrogenase activity which is inhibited by L-threonine. Another derivative of this class, the Co(III) substrate and inhibitor adducts, have special utility in studying the complex interaction of substrate and inhibitor ligands with the bifunctional protein. These derivatives contain a substrate or inhibitor molecule bound to the enzyme via a metal bridge. Both Co(III) affinity-labeled complexes and the dehydrogenase proteolytic fragment were employed in this study to simplify the study of ligand interactions with the enzyme complex. In particular we have sought to: (1) study conformational transitions caused by threonine binding at the regulatory sites not located at the kinase active site; and (2) locate the sites responsible for aspartate and ATP inhibition of the dehydrogenase.

Materials and Methods

Enzymes. Aspartokinase–homoserine dehydrogenase was isolated from *E. coli* K12 Tir-8 as previously described (Ehrlich and Takahashi, 1973). Purified enzyme was stored as an ammonium sulfate (50% saturation) slurry at 4 °C. Aspartokinase activity was assayed by a coupled assay (Wampler and Westhead, 1968). The homoserine dehydrogenase activity was assayed in the forward direction (Patte et al., 1963). This assay mixture contained: 0.05 M tris(hydroxymethyl)aminomethane, 0.63 M KCl, 2 mM Mg(II)–EDTA,[1] 0.3 mM NADPH, and 0.62 mM aspartate-β-semialdehyde, pH 7.5; assays were conducted at 30 °C. Both activities were tested for maximal feedback inhibition in the presence of 7 mM threonine. Since the residual aspartokinase activity is always totally inhibited by threonine, all subsequent references to threonine sensitivity refer solely to that of homoserine dehydrogenase.

The cooperativity of the inhibition of homoserine dehydrogenase activity in the various proteins was determined from a modified Hill plot:

$$\ln \frac{v_0 - v}{v - v_\infty} = (\ln K) - n_H(\ln (A))$$

where K is the appropriate steady-state dissociation constant, n_H is the Hill coefficient, v_0 is the velocity in the absence of inhibitor, v is the velocity at inhibitor concentration [(A)], and v_∞ is the velocity at saturating concentration of inhibitor. The value of v_∞ was determined from a replot of the kinetic data in the following form (cf. Klotz and Hunston, 1971):

$$\frac{1}{(A)} = \frac{1}{K}(I_\infty - I)$$

where I is the inhibition at concentration of inhibitor [(A)], and I_∞ is the maximal inhibition.

Protein concentrations were determined by the method of Lowry et al. (1951). Bovine serum albumin, used as the standard, and aspartokinase have the same color yield. Adenine nucleotides were determined by their ultraviolet absorbances using published molar absorptivities (P-L Biochemicals, Inc., Circular OR-10, 1969).

The homoserine dehydrogenase fragment was prepared by tryptic digestion of native enzyme according to the procedure used for chymotryptic digestion (cf. below). The molecular weights of some derivatives thus prepared were determined by gel filtration on Sephadex G-200 (Pharmacia).

For all experiments protein solutions were exchanged into the appropriate buffers by dialysis for 1 to 2 h in stretched tubing against a 500-fold or greater volume excess of buffer (Englander and Crowe, 1965). Rabbit muscle pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (1.1.1.27), bovine pancreas trypsin (3.4.21.4), and α-chymotrypsin (3.4.21.1) were obtained from Sigma Chemical Co.

Spectra. Absorbance measurements were made with Cary 118 or Gilford 240 spectrophotometers. Circular dichroism spectra were obtained on a Cary 61 spectropolarimeter. The optical activity of native and modified aspartokinase is reported as the molar ellipticity $[\theta]$ in deg cm^2/dmol of subunit using the relation, $[\theta] = x/10lc$, where x is the observed ellipticity in degrees, l is the path length in cm, and c is the molar concentration of subunits. The reported ellipticities are average values computed from three repetitive scans. Solutions for these studies contained 0.15 M KCl, 10 mM tris(hydroxymethyl)-aminomethane, and 50 μM dithioerythritol, pH 7.6. A solution from which protein was omitted was used as a reference. No correction was made for the solvent refractive index.

Binding Studies. The binding of threonine to aspartokinase was followed using the Kontron Diapack with Teflon minicells. Compartments of 250-μL volume were separated by membranes which had been boiled in water, rinsed, and soaked in buffer for 2 h before use. Solutions of enzyme plus threonine or threonine were introduced on either side, and sealed cells were placed in a water bath and allowed to dialyze with rotation (ca. 5 rpm) overnight. Samples were removed for protein determination and activity assay. Samples (100 μL) were counted in 10 mL of Aquasol (New England Nuclear) in a Packard TriCarb Model 3310 spectrometer. The threonine concentration was varied between 25 μM and 1 mM. The protein concentrations were ca. 15 or 40 μM at low and high KCl concentrations, respectively. The buffers employed were 10 mM tris(hydroxymethyl)aminomethane, 50 μM dithiothreitol, and 10 mM or 150 mM KCl, pH 7.6. L-[U-^{14}C]Threonine was purchased from New England Nuclear. The modified proteins used in these binding studies contained no radioactive ligands. Therefore, substrate or inhibitor stoichiometries for these proteins were independently determined by analysis of protein samples which had been identically affinity labeled except for the presence of radioactive isotopic species.

Aspartokinase was affinity labeled with ATP, aspartate, or

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); AK, aspartokinase; otherwise follow accepted abbreviations [(1966), *Biochemistry 5*, 1445].

TABLE I: L-Threonine Inhibition of Homoserine Dehydrogenase.

Enzyme[a]	Max Threonine Inhibition (%)	Hill Coefficient, n_H
AK/native	88	2.9
DTNB-treated AK	0	
Dehydrogenase fragment	0	
AK-Co(III)	36	2.5
AK-Co(III)-Thr	31	2.6
AK-Co(III)-Asp	35	2.2
AK-Co(III)-ATP	32	2.4

[a] Homoserine dehydrogenase activity assayed in native enzyme (AK/native), Co(III) affinity-labeled derivatives (AK-Co(III)) with the immobilized ligand indicated where present, DTNB-treated enzyme, and the dimeric dehydrogenase fragment produced by tryptic or chymotryptic digestion.

threonine by H_2O_2 oxidation of the corresponding Co(II) complex (Wright et al., 1976a). The incorporation of metal and ligands into aspartokinase is reported as $\bar{\nu}$, the number of equivalents incorporated per subunit. A value of 360 000 g/mol was employed for the molecular weight of the tetramer.

Chemical Modification. Thiol groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) by a modification of the procedure of Ellman (1959). A stock solution of titrant (4 mg/mL) was made in 0.1 M potassium phosphate, pH 7.0. Titrations were initiated by the addition of 15 μL of stock solution per mL of protein sample in 0.1 M potassium phosphate buffer, pH 7.6. A molar absorptivity of 13 300 M^{-1} cm^{-1} at 412 nm was determined for the nitrothiobenzoate anion based on a value of 13 600 M^{-1} cm^{-1} at pH 8.0. The completion of reaction was judged to occur when the rate of absorbance increase at 412 nm of protein samples was the same as that in blanks lacking protein.

Acetylation was accomplished with *N*-acetylimidazole (Riordan and Vallee, 1972). *N*-Acetylimidazole (Pierce) was dissolved in dry, distilled benzene, washed with anhydrous Na_2SO_4, and recrystallized in the cold in a desiccator. This reagent was added to the protein in 0.5 M KCl-0.1 M *N*-ethylmorpholine (pH 7.5) to give a final concentration of 2 mg/mL. Control enzyme was incubated in buffer alone. Samples were withdrawn for analysis of activity and inhibition. A solution of buffer containing 2 mg/mL of the modification reagent did not affect the coupled assay for aspartokinase, nor did the system used for the dehydrogenase assay when added at twice the level present during the assay of modified enzyme.

Methylacetimidate hydrochloride (Pierce) was dissolved in 0.5 M KCl-0.1 M potassium phosphate and the pH of the solution was readjusted to pH 8.0 with KOH. Aspartokinase was incubated in the above buffer with 10 or 50 mM methylacetimidate (cf. Wofsy and Singer, 1963). The pH of the reaction mixture was maintained near 8 by the addition of small amounts of 1 M potassium phosphate buffer (pH 9.5). A control which lacked the modifying reagent was run to follow the stability of the enzyme at this high pH.

Proteolytic digestion was conducted in 0.3 M KCl-10 mM tris(hydroxymethyl)aminomethane-5 mM $Ca(NO_3)_2$ (pH 7.8). Trypsin or α-chymotrypsin (10 mg/mL in 1 mM HCl) was added to solutions of protein in the above buffer to a final concentration of 2% of the total protein. Digestion was at 30 °C and was conducted for the indicated length of time or until complete desensitization occurred. Preparations of affinity-labeled aspartokinase or homoserine dehydrogenase fragments used for kinetic studies contained less than 2% of their initial aspartokinase activity.

Results

The threonine sensitivities of the homoserine dehydrogenase activity of native enzyme and two classes of derivatives are presented in Table I. Only native enzyme and various affinity-labeled adducts possessed dehydrogenase activity which was cooperatively inhibited by threonine. The cooperativity of this inhibition in unmodified enzyme and the Co(III) complexes was comparable, although the threonine sensitivity of the derivatized enzymes was reduced. Native enzyme alone of all these proteins had aspartokinase activity.

The binding of threonine and aspartate to enzyme which contains immobilized substrate or inhibitor was followed spectroscopically (Table II). The side-chain Cotton effects observed for the affinity-labeled derivatives in the absence of added ligands do not differ significantly from those observed for native enzyme. The chief effect of threonine on the circular dichroism of native enzyme is a decrease in the negative ellipticity of all wavelengths studied. The effects of aspartate on the native enzyme are less dramatic and confined to the longer wavelength troughs. The spectrum of the aspartokinase-Co(III)-threonine derivative in a medium lacking added free threonine differs from that of native enzyme in the presence of threonine. The aspartokinase-Co(III) derivative containing stably bound aspartate does not exhibit the circular dichroism of the native enzyme in the presence of aspartate. This suggests that, while the aspartate molecule bound at the kinase active site prevents access of other free aspartate molecules to the kinase site, the immobilized ligand is inappropriately positioned to induce a conformational change. Addition of aspartate to solutions of this ternary complex still altered the observed circular dichroism, however. The aspartokinase Co(III)-ATP derivative has a distinctive feature in its spectrum. A strong shoulder at 265 nm ($[\theta] = -17\,000$ deg cm^2/dmol subunit) is presumably an extrinsic Cotton effect of bound ATP. An extrinsic Cotton effect in the 500-650-nm region assignable to bound Co(III) could not be observed for any derivative.

Heck and Truffa-Bachi (1970) have used the ellipticity at 280 nm to follow the threonine titration of the enzyme from the active to the inhibited form. Addition of 5 mM L-threonine to solutions of Co(III) affinity-labeled aspartokinase resulted in a decrease in the negative ellipticity at 280 nm for all derivatives. Even the ternary complex which contains one threonine molecule bound at each kinase site underwent the threonine-induced spectroscopic change.

The binding of threonine to modified aspartokinase possessing 0.93 equiv of threonine per subunit was followed by equilibrium dialysis. Threonine binding to aspartokinase-homoserine dehydrogenase is noncooperative, yielding linear Scatchard plots (Scatchard, 1949) at low potassium ion levels (Janin and Cohen, 1969; Véron et al., 1973).

At 10 mM KCl the binding of L-threonine to aspartokinase exposed to Mg(II) and threonine in the presence of H_2O_2 and to the aspartokinase-Co(III)-threonine adduct produced by H_2O_2 oxidation of the Co(II) complex is noncooperative (Figure 1). The process of affinity labeling has, however, reduced the number of binding sites from 7.5 to 3.8 per tetramer. The dimeric homoserine dehydrogenase fragment does not bind threonine under equilibrium conditions at low potassium ion concentrations (data not shown) or steady-state assay conditions (Table I).

At higher potassium ion levels, threonine binding is coop-

TABLE II: Side-Chain Cotton Effects in Labeled and Native Aspartokinase ($[\theta] \times 10^{-3}$ (deg cm^2/dmol Subunit)).

	Aspartokinase-Co(III)-X[a]											
λ (nm)	H_2O		Aspartate			ATP		Threonine		Native Enzyme		
		+Thr[b]		+Thr	+Asp		+Thr		+Thr		+Thr	+Asp
262	−13.2	−14.0	−15.3	−14.7	−14.9	−17.1[c]	−17.0[c]	−15.7	−15.5	−15.8	−14.8	−15.7
268	−28.2	−19.6	−24.8	−21.4	−28.0	−25.6	−20.0	−26.2	−18.9	−28.6	−20.4	−28.3
274	−32.1	−23.5	−29.7	−24.1	−28.7	−36.3	−22.8	−27.4	−23.2	−31.1	−22.3	−27.3
280	−32.5	−22.9	−29.6	−25.4	−28.5	−31.3	−24.7	−25.9	−25.1	−32.0	−24.5	−27.9

[a] The degrees of label incorporated per subunit for the modified aspartokinase studied are: X = H_2O, 0.89 (Co); X = Asp, 1.06 (Co), 0.83 (Asp); X = ATP, 0.96 (Co), 1.06 (ATP); and X = Thr, 0.92 (Co), 1.03 (Thr), respectively. [b] Circular dichroism spectra were obtained with protein in the buffer stated in Materials and Methods which also contained 5 mM threonine (+Thr) or 10 mM aspartate (+Asp) for some spectra. [c] Strong shoulder at 265 nm.

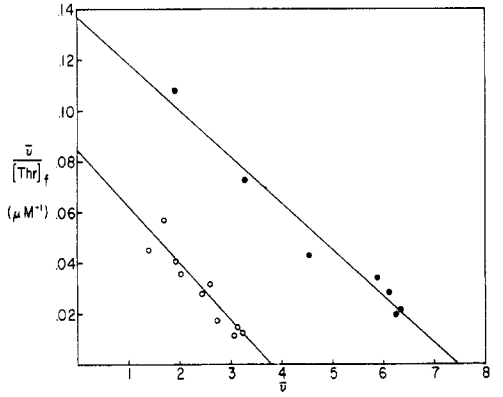

FIGURE 1: The binding of threonine to affinity-labeled aspartokinase and native enzyme in the presence of 10 mM KCl. The aspartokinase-Co(III)-threonine complex contained 0.93 equiv of threonine/subunit (●). The native enzyme was treated with H_2O_2 in the presence of Mg(II) and threonine (○). The lines are least-square fits to the data.

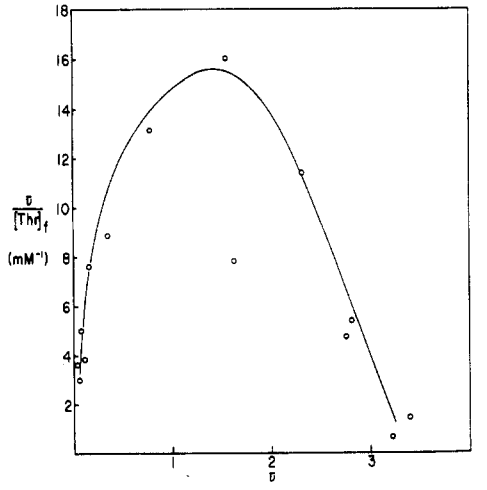

FIGURE 2: The cooperative binding of threonine to the aspartokinase-Co(III)-threonine complex in the presence of 0.15 M KCl. The affinity-labeled complex contained 0.85 equiv of threonine/subunit. The Hill coefficient was 2.2; the concentration of threonine at half saturation was 0.11 mM. The temperature was 29.1 °C.

erative (Janin et al., 1969). In buffer containing 0.15 M KCl, the binding of threonine to a ternary Co(III) adduct (0.85 threonine/subunit) is cooperative (Figure 2). As noted above the number of regulatory sites is reduced approximately by one-half to 3.5 sites per tetramer. The Hill coefficient is 2.2,

TABLE III: Chymotryptic Digestion of Native and Affinity Labeled Enzymes.

		Residual Threonine Inhibition[b] Time (h)			
Enzyme[a]	Thr (mM)	0.5	1	2	4
AK native	0	72	57	39	15
	5	100	100	102	101
AK-Co(III)	0	50	26	5	0
	5	98	102	99	96
AK-Co(III)-Thr	0	69	34	11	0
	5	97	99	97	95

[a] Proteins are native aspartokinase, AK, the binary Co(III) complex, AK-Co(III); (Co) = 0.89; and the ternary complex, AK-Co(III)-Thr, (Thr) = 1.03; (Co) = 0.92. The concentration of kinase was 2 μM in each case. Chymotrypsin concentration was 2% of total protein by weight. Samples incubated at 30 °C. [b] Residual threonine inhibitions are expressed as percent of original values: 87%, AK; 36%, AK-Co(III); and 31%, AK-Co(III)-Thr. Controls incubated without chymotrypsin and threonine were not desensitized to threonine during the course of the experiment.

and the concentration of threonine required for half saturation is 0.11 mM. These latter values are comparable to those for unmodified aspartokinase-homoserine dehydrogenase (Janin et al., 1969).

Threonine binding to native aspartokinase reduces its susceptibility to proteolytic digestion (Véron, et al., 1972; Mackall and Neet, 1974). The degradation of native enzyme and the Co(III)-aspartokinase and threonine-Co(III)-aspartokinase complexes by chymotrypsin was investigated to determine the protective effects of immobilizing threonine at the kinase regulatory site. The sensitivity of the dehydrogenase to threonine inhibition was followed as an indicator of the intactness of the tetramer. In the absence of threonine, the binary aspartokinase-Co(III) complex is more readily attacked than native enzyme (Table III). The enzyme derivative labeled with threonine is also digested, though at a slightly reduced rate in comparison to the binary complex. The addition of threonine to the samples causes all the enzyme Co(III) derivatives to be protected. These observations suggest that threonine binding at each of two classes of sites shows differential effects on rate of proteolytic degradation.

Chemical Modification of Intact and Affinity-Labeled Enzymes. Aspartokinase thiol groups may be titrated with DTNB. Threonine is able to completely prevent thiol modification which causes inactivation of aspartokinase and de-

TABLE IV: DTNB Titration of Enzyme Thiols.

Enzyme[a]	Thiols per Subunit[b]	Residual Thr Sensitivity of DTNB-Treated Samples[c]
AK	7.5 ± 0.1	89
AK + Mg(II)	7.3 ± 0.2	88
AK-Co(III)-H_2O	7.1 ± 0.2	31-37
AK-Co(III)-Thr	5.9 ± 0.2	35-37
AK-Co(III)-Asp	7.2 ± 0.2	30-31
AK-Co(III)-ATP	5.6 ± 0.2	33-35

[a] Enzymes treated with DTNB include native aspartokinase-homoserine dehydrogenase, AK, and affinity-labeled aspartokinase, AK-Co(III)-X, where X represents bound ligand. All Co(III) adducts contained between 0.93 and 1.05 equiv of the indicated ligand per subunit. AK + Mg(II) is a fully active, threonine-sensitive enzyme control which was exposed to H_2O_2 in the affinity-labeling procedure but with Mg(II) in place of Co(II). [b] Titrations were conducted in the absence and presence (5 mM) of threonine. Each determination was run in duplicate. In each case several independently prepared labeled enzymes were tested. In the presence of threonine virtually all thiol groups were protected. [c] Threonine inhibition of homoserine dehydrogenase exposed to DTNB in the presence (5 mM) of threonine during the modification. The maximal feedback inhibition is determined from assays in the presence of 7 mM threonine. In the absence of threonine, the enzyme was completely desensitized.

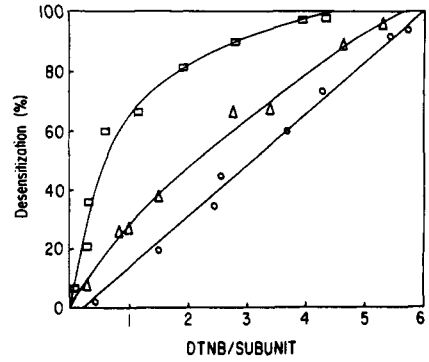

FIGURE 3: The loss of threonine inhibition of the homoserine dehydrogenase activity in native enzyme (○), the aspartokinase-Co(III) complex (△), and the aspartokinase-Co(III)-threonine complex (□) by DTNB treatment. The protection of 1 to 2 thiols by immobilized threonine (1.06/subunit) abolishes the linear dependence of desensitization upon modification observed for native enzyme and the aspartokinase-Co(III) complex.

sensitization of homoserine dehydrogenase (Truffa-Bachi et al., 1968). The effect of ligand immobilization at the kinase site was investigated. The results (Table IV) indicate that a slight protection is afforded by some ligands. Native aspartokinase, aspartokinase treated with H_2O_2, and the aspartokinase-Co(III) binary adduct all have 7.0-7.5 thiols available per subunit. These observations indicate that H_2O_2 treatment during the affinity-labeling procedure does not reduce the number of readily titratable thiols in comparison to native enzyme and that Co(III) incorporation alone does not protect thiols due to direct coordination or indirect shielding. The inability to detect significant absorption bands of the Co(III) adducts in the 500-700-nm region also indicates the absence of direct thiol-Co(III) coordination. For ternary adducts, threonine and ATP but not aspartate protect a small number of thiols (1.5-2.0 per subunit). If 5 mM threonine is present during the DTNB treatment, no thiols are modified, and the threonine sensitivity of the homoserine dehydrogenase activity is retained. In the absence of threonine, all threonine sensitivity is lost (Table IV), indicating that the integrity of thiols protected by threonine or ATP at the kinase site is not responsible for the ability of threonine to inhibit the dehydrogenase activity.

Since immobilized threonine rendered a few thiols nontitratable, the correspondence between thiol modification and residual dehydrogenase threonine sensitivity was reexamined. While a linear correlation between thiol modification and dehydrogenase threonine sensitivity exists when native enzyme is titrated with DTNB, deviations are observed with Co(III) affinity-labeled adducts (Figure 3). While native aspartokinase and the Co(III) binary adduct show similar thiol reactivity (Table III), the dehydrogenase threonine inhibitability in aspartokinase-Co(III) is more sensitive to DTNB. A more dramatic departure from the linear correlation is observed in the case of the aspartokinase-Co(III)-threonine complex. The point of complete desensitization occurs when approximately two thiols fewer are modified than in the the case with native aspartokinase. Two thiols also appear to be protected by the immobilized threonine (Table IV). The difference in DTNB desensitization of the dehydrogenase feedback inhibition in these proteins did not result from different reaction time courses since inactivation was complete in 70-85 min.

The DTNB modification of the ternary complex demonstrated that the coincidence of thiol modification and dehydrogenase desensitization is abolished by affinity labeling. The interrelationship between kinase activity and dehydrogenase feedback inhibition using a different chemical modification was investigated.

The acetylation of proteins by *N*-acetylimidazole occurs chiefly at tyrosyl residues, although some modification of amino groups may occur (Riordan and Vallee, 1972). While this modification does not formally introduce an additional charge and the acetyl group is small, the modified tyrosyl residue is unable to form the phenolate anion or act as a hydrogen bond donor.

The effects of acetylation on native aspartokinase homoserine dehydrogenase are presented in Figure 4. The most salient feature of the time course of the modification in comparison to the DTNB modification is the noncoincidence of the loss of aspartokinase activity and the homoserine dehydrogenase threonine sensitivity. The kinase activity is most sensitive while its threonine inhibition is not affected. The kinase activity is always completely inhibited. The sites of modification are probably tyrosyl residues since treatment of aspartokinase with methylacetimidate (an amino-group specific reagent) does not result in any alteration of catalytic properties or of threonine sensitivity in comparison with controls (data not shown). The acetylation of the protein yields an active dehydrogenase which is still threonine sensitive and a nearly inactivated kinase which is still completely inhibited by threonine.

Dehydrogenase Inhibition by Kinase Substrates. In addition to threonine, the kinase substrates are inhibitors of homoserine dehydrogenase. Aspartate inhibition is Michaelian, while ATP inhibition is cooperative, $n_H = 1.8$ (Patte et al., 1966). This inhibition was studied in the simpler systems afforded by both classes of derivatized aspartokinase described above.

Aspartate is still an inhibitor of the residual homoserine dehydrogenase activity of affinity-labeled protein containing aspartate immobilized at the kinase active site (Wright, 1976). This inhibition is noncooperative in the presence and absence

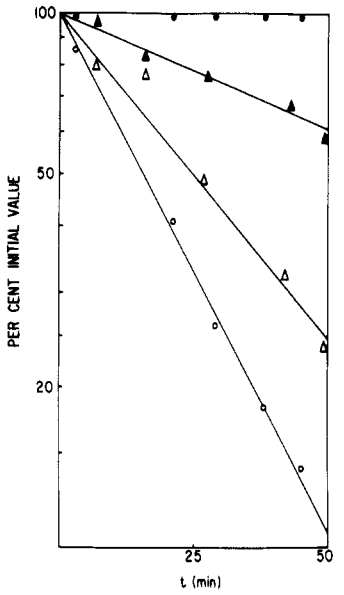

FIGURE 4: Acetylation of native aspartokinase. Enzyme (9.8 μM) was incubated with *N*-acetylimidazole (2 mg/mL) at pH 7.5. At the indicated times, samples were withdrawn for determinations of aspartokinase and homoserine dehydrogenase activity. Inhibition was determined in the presence of 7 mM threonine. Homoserine dehydrogenase (△) and aspartokinase (○) activities and dehydrogenase (▲) and kinase (●) threonine inhibitions are reported as percent of initial values. The initial specific activities were 97 and 12.8 μmol min^{-1} (mg of protein)$^{-1}$ for dehydrogenase and kinase, respectively. Their threonine inhibitions were 87 and 100%, respectively.

of threonine (n_H = 1.0). The inhibition constants are 15 mM (no threonine) and 12 mM (7 mM threonine). The extrapolated maximal aspartate inhibition is identical in the presence and absence of threonine, namely, 80 ± 1%. Similarly, the residual dehydrogenase activity of the aspartokinase-Co(III)-ATP complex is still inhibited by ATP (Wright, 1976). The inhibition is cooperative (n_H = 2.0), and the cooperativity is not affected by the presence of threonine. The ATP inhibition constant is 26 mM and increases to 35 mM in the presence of 7 mM threonine. The maximal inhibition is 65–75% and is probably independent of the threonine concentration; the uncertainty in this extrapolation derives from the cooperative nature of ATP inhibition which leads to curvature of kinetic data replots.

These results certainly suggested that ATP and aspartate inhibition of the dehydrogenase activity do not arise from the binding of these molecules at the kinase active site. Confirmatory evidence was obtained by studying the inhibition by kinase substrates on the activity of the dehydrogenase fragment produced by proteolysis and the specificity of this inhibition. The results obtained with the dimeric homoserine dehydrogenase from α-chymotrypsin digestion of native enzyme are presented in Table V. Estimates by gel filtration of the molecular weight of several preparations of these fragments were in the range 112 000–117 000. The residual activity is not threonine sensitive, but ATP remains a cooperative inhibitor of the dehydrogenase. This inhibition is not specific for ATP since both ADP and AMP are nearly as potent inhibitors. The inhibition is not enhanced by added Mg(II) or threonine. The residual homoserine dehydrogenase activity is also still inhibited by aspartate. Significantly, asparagine gives comparable inhibition. Asparagine is *not* an inhibitor of the kinase activity of intact aspartokinase. Both ATP and aspartate inhibitions do not require intact thiol groups.

TABLE V: Inhibition of Dehydrogenase Fragment.

Additions	Inhibition
7 mM Thr	0
50 mM ATP	47 (n_H = 1.9)
50 mM Mg(II) ATP	52
50 mM ATP + 7 mM Thr	48
50 mM ADP	38
50 mM Mg(II) ADP	40
50 mM AMP	43
50 mM Mg(II) AMP	44
60 mM Asp	77 (n_H = 1.0)
40 mM Asn[a]	76
DTNB[b] + 50 mM ATP	41
DTNB[b] + 60 mM Asp	72
20 mM homoserine	62
40 mM L-α-aminobutyrate	37
70 mM L-α-aminobutyrate	70

[a] This concentration of asparagine does not inhibit the aspartokinase activity of intact enzyme at either 10 or 1 mM aspartate concentration. [b] The dehydrogenase fragment was treated with DTNB for 1 h and then assayed.

To complete the investigation of the interaction of inhibitors with the enzyme, dehydrogenase inhibition by L-α-aminobutyrate was investigated. Homoserine dehydrogenase was weakly inhibited by L-α-aminobutyrate, which was considered to be a threonine analogue (Patte et al., 1963). This compound, a noncompetitive-noncooperative inhibitor of the native dehydrogenase (Wright, unpublished data) is found to be an inhibitor of the threonine-insensitive activity of the fragment (Table V). As anticipated, homoserine acts as a product inhibitor of the reaction. We feel that L-α-aminobutyrate is most likely functioning as a homoserine and not a threonine analogue in its inhibition of homoserine dehydrogenase.

Discussion

A homoserine dehydrogenase active fragment and an aspartokinase specifically affinity labeled with Co(III) have been used to study the interactions of effectors with aspartokinase-homoserine dehydrogenase. We have been able to study the effects of threonine binding which is restricted to one of the two subclasses of threonine sites. In the ternary aspartokinase-Co(III)-threonine complex, the feedback regulatory site at the kinase active site is occupied by the immobilized threonine. Occupation of this site reduces the number of sites observed by equilibrium dialysis to one-half that of the native enzyme. This is the first demonstration of an enzyme with an intermediate threonine-binding capacity. The binding of threonine at this second or truly allosteric, regulatory site cooperatively inhibits the dehydrogenase, causes a distinct conformational change detected by the circular dichroism of the complex, and protects the protein against thiol modification and proteolytic digestion. These effects correspond to those observed for the interaction of threonine with the native enzyme. In contrast, threonine immobilized at the kinase site protects only a small number of thiol groups and only slightly retards proteolytic digestion. This immobilized threonine is either unable to induce the conformational change detected by circular dichroism measurements or else is inappropriately positioned to do so. Our observations support the contention that the two classes of threonine regulatory sites are topologically distinct. Furthermore, our data are consistent with independent regulation of the two activities (Mackall and Neet, 1974; Ryzewski and Takahashi, 1975; Wright et al., 1976a).

Another model (Véron et al., 1973) was based on the apparent linkage of the inhibition of the dehydrogenase activity with the intactness of the kinase site. The correlation previously observed with thiol reagents, limited proteolysis, and thermal denaturation (Véron et al., 1973) was probably a product of the removal or rearrangement of the polypeptide chain in the kinase domain with consequent loss of an active kinase site and the two threonine binding sites per monomer. The time course of the acetylation of native aspartokinase clearly demonstrates that the two activities and their inhibitions are not necessarily linked.

We feel, however, that both regulatory sites reside in the kinase domain. Previously we had demonstrated the existence of an "isosteric" component of the feedback inhibition due to direct binding of threonine at the kinase site (Tilak et al., 1976; Wright et al., 1976a). No corresponding mechanism exists for the dehydrogenase inhibition since the active dimer produced by trypsinolysis does not bind threonine under equilibrium or steady-state assay conditions. Thus, at least a portion of the second (nonkinase) threonine regulatory site must be located in the kinase region. Modification of even a single thiol per subunit can inactivate the kinase and desensitize the dehydrogenase (Truffa-Bachi and Heck, 1971). The second, functional, threonine binding site is partially destroyed, or inhibitor binding becomes futile because the modified residue cannot be buried in the protein. We are attempting to further elucidate the mechanism of feedback regulation by an examination of the relative location of the two active sites.

We have also examined the binding of the kinase substrates to enzymes which contain ATP or aspartate immobilized at the kinase site or which lack the kinase region because of proteolytic digestion. Ternary aspartate-Co(III)-(aspartate or ATP) adducts exhibit homoserine dehydrogenase activity which is cooperatively inhibited by threonine. The presence of aspartate or ATP immobilized at the kinase site in these derivatives does not prevent added aspartate or ATP from inhibiting the dehydrogenase activity. The binding sites for kinase substrates which cause inhibition of homoserine dehydrogenase must reside in the dehydrogenase domain. Originally this inhibition was cited as part of the indirect evidence used to conclude that the two activities resided on a single polypeptide chain (Patte et al., 1966). The Hill coefficient for the ATP inhibition of the dehydrogenase activity of the ATP adduct was 2.0. Proteolytic digestion of the aspartokinase-Co(III)-ATP complex destroys the kinase active site and releases an ATP-Co(III)-peptide complex (Wright et al., 1976b). Both aspartate and ATP ($n_H = 2.0$) are still inhibitors of the threonine-insensitive dehydrogenase activity of the dimer resulting from this treatment. The ATP inhibition is not specific as both ADP and AMP are inhibitors. Mg(II), the metal cofactor of the kinase activity, does not appreciably enhance the inhibition. The data indicate that ATP inhibition of homoserine dehydrogenase is *not* due to nucleotide binding at the kinase site. Eby and Kirtley (1971) have noted that ADP and AMP function as an NAD analogue in inhibiting glyceraldehyde-3-phosphate dehydrogenase. While nucleotide binding within the dinucleotide pocket is a reasonable explanation for the observed inhibition, another site responsible for ATP inhibition may exist. Dinucleotide binding is not cooperative (Patte et al., 1963; Janin et al., 1969; Véron et al., 1973). The apparently cooperative ATP inhibition may be explicable by ATP binding to more than one form of the enzyme in the kinetic pathway; NADPH forms both catalytic and abortive complexes since substrate inhibition is observed.

Investigation of the aspartate inhibition of homoserine dehydrogenase produced parallel results. The dehydrogenase activities of the aspartokinase-Co(III)-aspartate complex and the dehydrogenase fragment were inhibited by aspartate. Asparagine, an analogue of both aspartate and aspartate-β-semialdehyde, inhibits the dehydrogenase activity of native enzyme and not the kinase activity. The inhibition of the desensitized activity of the proteolytically produced dimer by L-α-aminobutyrate suggests that this compound functions as a homoserine analogue. Thus, aspartate, asparagine, and aminobutyrate inhibitions of homoserine dehydrogenase are most readily accounted for by the binding of these amino acids at the homoserine/aspartate semialdehyde site; the data presented, however, would be equally compatible with a site in the dehydrogenase domain which is distinct from the active site.

The evidence presented here clearly suggests there are two aspartate and two ATP sites per subunit. Truffa-Bachi and Heck (1971) have previously cited aspartate and ATP inhibition of the residual dehydrogenase of enzyme modified by 6-mercapto-9-β-D-ribosylpurine 5′-triphosphate as partial evidence that modification does not occur at the kinase site; this conclusion must be further questioned (Truffa-Bachi et al., 1974b) in view of the findings discussed above.

The maximal inhibitions by aspartate or ATP of the threonine-sensitive dehydrogenase in the respective aspartate or ATP ternary Co(III)-aspartokinase complexes are nearly identical in the presence (7 mM) or absence of threonine. Threonine is a competitive inhibitor of both kinase substrates (Stadtman et al., 1961; Ehrlich and Takahashi, 1973). Threonine binding is antagonized by the presence of the kinase substrates (Wampler and Westhead, 1968; Janin et al., 1969). Previous work has shown that threonine and ATP can occupy the kinase site simultaneously (Ehrlich and Takahashi, 1973; Wright et al., 1976a). Titration of the thiol groups of affinity labeled complexes shows that immobilized ATP or threonine protect 1 to 2 residues while aspartate does not. The protection of these thiols in the threonine adduct altered the dependence of the threonine sensitivity of the dehydrogenase activity on thiol integrity. Native aspartokinase is similarly protected by threonine or a combination of Mg(II)-ATP and homoserine at low potassium concentration. These ligands cause approximately 5 thiols per subunit to become unavailable for DTNB titration (Truffa-Bachi et al., 1974a). Immobilized threonine and aspartate are apparently bound differently to the enzyme; our previous findings indicated that aspartate and threonine binding sites at the kinase active site overlap (Wright et al., 1976a). If the same thiols are protected by immobilized ATP and threonine, then the ATP and threonine sites must partially overlap also. Thus, the effect of threonine binding at the kinase site may be a simultaneous rearrangement of ATP and aspartate at the active site. These suppositions would be consistent with the competitive nature of the threonine inhibition of aspartokinase and the antagonism of threonine binding by kinase substrates.

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Fluorescence Energy Transfer between Heterologous Active Sites of Affinity-Labeled Aspartokinase of *Escherichia coli*†

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ABSTRACT: The distance between aspartokinase and homoserine dehydrogenase active sites was determined using fluorescence energy transfer between modified substrates. The fluorescent 1,*N*(6)-ethenoadenosine 5′-triphosphate was bound at the kinase active site by Co(III) affinity labeling. Reduced thionicotinamide adenine dinucleotide phosphate quenched the fluorescence of bound nucleotide. Fluorescence depolarization measurements led to a delimitation of the value of the dipolar orientation factor to the range 0.3 to 2.8. The distance between the fluorescent probe and the quencher was 29 ± 4 Å. In the presence of threonine, this distance increased to 36 ± 5 Å. Threonine binding either increased the intersite distance by ca. 7 Å or caused a reorientation of the probe at the dehydrogenase site.

The two catalytic activities of the threonine-sensitive aspartokinase–homoserine dehydrogenase are sequentially located along each of four identical polypeptide chains (Véron et al., 1972, 1973). Each subunit is organized into two discrete domains of enzymatic activity (Véron et al., 1972; Mackall and Neet, 1974). The aspartokinase activity (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) is located in the amino-terminal region. The homoserine dehydrogenase activity (L-homoserine:NADP oxidoreductase, EC 1.1.1.3) resides in a domain at the carboxyl terminus (Véron et al., 1972). Véron et al. (1973) and Mackall and Neet (1974) have proposed models for the quaternary structure of the complex. Both activities are inhibited by threonine, a product of aspartate metabolism (Cohen, 1969). The aspartokinase active site can be affinity labeled with Co(III) (Ryzewski and Takahashi, 1975). This labeling procedure can also be used to attach threonine, aspartate, and ATP (Wright et al., 1976a); such enzyme derivatives lack aspartokinase activity but still retain a partially threonine-sensitive dehydrogenase activity (Ryzewski and Takahashi, 1975; Wright et al., 1976a). The native enzyme complex contains eight binding sites per tetramer for threonine the feedback inhibitor (Falcoz-Kelly et al., 1972; Véron et al., 1973). The threonine–Co(III)–aspartokinase ternary adduct binds only four additional molecules of threonine, one-half the number bound to native enzyme. These affinity-labeled enzymes are, however, appropriate derivatives for investigating the threonine-induced conformational changes in the enzyme complex. Threonine binding to the subclass of regulatory sites not modified by the labeling procedure is apparently respon-

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