Cobalt(III) Labeled Aspartokinase–Homoserine Dehydrogenase of *Escherichia coli*†

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ABSTRACT: The kinase activity of the threonine-sensitive aspartokinase–homoserine dehydrogenase enzyme complex of *Escherichia coli* was selectively inactivated by Co(III) incorporation. Incubation of the enzyme with Co(II) in the presence of oxygen or H_2O_2 resulted in incorporation of one Co(III) per subunit. The cobalt(III) bound to the enzyme was not removable by dialysis and presumably results from formation of "inert" coordination complexes with ligands contributed by the enzyme. Cobalt was released from the enzyme by incubation with dithiothreitol but not by metal chelating agents. The Co(III)-labeled enzyme was aspartokinase inactive but still retained 60% of its original homoserine dehydrogenase activity. Studies of the time course of inactivation showed aspartokinase inactivation paralleled Co(III) incorporation. The residual dehydrogenase activity of aspartokinase inactive enzyme was still inhibited by threonine Thus, Co(III) incorporation seems to result in a specific inactivation of kinase activity which permits enumeration of the number of aspartokinase sites. Limited α-chymotrypsin digestion of Co(III)–enzyme produced homoserine dehydrogenase-active fragments devoid of Co(III), further confirming the specificity of the labeling procedure. Aspartokinase inactivation obtained without concomitant desensitization of homoserine dehydrogenase to threonine inhibition suggests that kinase active site integrity is not required for threonine binding and inhibition of homoserine dehydrogenase.

Three aspartokinases catalyze the conversion of aspartate to aspartyl phosphate which is used for the production of L-lysine, L-methionine, and L-threonine by three diverging pathways in *Escherichia coli* (Truffa-Bachi et al., 1968; Janin et al., 1969). The three enzymes can be distinguished on the basis of their differing sensitivities to the three amino acids as end-product inhibitors (Patte et al., 1966). Aspartokinase L-homoserine dehydrogenase I (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3) is sensitive to L-threonine which can competitively and completely inhibit aspartokinase activity and shows noncompetitive inhibition (80–85%) of the homoserine dehydrogenase activity.

The revised subunit structure of the enzyme indicates that there are four instead of six subunits per aspartokinase molecule of molecule weight 360,000 (Falcoz-Kelly et al., 1972, Wampler, 1972). Each of the four subunits is believed to be identical and possesses two independent polypeptide regions which carry the two catalytic activities (Janin et al., 1969). Veron et al. (1972) have proposed a model for the native tetrameric molecule based upon studies of limited proteolytic digestion of the enzyme by α-chymotrypsin. Digestion with α-chymotrypsin produces a homoserine dehydrogenase active dimeric fragment of 110,000 molecular weight which shows no aspartokinase activity. Their model for the native enzyme proposes independent polypeptide domains for the two enzymic activities with primary stabilization of tetrameric quaternary structure derived from interaction of the aspartokinase moieties in each subunit. Binding studies have shown that there are four NADPH binding sites, hence four homoserine dehydrogenase sites, and eight threonine binding sites per enzyme molecule (Veron et al., 1973). Since the K_m for aspartate is 1.8 m*M* (Wampler and Westhead, 1968) and 0.8 m*M* for metal-ATP (Ehrlich and Takahashi, 1973), accurate binding data for the estimation of the number of aspartokinase sites is difficult to obtain. Hence an alternate method for the enumeration of aspartokinase sites was sought.

Numerous studies have confirmed the essential role of sulfhydryl groups in the maintenance of activity, threonine inhibition, and quaternary structure of aspartokinase (Truffa-Bachi et al., 1966). There are 11 sulfhydryl groups per subunit (Cohen, 1969); sulfhydryl reactive agents such as PCMB[1] or Nbs_2 generally destroy both enzymatic activities and the resultant inactive molecule does not bind threonine (Truffa-Bachi et al., 1968). More specific reaction of just four sulfhydryl groups (one per subunit) occurs when the enzyme reacts with a sulfhydryl analog of ATP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (Truffa-Bachi and Heck, 1971). The enzyme produced is aspartokinase inactive, but retains approximately 80% of the native homoserine dehydrogenase activity—the residual homoserine dehydrogenase activity is not inhibited by threonine. All the studies cited above would seem to indicate that an intact aspartokinase site is necessary to obtain threonine inhibition of homoserine dehydrogenase.

Although the threonine-sensitive aspartokinase is not a metalloenzyme, aspartokinase activity requires divalent magnesium ion as a metal cofactor. Recent nuclear magnetic resonance studies using manganese indicate that the metal serves as a bridge between the enzyme and the ATP (Ehrlich and Takahashi, 1973). Since Co(II) will substitute for magnesium in aspartokinase activity the methods reported by Kang et al. (1972) for the formation of exchange-

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[1] Abbreviations used are: PCMB, *p*-chloromercuribenzoate; Nbs_2, 5,5-dithiobis(2-nitrobenzoic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid; otherwise as stated in *Biochemistry 5*, 1445 (1966).

inert cobalt–enzyme complexes by oxidizing Co(II) to Co(III) appeared to be applicable to aspartokinase. In addition to providing a means for specifically labeling the aspartokinase active site, the method promised to produce a derivatized enzyme with unique properties for further study.

Experimental Procedures

Aspartokinase–homoserine dehydrogenase I from *E. coli* K12 strain Tir-8 was prepared by previously described methods (Truffa-Bachi et al., 1968, Takahashi and Westhead, 1971). The enzyme was stored at 4° in buffer B (20 m*M* potassium phosphate (pH 7.2) containing 0.15 *M* KCl, 2 m*M* magnesium acetate, 1 m*M* L-threonine, 1 m*M* dithiothreitol, and 2 m*M* EDTA). Homoserine dehydrogenase activity was assayed in the forward direction by the method of Patte et al. (1966). Aspartokinase activity was measured by a coupled assay employing pyruvate kinase and lactate dehydrogenase (both purchased from Sigma Chemical Company) or using the hydroxamate assay as described by Wampler and Westhead (1968). Both activities were tested for inhibition using 5 m*M* L-threonine. Retention or inhibition of activity for the cobalt-derivatized enzyme is expressed as percent of initial activity or initial inhibition.

Determination of Protein Concentration. For calculations of specific activity the protein concentration was determined by absorbance at 278 nm against a water blank using a molar extinction coefficient of 208,000 M^{-1} cm^{-1} (M. T. Takahashi, unpublished data) and an enzyme molecular weight of 360,000 (Truffa-Bachi et al., 1968). Since incorporation of cobalt alters the extinction coefficient of aspartokinase–homoserine dehydrogenase I, either the Lowry method (Lowry et al., 1951) or the biuret method (Jornall et al., 1949) was used to determine the enzyme concentration using a bovine serum albumin standard.

Incorporation of Cobalt. The enzyme was equilibrated with buffer W (0.5 *M* KCl, 0.1 m*M* dithiothreitol, and 25 m*M* Hepes (pH 7.2)) by dialysis for at least 4 hr. The enzyme solution was cleared of insoluble materials by centrifugation prior to cobalt incorporation. Cobaltous chloride (purchased from Matheson Coleman and Bell, Baker, or Johnson Mathey Chemicals Limited, Specpure) and hydrogen peroxide were added consecutively as concentrated solutions in buffer W.

Two controls were used for the air oxidation experiments; one in which the enzyme was incubated without cobalt and the other in which cobalt was added but the reaction mixture kept under nitrogen. As a control for experiments in which H_2O_2 was included, the $CoCl_2$ was omitted in one sample.

The cobalt incorporation was stopped and the unbound cobalt removed by passing the reaction mixture through a Sephadex G-25 column (1 × 23 cm) equilibrated with buffer D (0.1 *M* KCl, 0.15 m*M* L-threonine, 1 m*M* EDTA, and 20 m*M* Tris-HCl (pH 7.2)). As a further precaution the enzyme was dialyzed for at least 12 hr against buffer D.

Determination of Cobalt Concentration. Cobalt concentration was determined by atomic absorption employing a Varian Instruments Model AA-4 spectrophotometer.

Reversal of Cobalt Incorporation by Dithiothreitol. Aspartokinase was first incubated with 5 m*M* $CoCl_2$ and 30 m*M* H_2O_2 for 6 hr at 30°. Combined Sephadex G-25 column fractions having the highest optical density at a wavelength of 278 nm were divided into two fractions. These fractions of Co(III) enzyme were then dialyzed against buffer D in separate vessels with dithiothreitol added to one vessel. After dialysis for 16 hr at 23°, both fractions were assayed for enzymic activities, threonine sensitivity, and for cobalt incorporated.

α-Chymotrypsin Digestion of Cobalt-Derivatized Aspartokinase. Conditions for α-chymotrypsin digestion are similar to those described by Veron et al. (1973). Cobalt-derivatized enzyme was prepared by incubation of the enzyme with 5 m*M* $CoCl_2$ and 30 m*M* H_2O_2 for 6 hr at 30°. For this experiment the Sephadex G-25 column was equilibrated with buffer T (0.15 m*M* KCl–20 m*M* sodium phosphate (pH 7.2)). The enzyme concentration in the pooled Sephadex G-25 column fractions was between 4 and 5 mg/ml. α-Chymotrypsin was added in buffer T to a concentration of 1.2% w/w of cobalt(III)–enzyme. Digestion was allowed to proceed for 1.5 hr. A preliminary experiment involving α-chymotrypsin digestion of unmodified enzyme under these conditions showed that 1 hr was sufficient to destroy 99.6% of the aspartokinase activity. After stopping proteolysis by the addition of phenylmethanesulfonyl fluoride dissolved in isopropyl alcohol to a final concentration of 2 m*M* in the digestion mixture, the mixture was concentrated to a 1-ml volume by pressure dialysis against desalting buffer. The larger molecular weight enzyme fragments were isolated by chromatography of the concentrated digestion mixture using an upward flow Sephadex G-75 (medium grade) column (1.5 × 83 cm) eluted at a rate of 12 ml/hr with buffer D. Fractions with the highest optical density of 278 nm were pooled and concentrated by pressure dialysis against buffer D. Enzyme activities, threonine sensitivity, and the cobalt/subunit ratio of the remaining undigested cobalt-derivatized enzyme were compared with that of the cobalt-derivatized enzyme used for digestion.

Results

Enzyme Activity with Co(II) vs. Mg(II). Cobalt(II) can be substituted for Mg(II) in the aspartokinase reaction. The values for K_m and V_m were determined using the hydroxamate assay for aspartokinase. The apparent K_m for Co(II) is 0.077 m*M* (at an ATP concentration of 10 m*M*) is lower than that for Mg(II), 0.11 m*M*, while the maximal velocity V_m is reduced to 32% of the value for Mg(II).

Cobalt Incorporation by Air Oxidation. When aspartokinase was incubated with the 1 m*M* cobalt and exposed to air with gentle shaking at room temperature (23°), significant losses occurred in aspartokinase activity and threonine sensitivity during a 28-hr incubation period. Since all residual aspartokinase activity was inhibited 100% by L-threonine, subsequent references to threonine sensitivity will refer solely to the inhibition of residual homoserine dehydrogenase activity by L-threonine. The control enzyme incubated without cobalt showed no significant losses of activity or inhibition by threonine. Analysis for cobalt and protein showed 0.39 cobalt atom incorporated per subunit after 28 hr of incubation. Exclusion of oxygen by placing the reaction mixture under nitrogen resulted in no significant losses in the three enzyme parameters and in no significant incorporation of cobalt. These preliminary experiments thus demonstrated the requirement of both Co(II) and an oxidant to effect changes in enzyme activities. Since the rate of air oxidation was slow, it was decided to utilize H_2O_2 instead in order to reduce the possibility of adventitious inactivation and desensitization caused by prolonged incubation of the enzyme in buffers lacking threonine.

Cobalt Incorporation Using Hydrogen Peroxide. Figure

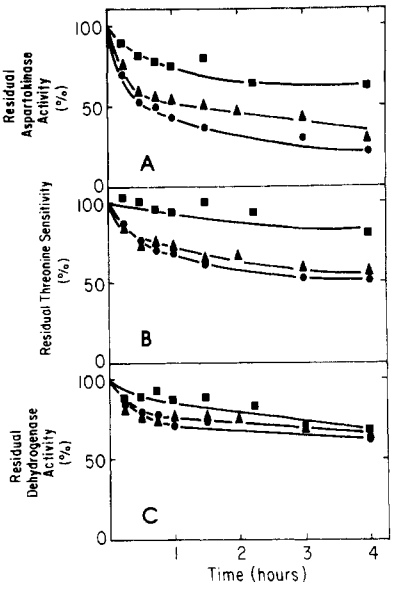

FIGURE 1: Percent residual aspartokinase activity (A), threonine sensitivity (B), and homoserine dehydrogenase activity (C) of enzyme incubated in the presence of 3 mM $CoCl_2$ and 9 mM (■), 15 mM (▲), and 20 mM (●) H_2O_2 at 25°.

1 shows the effects of incubating aspartokinase with 3 mM $CoCl_2$ in varying concentrations of H_2O_2. The loss of aspartokinase activity was enhanced most strongly by increasing the concentration of H_2O_2 while the loss of homoserine dehydrogenase was least affected; loss of threonine inhibition showed as intermediate sensitivity to increases in H_2O_2 concentrations.

The cobalt/subunit ratios determined after selected time intervals of incubation of the enzyme with 3 mM $CoCl_2$ and 20 mM H_2O_2 are presented in Figure 2A. There is a gradual increase in the number of cobalt atoms incorporated which appears to level off after 5 hr of incubation at a limiting value of approximately one per subunit. The 15-hr time point for cobalt incorporation in Figure 2 was obtained in a separate experiment and shows that one cobalt per subunit is still the limiting value. Figure 2B–D show the aspartokinase activity, threonine sensitivity, and homoserine dehydrogenase activity during cobalt incorporation for this experiment, and during the incubation of the enzyme in the presence of only 20 mM H_2O_2. No significant losses in the two activities and threonine sensitivity were observed for the control enzyme. This was found to be true on incubation of aspartokinase with concentrations of H_2O_2 as high as 40 mM. For enzyme incubated with cobalt and H_2O_2, the loss of threonine sensitivity was intermediate between the loss of aspartokinase activity (highest) and the loss of homoserine dehydrogenase activity.

Figure 3 represents plots of cobalt/subunit ratio vs. percent loss in aspartokinase activity, homoserine dehydrogenase activity, and threonine sensitivity. From this analysis it appears that aspartokinase activity losses approach 1:1 correspondence with cobalt incorporation. It is also apparent that as the number of cobalt atoms incorporated per subunit approaches a value of 1, aspartokinase activity is almost completely destroyed.

These data illustrates that the fully aspartokinase inactive enzyme which contains one Co(III) per subunit still retains substantial threonine inhibition of residual homoserine dehydrogenase activity.

Effects of Threonine upon Cobalt Incorporation. Effects

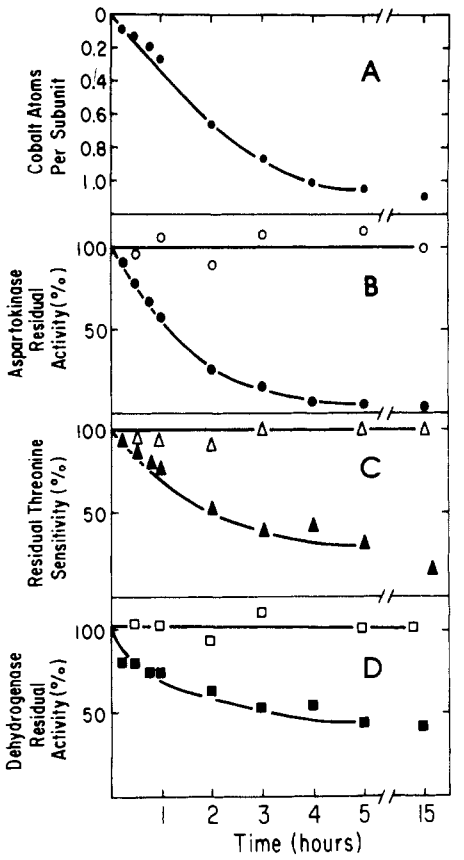

FIGURE 2: Time course of cobalt incorporation per subunit (A) during incubation of aspartokinase in the presence of 3 mM $CoCl_2$ and 20 mM H_2O_2. For this experiment, percent residual aspartokinase activity (B), threonine sensitivity (C), and homoserine dehydrogenase activity (D) in the presence (closed symbols) and absence (open symbols) of 3 mM $CoCl_2$ and in the presence of 20 mM H_2O_2.

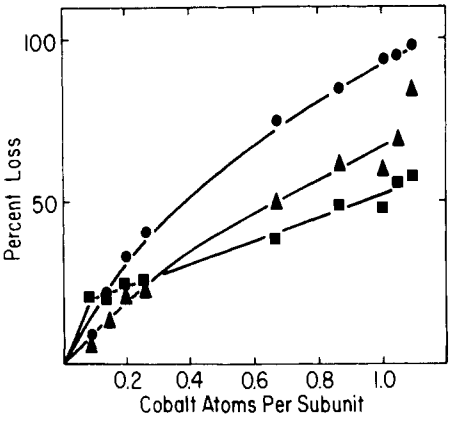

FIGURE 3: Percent loss in aspartokinase activity (●), threonine sensitivity (▲), and homoserine dehydrogenase activity (■) is shown plotted as a function of number of cobalt atoms incorporated per subunit during incubation of enzyme (18.3 mg/ml in buffer W) in the presence of 3 mM $CoCl_2$ and 20 mM H_2O_2 at 25° over a 15-hr period.

upon the two enzymic activities and threonine sensitivity of the enzyme incubated with 3 mM $CoCl_2$ and 20 mM H_2O_2 in the presence and absence of 5 mM L-threonine are shown in Figure 4. No significant losses were found in the two activities and threonine sensitivity on incubation of enzyme with 20 mM H_2O_2 and 5 mM L-threonine in the absence of Co(II). The presence of threonine increases the initial rate of loss of all three parameters. After 5 hr of incubation, aspartokinase activity and threonine sensitivity were only

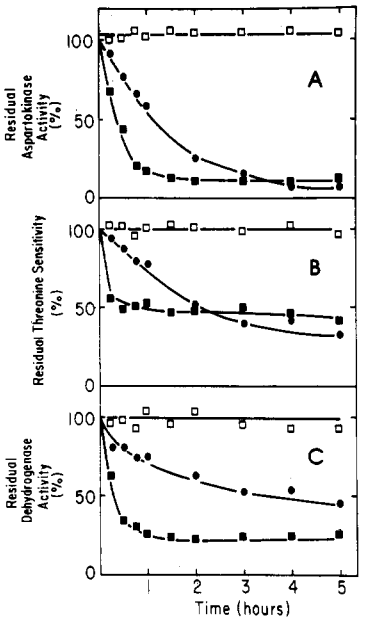

FIGURE 4: Percent residual aspartokinase activity (A), threonine sensitivity (B), and homoserine dehydrogenase activity (C) of enzyme incubated with 20 m*M* H_2O_2 (□) control in the presence of 5 m*M* L-threonine without cobalt, (■) in the presence of 5 m*M* L-threonine and 3 m*M* $CoCl_2$, and (●) in the presence of 3 m*M* $CoCl_2$ without threonine.

slightly higher than when threonine was excluded. Losses in homoserine dehydrogenase activity are consistently greater in the presence of threonine (Figure 4C). This acceleration in the loss of enzyme activity and inhibition is in sharp contrast to the protective effects afforded the enzyme by threonine toward attack by sulfhydryl reactive agents such as PCMB (Truffa-Bachi et al., 1966) and Nbs_2 (Truffa-Bachi et al., 1968).

Effects of Dithiothreitol Treatment upon Co(III)-Aspartokinase. Danchin and Buc (1973) have reported that the labeling of phosphorylase *b* with an adenosine 5′-monophosphate-Co(III) complex is reversed by treatment with dithiothreitol. Since our labeling procedure is based upon the same basic principle we attempted to reverse Co(III) incorporation using dithiothreitol. Co(III)-enzyme solutions were dialyzed against 10 and 20 m*M* concentrations of dithiothreitol over a period of 16 hr. Comparison of kinetic parameters of both treated and control enzyme are presented in Table I. It can be seen that substantial removal of bound Co(III) is effected by dithiothreitol treatment. Slight regain of aspartokinase activity results from dithiothreitol treatment, however, complete regain of aspartokinase activity is not achieved. Reactivation of homoserine dehydrogenase activity and regain of threonine inhibition seem to be variable and are not uniformly restored by dithiothreitol treatment. The reason for this lack of complete reversibility is unexplained at this time.

Limited Proteolytic Digestion of Co(III)-Aspartokinase. Since the data presented above are strongly suggestive of Co(III) labeling at or very close to the aspartokinase active site, an attempt was made to determine whether the Co(III) was bound to the aspartokinase, amino terminal, polypeptide segment of each subunit. Co(III)-aspartokinase was prepared and subjected to limited proteolytic digestion with α-chymotrypsin. The results of this treatment upon kinetic parameters and upon the number of cobalt atoms bound to the resulting homoserine dehydrogenase fragment are

Table I: Reversal of Cobalt Incorporation with Dithiothreitol.

	Aspartokinase Activity[a]	Homoserine Dehydrogenase Activity[a]	Threonine Sensitivity[b] (%)	Co/Subunit
6-hr incubation[c]	1.3	31.3	42	
Control (0 dithiothreitol)[d]	2.3	43.7	36	0.71
10 m*M* dithiothreitol[d]	3.2	44.5	38	0.20
6-hr incubation[c]	1.8	46	23	
Control (0 dithiothreitol)[d]	1.7	46	24	0.89
20 m*M* dithiothreitol[d]	2.35	46	38	0.34

[a] Enzyme activities in this and subsequent tables are expressed in enzymic units defined as the amount of enzyme required to form 1 μmol of product per min at 30°. Activities here reflect specific activity per milligram of protein. [b] Threonine sensitivity is expressed as the percent of homoserine dehydrogenase activity remaining after the addition of 5 m*M* L-threonine. [c] Enzyme activities and threonine sensitivity were determined after 6 hr of incubation of the enzyme under the conditions described in Experimental Procedures. [d] Enzyme activities, threonine sensitivity, and the number of cobalt atoms per subunit were determined for the cobalt derivatized enzyme after dialysis against desalting buffer in the presence and absence of dithiothreitol as described in Experimental Procedures.

Table II: Effects of α-Chymotrypsin Digestion on Cobalt-Derivatized Aspartokinase.

	Aspartokinase Activity	Homoserine Dehydrogenase Activity	Threonine Sensitivity (%)	Co/Subunit
Control[a]	2.17	48.9	32	0.67
Digested[a]	1.17	55.9	15	0.11

[a] Enzyme activities, threonine sensitivity, and the number of cobalt atoms per subunit were determined for cobalt derivatized aspartokinase before (control) and after (digested) digestion with α-chymotrypsin as described in Experimental Procedures.

shown in Table II. The proteolysis was evidently not complete since there is residual aspartokinase activity even after 1.5 hr of proteolytic digestion. However, it can be seen that removal of the aspartokinase terminus of each subunit does substantially remove bound Co(III). The Co(III) is apparently bound within the approximately 30,000 dalton amino terminal fragment of each subunit which contains the aspartokinase active site destroyed by proteolysis.

Discussion

The incorporation of Co(III) into aspartokinase-homoserine dehydrogenase via oxidation of Co(III) by H_2O_2 results in the binding of one metal atom per subunit. The cobalt(III) ion appears to be bound as an inert sphere complex (Taube, 1952) which is not removable either by prolonged dialysis or by passage through Sephadex G-50 columns. Cobalt incorporation results in concomitant inactivation of the aspartokinase activity of the enzyme—the apparent one for one corrrespondence of cobalt incorporation with destruction of aspartokinase activity permits enumeration of the number of kinase active sites per tetrameric enzyme molecule. There appear to be four aspartokinase active sites inactivated per 360,000 daltons—confirming the

subunit structure determined by other workers (Falcoz-Kelly et al., 1972). This technique would seem to be useful to enumerate numbers of binding sites for metal activated enzymes where the following conditions obtain: first, the metal must serve as a bridge between substrate and enzyme, or at least bind directly to enzyme amino acid side chains; secondly, cobalt(II) or other metals such as chromium(II) must function as catalytically active cofactors.

Previous work with sulfhydryl group reagents has also shown that threonine protects against activity and threonine sensitivity losses (Truffa-Bachi et al., 1968). This work on the effects of incubation of the enzyme with cobalt(II) and H_2O_2 in the presence and absence of threonine indicates that threonine has an *opposite* effect on cobalt incorporation. These differential effects of threonine upon enzyme inactivation draw the most dramatic distinction between the effects of sulfhydryl reagents and cobalt incorporation. Cobalt incorporation thus produces a unique enzyme derivative in which aspartokinase inactivation does not produce simultaneous homoserine dehydrogenase desensitization to threonine. Threonine inhibition is not obligatorily linked to the presence of functionally intact aspartokinase site. These conclusions are consonant with the authors' current picture of the nature of the inhibition of the enzyme complex by threonine. We propose that the eight threonine binding sites are composed of two separate classes of four sites each. One set of four inhibiting sites is at the kinase active sites and is responsible for aspartokinase inhibition, while another set of four "allosteric" sites is responsible for homoserine dehydrogenase inhibition. A similar proposal that only four of the eight threonine binding sites is involved in allosteric inhibition has been made previously (Veron et al., 1973).

Results of studies with [^{13}C]threonine (90% enriched in the carboxyl carbon) with aspartokinase and paramagnetic Mn^{2+} in place of Mg^{2+} as the metal cofactor shows that the carboxyl carbon in threonine is probably in the first coordination sphere of the Mn^{2+} at the active aspartokinase site (unpublished data, manuscript in preparation). Direct competitive inhibition by threonine at the aspartokinase active site is also in agreement with kinetic data on the inhibition of aspartokinase. This threonine binding site at or near the aspartokinase active site is also presumably responsible for maintenance of the quaternary structure of the enzyme. Destruction of this site via sulfhydryl reagents or by incubation of the enzyme at high pH in the absence of threonine and K^+ is presumed to result in dissociation of the enzyme. The location of the other set of four threonine binding sites is as yet undetermined but may be those sites blocked by derivatization with the sulfhydryl ATP analog.

Acknowledgments

We gratefully acknowledge the technical assistance of Ms. Linda Kovachs in this work and the help and ideas provided by Mr. Keith Wright.

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