Cobalt(III) Affinity-Labeled Aspartokinase. Formation of Substrate and Inhibitor Adducts†

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ABSTRACT: The kinase active site of the aspartokinase-homoserine dehydrogenase enzyme complex of *Escherichia coli* has been affinity labeled both with substrates aspartate and adenosine triphosphate and feedback inhibitor threonine. Co(III) exchange-inert adducts of aspartokinase and inhibitor or substrates were produced in situ by oxidation of Co(II) with H_2O_2. Enzyme-Co(III)-adenosine 5′-triphosphate (ATP), enzyme-Co(III)-aspartate, and enzyme-Co(III)-threonine ternary adducts were produced in this manner. The formation of the enzyme-Co(III)-threonine adduct leads us to conclude that threonine inhibits the kinase activity of this enzyme complex by binding in the first coordination sphere of the catalytic metal ion cofactor, a conclusion which is consistent with evidence derived from previous nuclear magnetic resonance data obtained in this laboratory. The quaternary adducts formed by H_2O_2 oxidation in the presence of aspartokinase, Co(II), ATP, aspartate, and threonine comprised a mixture of both enzyme-Co(III)-ATP-aspartate and enzyme-Co(III)-ATP-threonine adducts. The formation of the quaternary aspartate-containing adduct was unexpected, since the presence of threonine was expected to prevent access of the aspartate to the active site; most significantly however, the sum of the numbers of aspartate plus threonine molecules incorporated per active site is one. We believe that this shows direct steric overlap between the metal-adjacent binding sites for aspartate and threonine. Aspartate or threonine can not occupy the kinase active site simultaneously; this conclusion is consistent with the direct competitive inhibition of aspartate by threonine observed in steady-state kinetic studies.

Aspartokinase 1-homoserine dehydrogenase I (ATP[1]:L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine: NADP oxidoreductase, EC 1.1.1.3) of *Escherichia coli* K12 is a regulatory enzyme in L-threonine biosynthesis. This protein carries two activities in discrete domains on each of its four polypeptide chains (Veron et al., 1972). These activities are modulated by L-threonine binding at eight sites on the tetramer (Veron et al., 1973). While Mg(II) is the physiological activator of aspartokinase, both Mn(II) and Co(II) function as catalytically active replacements (Ehrlich and Takahashi, 1973; Ryzewski and Takahashi, 1975). A useful property of Co(II) is that oxidation of the d^7 ion to the d^6 Co(III) species renders exchange-inert the previously exchange-labile ligands in the metal's first coordination sphere (Taube, 1952). Co(III)-protein complexes exhibiting this inertness have been prepared. Co(III) metalloenzymes have been prepared from Co(II) precursors in the cases of carboxypeptidase A (Kang et al., 1972), carbonic anhydrase (Shinar and Navon, 1974), and alkaline phosphatase (Anderson and Vallee, 1975). In the case of carbonic anhydrase, spectroscopic evidence suggested that enzyme inhibitors were immobilized in stable, inner-sphere complexes. Co(III) complexes of metal-activated proteins have been produced in phosphorylase b (Danchin and Buc, 1973) and myosin (Werber et al., 1974) through ligand exchange with Co(III)-nucleotide complexes and in aspartokinase by oxidation of the Co(II) complex (Ryzewski and Takahashi, 1975). Co(III) affinity-labeling studies have called into question previous suggestions that a catalytically active aspartokinase site is required for the feedback inhibition of homoserine dehydrogenase (Ryzewski and Takahashi, 1975). Observation of the effects of enzyme-bound Mn(II) on the nuclear relaxation rates of H_2O protons and [1-^{13}C]threonine (Tilak et al., manuscript submitted for publication) suggested that the metal site and one threonine site were adjacent and stimulated a closer investigation of the kinase active site and the mechanism of threonine inhibition of aspartokinase. In this study, we exploited the exchange inertness of protein, substrate, and inhibitor ligands bound as Co(III)-enzyme complexes to investigate the involvement of the kinase site in threonine regulation.

Materials and Methods

Aspartokinase was isolated from *E. coli* K12, Tir-8 using previously described procedures (Ehrlich and Takahashi, 1973). The enzyme was stored as a slurry in 50% saturated ammonium sulfate at 4 °C. Homoserine dehydrogenase activity was measured by the method of Patte et al. (1966). Aspartokinase activity was determined by the coupled assay of Wampler and Westhead (1968). Co(II) must be excluded from the assay via prior dialysis, since it produces spurious apparent enzymatic activity due to the decomposition of phosphoenolpyruvate. Both enzymatic activities were tested for threonine inhibition at saturating (7 mM) levels. Homoserine dehydrogenase activity had a specific activity of 100 μmol min^{-1} mg^{-1} of protein and threonine inhibition of 85–88%. Aspartokinase had a specific activity of ca. 13 μmol min^{-1} mg^{-1} of protein. Since the aspartokinase in native and labeled protein was always 100% inhibited by L-threonine, subsequent references to residual threonine sensitivity refer solely to that of homoserine dehydrogenase.

Scintillation fluid, carrier-free ^{57}Co, and ^{14}C-labeled amino acids and ATP were obtained from New England Nuclear. D,L-[G-^{3}H]threonine was purchased from Amersham-Searle. Threonine deaminase was a gift of Dr. D. E. Wampler.

All other enzymes were obtained from Sigma Chemical Co. Spectrographically pure cobaltous chloride was obtained from

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[1] The abbreviations used follow those stated in *Biochemistry 5*, 1445 (1966).

TABLE I: Co(III)-Aspartokinase Complexes.

Label[a]	Concn (mM)[b]	Incubation (h)	$\tilde{\nu}$	Kinase[c] (sp act.)	Dehydrogenase[c] (sp act.)	Dehydrogenase[c] I (%)
Co	3.0	6	0.75	3.17	40.7	35
Co	3.0	12	0.83	1.03	37.3	30
ATP	4.9	18	1.12	0.02	39.2	42
Co/ATP	4.3/4.3	12	0.73/0.73	2.19	42.0	39
Thr	3.1	18	0.90	0.80	45.6	37
Asp	4.0	12	0.69	1.43	33.7	35
Co/Asp	2.9/6.3	18	1.16/0.93	0.11	32.6	23
Thr[d]	3.0	18	-	13.2	97.4	87

[a] Radioactive label whose incorporation was followed. Double entries indicate experiments in which the incorporation of each of two labels was followed in parallel experiments. [b] Concentration of species in oxidation sample, concentration of H_2O_2, aspartokinase, and $CoCl_2$, where not specified, as reported in Methods. [c] Residual specific activities for aspartokinase and homoserine in μmol min^{-1} mg^{-1} of protein, I is the maximum threonine inhibition of homoserine dehydrogenase in percent. [d] Native enzyme control sample. Threonine but not Co(II) or H_2O_2 were present.

TABLE II: Specificity of Incorporation.

Ligand	Incubation (h)	$\tilde{\nu}$	Kinase[a] (sp act.)	Dehydrogenase[a] (sp act.)	Dehydrogenase[a] I (%)
D-Asp[b]	6	0.03	4.02	45	39
D-Thr	6	0.08	3.62	42	40
L-Ser	6	0.09	5.16	62	35
L-Glu	6	0.18	3.97	43	37
Co	6	0.65	4.43	38	42
Mg-ATP[c]	8	0.10	11.2	78	84
Mg-L-Thr[d]	18	0.07	12.9	87	86

[a] Activities as reported as in Table I. [b] The concentration of each analogue amino acid was 5 mM. [c] Samples contained 4.3 mM Mg(II)-ATP instead of Co(II)-ATP. [d] Sample contained 3 mM $MgSO_4$.

Johnson Mathey Chemicals Ltd. All other biochemicals, salts, buffers, and chemicals were the best commercially available grades.

Preparation of ^{14}C-Labeled D-Amino Acids. L-Aspartate β-decarboxylase (L-aspartate 4-carboxylyase EC 4.1.1.12) present in an acetone powder preparation of *C. welchii* glutamate decarboxylase (EC 4.1.1.15) was used to remove L-aspartate from D,L-[4-^{14}C]aspartate. The procedure of Meister et al. (1951) was followed. The decarboxylation was conducted in 0.2 M sodium acetate buffer, pH 4.9. Pyridoxal 5'-phosphate was added to stimulate activity. This preparation had no activity against samples of D-aspartate. Exhaustive treatment of the racemic mixture resulted in the production of alanine and 49% loss in aspartic acid as determined by amino acid analysis and a 47% loss in total radioactivity as determined by scintillation counting after lyophilization. D-[G-^3H]Threonine was prepared from a racemic mixture by the action of threonine deaminase (L-threonine hydro-lyase, deaminating, EC 4.2.1.16). The reaction was driven to completion by reducing the 2-oxobutanoate produced with NADH in the presence of beef heart lactate dehydrogenase (type III), EC 1.1.1.27. The reaction was allowed to proceed until the addition of NADH and enzymes produced no further loss in NADH. This system had no activity against samples of D-threonine as determined by unchanged paper chromatographic behavior and by lack of utilization of NADH.

Buffers. The buffer employed during the oxidation of Co(II) to Co(III) contained 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.5 M KNO_3, and 50 μM dithiothreitol, pH 7.2. Dialysis buffer consisted of 10 mM tris(hydroxymethyl)aminomethane, 0.15 M KCl, 50 μM dithiothreitol, and 0.5 mM L-threonine, pH 7.6. All buffers were made with triply deionized high-purity water and were also extracted with 0.1 g/l. solution of diphenyl thiocarbamate (Eastman) in CCl_4.

Labeling of Aspartokinase. The ammonium sulfate slurry of aspartokinase was centrifuged, and the pellet was dissolved in oxidation buffer. This solution was dialyzed against a 500-fold volume excess of oxidation buffer for 2 h and cleared of insoluble material by centrifugation. To this solution of aspartokinase were then added 50–100-fold concentrated solutions of metal and ligands (substrates and/or inhibitor) dissolved in oxidation buffer. The pH of the Co(II)-ATP solution was readjusted to pH 7.2 prior to addition to the enzyme solution. When both substrates (ATP and aspartate) and the inhibitor were present in the final incubation mixture, ATP and L-threonine were added to the protein solution first, and the mixture was allowed to stand for 10 min before the subsequent addition of L-aspartate. The composition of ternary and quaternary complexes was determined by the incorporation of radiolabels in parallel experiments (e.g., enzyme-^{57}Co-ATP vs. enzyme-Co-[^{14}C]ATP). The solutions employed in these analyses were identical except for the presence of different isotopic labels. The incubation mixture for the affinity labeling experiments contained 25–30 μM aspartokinase. The final concentration of $CoCl_2$ was 3 mM except in samples containing ATP where equivalent amounts of metal and nucleotide were present. Finally, sufficient H_2O_2 in oxidation buffer was introduced to yield a final concentration of 20 mM. The concentrations of amino acids and nucleotide in individual experiments are listed in the tables. Samples were allowed to incubate at room temperature for the specified lengths of time (cf. Tables I–III). When the time course of incorporation was followed, samples were removed from the incubation mixture and diluted into a fivefold excess of dialysis buffer containing 50 μg/ml of beef liver catalase to remove residual H_2O_2. Unbound labels were removed by dialysis in stretched dialysis tubing according to the method of Englander and Crowe (1965). The dialysis regimen consisted of three 3-h

TABLE III: Mixed Quaternary Complexes.

	Thr		Asp		ATP				Dehydrogenase	
expt.	$\bar{\nu}$	(mM)[a]	$\bar{\nu}$	(mM)[a]	$\bar{\nu}$	(mM)[a]	Asp + Thr $\bar{\nu}$	Kinase (sp act.)[b]	(sp act.)[b]	I[c] (%)
1	0.66	2.1	-	-	0.89	4.3	(0.74)[d]	1.37	43.9	37
2	0.07	1.0	1.10	7.6	-	-	1.17	0.10	47.2	34
3	0.31	4.0	0.72	6.4	-	4.8	1.03	0.14	41.6	30
4	0.38	3.8	0.76	6.0	0.97[e]	7.3	1.14	0.03	33.7	35
5	0.85	12.5	0.18	1.0	1.17[e]	7.5	1.03	-	-	-
6	0.55	12.3	0.40	6.7	-	7.4	0.95	0.07	45.1	37
7a	0.72	4.0	-	0.0[f]	0.83	7.3	(0.72)[f]	2.52	46.9	34
7b	0.65	0.0[g]	0.11	6.6	0.79	0.0	(0.76)[g]	2.40	42.7	36

[a] Concentrations of ligands present during the oxidation. [b] Enzyme specific activities in μmol min^{-1} mg^{-1} of protein. [c] Inhibition (I) of dehydrogenase activity in percent. [d] Based on linear extrapolation to 1 ATP/subunit. [e] Calculated from ^{57}Co incorporation assuming 1:1 ratio of Co and ATP. [f] Aspartate not present during H_2O_2 treatment. [g] Threonine-Co(III)-aspartokinase complex (7a) exposed to aspartate in the absence of threonine, Co(II), and H_2O_2.

dialyses against 500-fold volume excess of buffer, followed by dialysis overnight against an additional 500-fold volume excess of buffer. Protein samples were centrifuged at 20 000*g* for 20 min. More than 85% of the original protein was recovered.

Determination of Stoichiometry. The labeling of aspartokinase alters the molar absorptivity at 278 nm. Protein concentrations were therefore determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Aspartokinase and albumin have identical color yields in this determination. A value of 360 000 was employed for the molecular weight of the tetramer (Truffa-Bachi et al., 1968; Wampler et al., 1970; Falcoz-Kelly et al., 1972). Radioactive ligands were determined by ligand scintillation counting in a Packard Tricarb Model 3310 spectrometer. Protein solutions and samples of the final dialyzate were counted in 10 ml of scintillation fluid (Aquasol, New England Nuclear). All protein samples were counted to a precision of greater than ±1%. Radiolabeled ligands were added in sufficient concentration to ensure specific activities high enough to achieve this degree of precision in times less than 100 min of counting. Control experiments showed that neither ^{57}Co nor ^{14}C counting efficiency was reduced by the addition of aspartokinase. Similarly, the ^{14}C counting efficiencies of labeled ATP, Co(II)-ATP, and H_2O_2-generated Co(III)-ATP were nearly identical. No ^{57}Co but some ^{14}C quenching was noted in the H_2O_2-generated, binary Co(III)-threonine complex. Solutions of this complex differ from those of threonine- or aspartate-Co(III)-aspartokinase by their strong violet-blue color. At dilutions of Co(III)-threonine comparable to those used in the aspartokinase experiments, the quenching due to the absorbance of the chelate was not observed. The stoichiometry of Co incorporation determined by a ^{57}Co label also agrees with that determined by atomic absorption spectroscopy (Ryzewski and Takahashi, 1975). The amounts of ligands and metal incorporated into aspartokinase are reported as $\bar{\nu}$, the number of equivalents of metal, amino acid, or nucleotide incorporated per mole of subunit. This value is estimated to have an accuracy of ±7%.

Electrophoresis. The molecular weight of the modified aspartokinase was estimated by comparison of the mobilities of unmodified and modified aspartokinase in 7.5% polyacrylamide gels. A stacking gel was used, and 2 mM L-threonine was present in gels and buffer. Protein bands were visualized by staining with Coomassie brilliant blue.

Reversal of Ligand Incorporation. Samples of modified protein were dialyzed for 24 h against buffer containing the agent to be used for removing ligands incorporated by H_2O_2 oxidation of Co(II) complexes. This treatment was followed by dialysis against three changes of a 500-fold volume excess of dialysis buffer for 18 h. The stoichiometries of the remaining ligands were determined in the same manner used for the original complexes. When $NaBH_4$ was used, three changes of buffer were used containing freshly added $NaBH_4$. The buffer concentration in solutions containing this reagent was 20 mM.

Results

Oxidation of the Co(II)-aspartokinase complex produces a stable, binary complex that is presumably a Co(III) exchange-inert complex. Cobalt incorporation monitored by ^{57}Co label incorporation approaches a stoichiometry of nearly 1 atom of Co(III)/subunit after a 12-h incubation with H_2O_2 (Table I). Oxidation of the ternary ATP-Co(II)-aspartokinase complex results in a stoichiometric binding of the nucleotide. Parallel experiments measuring the simultaneous incorporation of metal and nucleotide by radioactive labels show in fact the ratio of bound Co to ATP is 1:1 (Table I).

The time course of ^{57}Co and [^{14}C]ATP incorporation into a ternary protein complex is presented in Figure 1. These parallel experiments show that metal and nucleotide are bound in approximately a 1:1 ratio. The complex after an 18-h incubation shows a stoichiometry of 0.96 equiv of Co(III) and 1.06 equiv of ATP/mol of subunit. Attempts to demonstrate additional incorporation of ^{57}Co or [^{14}C]ATP either by employing higher H_2O_2 concentrations or longer incubation periods were unsuccessful.

A tenfold increase in H_2O_2 levels does not result in a significant increase in bound ^{57}Co after a 12-h incubation ($\bar{\nu}$ = 0.71); however, the homoserine dehydrogenase activity becomes almost completely insensitive to L-threonine inhibition. This may reflect modification of essential residues, perhaps sulfhydryl groups, associated with catalysis and inhibition. Prolonged incubation initially results in no increase in Co(III) incorporation over the approximately 1 Co-ATP incorporated per subunit. After 28 h however, greater than 5 Co atoms/subunit are bound. This nonstoichiometric incorporation coincides with complete desensitization of homoserine dehydrogenase to L-threonine inhibition and probably corresponds to gross denaturation of the protein with subsequent nonspecific incorporation of label. When aged aspartokinase, partially desensitized to L-threonine inhibition, is used in these studies, similar nonspecific binding is observed. Native protein struc-

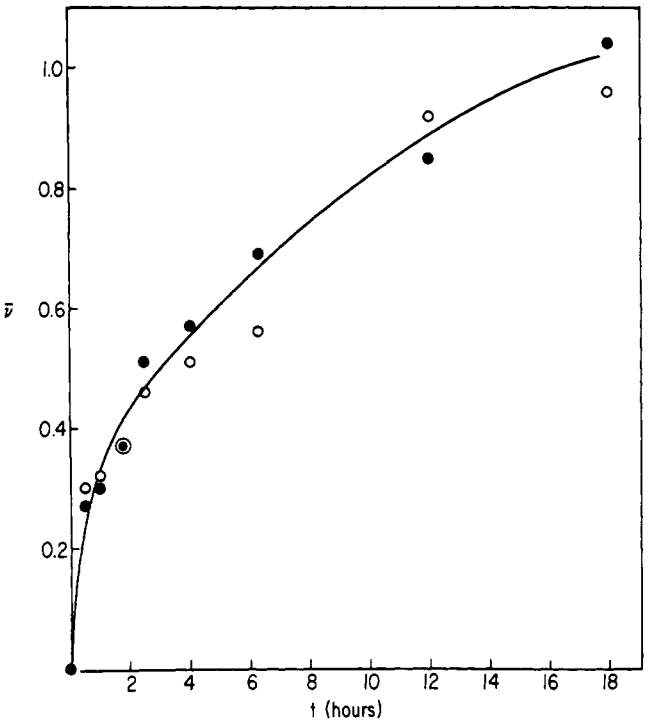

FIGURE 1: The time course of Co (○) and ATP (●) incorporation ($\bar{\nu}$) during incubation of 30 μM aspartokinase in the presence of 8.5 mM Co(II)-ATP and 20 mM H_2O_2.

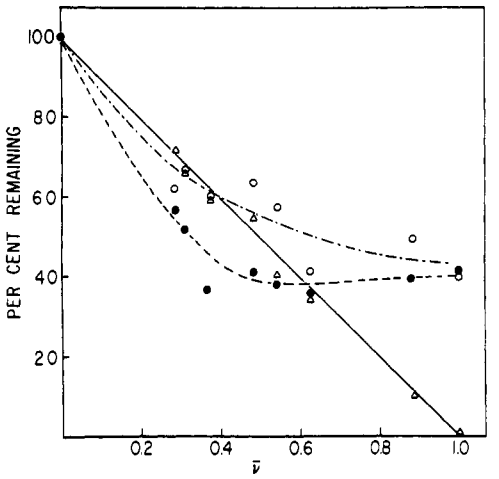

FIGURE 2: Residual aspartokinase activity (△), homoserine dehydrogenase activity (○) and L-threonine sensitivity (●) as a function of Co-ATP incorporation ($\bar{\nu}$). The value of $\bar{\nu}$ is the average of Co and ATP incorporations presented in Figure 1.

ture is thus a requisite for specific labeling. Similar observations have been made by Hirth et al. (1975) in studies of affinity labeling of the homoserine dehydrogenase activity of the same protein.

The loss of aspartokinase activity is linearly related to the average incorporation of Co(III) and ATP labels (Figure 2). The effect of the H_2O_2 treatment on the homoserine dehydrogenase activity and on its inhibition by saturating levels of the inhibitor threonine is also presented in Figure 2. Approximately 0.2–0.3 equiv of Co(III)-ATP per subunit is incorporated at the point at which approximately 50% of the changes in either dehydrogenase activity losses or threonine desensitization occur; this corresponds to about 1/tetramer. The loss in homoserine dehydrogenase activity does not completely level off. This subsequent continued loss of activity, which occurs at a much slower rate, may reflect nonspecific modification of essential residues. The residual aspartokinase is always completely inhibited by saturating levels of L-threonine. Controls containing Mg(II) instead of Co(II) reveal only 3–5% loss in enzymatic activities or threonine inhibition after 18 h exposure to H_2O_2.

Formation of Additional Ternary Complexes. Other stable ternary Co(III) complexes can be produced by H_2O_2 oxidation. Both L-threonine and L-aspartate are also incorporated into such complexes (Table I). As these results were somewhat unexpected, additional data are presented in Table II that delineate the specific requirements for ligand incorporation. H_2O_2 oxidation in the presence of an equivalent amount of Mg(II) as a Co(II) replacement does not result in ligand incorporation. No inactivation of aspartokinase or alteration in homoserine dehydrogenase activity or inhibition are noted. Similarly, incubation of aspartokinase and Co(II) in the presence of air but without H_2O_2 results in the incorporation of only 0.13 Co(II)/subunit after 6 h, indicating the requirement for a strong oxidizing agent for rapid incorporation. The formation of the Co(III)-enzyme complex is due to oxidation by dissolved O_2 (Ryzewski and Takahashi, 1975).

The effector analogues D-threonine and L-serine and the substrate analogues D-aspartate and L-glutamate were tested for potential incorporation into the Co(III) complex. None of these analogues forms catalytically significant complexes with the protein as judged by their inability to function as either substrates or inhibitors. A control sample containing no added ligands was employed to monitor ^{57}Co incorporation during the 6-h incubation. The concentrations of all substrate and inhibitor analogues were 5.0 mM during the oxidation. While 0.65 atom of Co was incorporated per subunit, negligible amounts of the ligand analogues D-aspartate, D-threonine, and L-serine were complexed to the enzyme (Table II). The L-glutamate label, however, appears to be incorporated at a somewhat higher level than the other analogues. In all cases, substantial inactivation of aspartokinase and desensitization of homoserine dehydrogenase due to Co(III) incorporation are observed. Thus, both Co(II) and ligands of specific structure and configuration are required for the production of stable, metal-bridged complexes. When the complexes of Table I are examined by gel electrophoresis in the presence of 2 mM threonine, these affinity-labeled proteins have mobilities similar to that of native aspartokinase, presumably indicating that the quaternary structure is maintained after labeling.

Formation of Mixed Quaternary Complexes. The previous results suggested that complexes of possible kinetic significance could be isolated by this technique. Oxidation of the Co(II) complexes formed in the presence of aspartokinase, ATP, aspartate, and threonine was undertaken. Solutions of enzyme, Co(II)-ATP, and threonine were mixed and permitted to stand for 10 min before aspartate was added. This preincubation allows the enzyme to complete conformational changes associated with the addition of the inhibitor (Barber and Bright, 1968; Janin and Iwatsubo, 1969). The levels of ATP and L-aspartate present in most experiments represent values that are several times the K_m of each substrate. Threonine levels utilized are fully inhibitory in the normal assay. The composition of the resulting complexes is detailed in Table III, experiments 2–6.

The incorporation of L-aspartate, L-threonine, and ATP independently into the Co(III)-aspartokinase complex was documented earlier (Table I). The simultaneous incorporation of L-threonine and ATP (Table III, experiment 1) demon-

strates that both ligands can be accommodated in the inner sphere of enzyme-bound Co(III). However, even at L-threonine concentrations which are completely inhibitory to aspartokinase activity under steady-state assay conditions, the presence of L-aspartate in the medium led, unexpectedly, to substantial incorporation of the substrate aspartate into the quaternary complex (Table III, experiments 2-6). It is considered highly significant that the combined total of bound L-threonine plus L-aspartate does not exceed 1/subunit in each case. There does not appear to be a correlation between the residual threonine sensitivity of homoserine dehydrogenase and the amount of threonine incorporated into the complex. When the ternary threonine-Co(III)-aspartokinase complex (Table III, experiment 7a) is exposed to aspartate in oxidation buffer (Table III, experiment 7b), no significant loss of threonine nor addition of aspartate is observed after 8 h. Thus, even in the absence of ATP, aspartate can not displace threonine. This observation suggests that ternary and quaternary complexes are most probably formed by a simultaneous immobilization of Co(III) and associated ligands.

Reversibility of Affinity Labeling. Danchin and Buc (1973) have noted that low-molecular-weight thiols were able to remove the Co(III)-ATP labels from rabbit muscle phosphorylase. Removal of Co(III) and regeneration of aspartokinase activity were attempted by procedures that involve either Co(III) ligand substitution or reduction of Co(III).

These treatments were applied to some binary and ternary complexes listed in Table I. As shown in Table IV, 20 mM dithiothreitol is capable of removing substrates, inhibitor, or metal. Reappearance of aspartokinase activity and of the threonine sensitivity of homoserine dehydrogenase was slight. The ability of 2,3-dimercaptopropanol, Fe(II), and Co(II) to reverse the labeling was examined by dialyzing samples of modified protein against buffer containing 0.2% (v/v) 2,3-dimercaptopropanol, 20 mM $K_4Fe(CN)_6$, or 20 mM $CoCl_2$. After 8 h, no significant regain of aspartokinase activity, threonine sensitivity, loss in [^{14}C]threonine, or ^{57}Co labels were observed with the threonine-Co(III)-aspartokinase complex. Further dialysis of samples under these conditions results in considerable loss of protein by precipitation.

The reversal of the affinity labeling of one of the mixed quaternary complexes (Table III, experiment 4) by exposure to 10 mM 2-mercaptoethanol plus 1 mM 1,10-*o*-phenanthroline or to 1 mM $NaBH_4$ in dialysis buffer for 24 h is detailed in Table V. Under these conditions regain of kinase activity and threonine sensitivity was more successful.

TABLE IV: Dithiothreitol Reversal.

Label	Untreated $\bar{\nu}$	Thiol-Treated $\bar{\nu}$	Kinase[a] (sp act.)	Dehydrogenase[a] (sp act.)	I%
Co	0.75	0.33	3.9	48.2	36
Co	0.83	0.21	1.74	36.5	35
ATP	1.12	0.30	0.1	45.0	45
Co/ATP	0.73/0.73	0.2/0.1	3.07	40.1	47
Thr	0.90	0.28	0.75	32.7	15
Asp	0.69	0.17	1.80	42.6	39

[a] Units of activities and inhibition (I) for dithiothreitol-treated samples are as reported in Table I; these values should be compared to those listed in Table I for untreated samples.

TABLE V: Affinity-Labeling Reversal in Quaternary Complexes.

	$\bar{\nu}$, I[a]	$\bar{\nu}$, II[b]	$\bar{\nu}$, III[c]
Ligands			
Co	0.97	0.84	0.69
Asp	0.76	0.59	0.38
Thr	0.38	0.27	0.15
Activities[d]			
Kinase	0.14	0.94	1.39
Dehydrogenase	37.2	31.3	36.8
I(%)	32	48	50

[a] Samples dialyzed against buffer with no reversal agents. [b] Samples treated with 10 mM 2-mercaptoethanol + 1 mM phenanthroline. [c] Samples treated with 1 mM $NaBH_4$. [d] Enzyme activities and threonine inhibition (I) as reported in Table I.

Discussion

Previous studies of the effect of the Mn(II)-aspartokinase complex on water proton relaxation rate enhancement suggested that the metal functioned as a bridge between the protein and the nucleotide substrate; Co(III) affinity-labeling studies have shown that H_2O_2 oxidation results in the incorporation of a single Co per subunit (Ryzewski and Takahashi, 1975). We have confirmed the presence of a metal-bridged nucleotide complex with aspartokinase by demonstrating stoichiometric incorporation of Co(III)-ATP consistent with a single metal-nucleotide site per monomer. Failure to detect a higher stoichiometry of ATP incorporation indicates that ATP at the second ATP binding site, whose existence was suggested by affinity labeling with the aspartokinase substrate analogue, 6-mercapto-9-β-D-ribofuranosylpurine 5'-triphosphate (Truffa-Bachi and d'A. Heck, 1971), is not bridged to the protein by Co(II).

In enzyme affinity labeled by a bridging Co(III), which has lost more than 99% of its aspartokinase activity and which has bound exchange-inert substrates and/or inhibitors, the homoserine dehydrogenase is still threonine inhibitable. Further description of the interaction of threonine with this modified enzyme will be the subject of a subsequent publication (manuscript in preparation). Threonine as well as the kinase substrates inhibit the dehydrogenase activity. The midpoint of the maximal desensitization of the dehydrogenase activity corresponds to the incorporation of approximately 1 Co(III)-ATP/tetramer. Furthermore, both kinase substrates inhibit the dehydrogenase reaction of the unmodified enzyme; the inhibition observed is additive, and ATP inhibition is cooperative, $n = 1.8$ (Patte et al., 1966). Co(III) affinity labeling of the aspartokinase site may cause a cooperatively transmitted conformational change that alters the dehydrogenase site and causes the observed decrease in dehydrogenase specific activity.

Oxidation of the ATP-Co(II)-aspartokinase complex resulting in the incorporation of ATP is understandable if the metal is a bridge between nucleotide and enzyme. The fact that both L-threonine and L-aspartate are also specifically incorporated into Co(III) enzyme complexes is a provocative observation. The simplest explanation is that each amino acid has a functional group in the inner coordination sphere of Co(II). The transfer of the γ-phosphate of ATP to the C-4 carboxylate of aspartate may occur by the juxtaposition of these two functional groups. The electrostatic barrier to approximation may be lowered by simultaneous coordination of both anionic groups to the divalent metal. This explanation, however, as-

cribes an unusually critical role to the kinase metal cofactor.

Alternative mechanisms may involve protonation of an outer sphere carboxyl group by an inner sphere H_2O or coordination of the carboxyl group to a protein functional group if polarization of the carboxyl moiety is necessary.

The threonine effect on the water proton relaxation rate enhancements of the enzyme-Mn and enzyme-Mn-ATP complexes (Ehrlich and Takahashi, 1973) is consonant with direct displacement of water from the Mn(II) inner sphere by threonine. Studies of the relaxation of the carboxyl carbon of [1-^{13}C]threonine by enzyme-bound Mn(II) suggest the threonine and metal binding sites are adjacent with the carboxyl carbon 4.4 Å removed from the metal (Tilak et al., submitted for publication). The formation of L-threonine-Co(III)-ATP-aspartokinase adducts suggests that threonine is likely bound as an inner-sphere complex. Inhibitors of carbonic anhydrase have been immobilized in this fashion; bound inhibitor is observed to be displaced by other inhibitors (Shinar and Navon, 1974). Dialysis of our samples is routinely made against buffer containing 0.5 mM L-threonine. Loss of L-[^{14}C]threonine is not observed over a 24-h period. Furthermore, L-aspartate, which is readily incorporated in quaternary complexes by H_2O_2 oxidation, is not able to displace incorporated L-threonine in significant amounts.

The ^{13}C nuclear relaxation studies (described above) have demonstrated that enzyme-bound threonine is in a state of fast exchange. The short residence time of any one threonine molecule at the kinase active site may explain the preponderance of aspartate incorporation despite the simultaneous presence of threonine at levels that are completely inhibitory to the macroscopically observed kinase enzymatic activity. This evidence would suggest either that the residence time of aspartate at the active site is longer than that for threonine or that the position of aspartate within the Co(II) inner coordination sphere permits more facile oxidation of the metal.

The sum of the numbers of threonine and aspartate incorporated into the quaternary complexes is approximately 1/subunit. A kinase active site with overlapping binding sites for threonine and aspartate in the vicinity of the metal binding site would be expected to lead to affinity-labeling patterns of this kind. Either aspartate or threonine, but not both, can be accommodated in the quaternary complex. Nuclear relaxation studies employing [4-^{13}C]aspartate are currently underway to delineate more definitively the geometry of the site overlap.

The affinity-labeling study described above differs from previously reported affinity-labeling studies of metal-nucleotide-dependent activities in that the Co(III) complexes are prepared by oxidation of the corresponding Co(II)-enzyme-ligand adduct in situ. When several Co(III)-ATP complexes were tested for their ability to inactivate myosin ATPase activities, ternary complexes possessing an additional bidentate ligand (1,10-*o*-phenanthroline and *N,N'*-dimethylethylenediamine) were observed to inactivate the ATPase activities more rapidly than the binary Co(III)-ATP complex (Werber et al., 1974). The structure of the binary metal-nucleotide complex in solution has been inferred by nuclear relaxation measurements. The metal coordinates one oxygen from each of the three phosphoryl residues and possibly a ligand donated by the adenine moiety (Glassman et al., 1971; Kuntz et al., 1972; Brown et al., 1973; Lam et al., 1974). The geometry of the metal-ATP complex may differ considerably when the complex is bound to a protein, especially where a metal bridge exists. The more rapid kinetics of myosin ATPase inactivation in the presence of the ternary Co(III) complexes compared to Co(III)-ATP inactivation (Werber et al., 1974) may reflect a greater similarity in the geometry of the former to that of myosin-bound metal-ATP. Our procedure does not require these ancillary ligands and permits us to observe the specific incorporation of substrate and inhibitor by direct H_2O_2 oxidation in a presumably catalytically significant configuration.

The immobilized ligands of other Co(III)-protein complexes have been substantially removed through exchange with thiols (Danchin and Buc, 1973) and through reduction of the Co(III) species by Co(II), Fe(II), and $NaBH_4$ (Kang et al., 1975; Werber et al., 1974). Rapid return of activity is concomitant with the loss of label in these cases. Treatment of binary and higher Co(III)-aspartokinase complexes to remove metal results in only a partial loss of Co(III) and ligand over a 24-h period and in only slight reactivation of aspartokinase activity and increase in threonine sensitivity. The recovery of enzymatic functional properties is not commensurate with metal or ligand loss. This affinity labeling may render certain protein functional groups more labile or the actual removal procedure may involve some deleterious alteration of the enzyme. The slow release of Co(III) or ligands from the Co(III)-protein complex may be due to steric inaccessibility of the reversal agents to the bound Co(III). Werber et al. (1974) have observed that while the affinity labeling of myosin by the Co(III)-ATP-*o*-phenanthroline complex is thiolytically reversed, the labeling by Co(III)-ATP-*N,N'*-dimethylethylenediamine is not. The methyl groups in the latter may shield the protein-bound metal from the thiols and consequently retard reversal. The reasons for the disappointing degree of reversibility observed in our technique are under continued investigation.

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References

Anderson, R. A., and Vallee, B. L. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 394.

Barber, E. D., and Bright, H. J. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 1363.

Brown, F. F., Campbell, I. D., Hensen, R., Hirst, C. W. J., and Richards, R. E. (1973), *Eur. J. Biochem. 38,* 54.

Danchin, A., and Buc, H. (1973), *J. Biol. Chem. 248,* 3241.

Ehrlich, R. S., and Takahashi, M. (1973), *Biochemistry 12,* 4309.

Englander, S. W., and Crowe, D. (1965), *Anal. Biochem. 12,* 579.

Falcoz-Kelly, F., Janin, J., Saari, J. C., Veron, M., Truffa-Bachi, P., and Cohen, G. N. (1972), *Eur. J. Biochem. 28,* 507.

Glassman, T. A., Cooper, C., Harrison, L. W., and Swift, T. J. (1971), *Biochemistry 10,* 843.

Hirth, C. G., Veron, M., Villar-Palasi, C., Hurion, N., and Cohen, G. N. (1975), *Eur. J. Biochem. 50,* 425.

Janin, J., and Iwatsubo, M. (1969), *Eur. J. Biochem. 11,* 530.

Kang, E. P., and Storm, C. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 621.

Kang, E. P., Storm, C. B., and Carson, F. W. (1975), *J. Am. Chem. Soc. 97,* 6723.

Kuntz, G. P. P., Glassman, T. A., Cooper, C., and Swift, T. J.

(1972), *Biochemistry 11,* 538.
Lam, Y. F., Kuntz, G. P. P., and Kotowycz, G. (1974), *J. Am. Chem. Soc. 96,* 1834.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Meister, A., Sober, H. A., and Tice, S. V. (1951), *J. Biol. Chem. 189,* 577.
Patte, J. C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128,* 426.
Ryzewski, C., and Takahashi, M. (1975), *Biochemistry 14,* 4482.
Shinar, H., and Navon, G. (1974), *Biochim. Biophys. Acta 334,* 471.
Taube, H. (1952), *Chem. Rev.* 50, 69.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *Eur. J. Biochem. 5,* 73.
Truffa-Bachi, P., and d'A. Heck, H. (1971), *Biochemistry 10,* 2700.
Veron, M., Falcoz-Kelly, F., and Cohen, G. N. (1972), *Eur. J. Biochem. 28,* 520.
Veron, M., Saari, J. C., Villar-Palasi, C., and Cohen, G. M. (1973), *Eur. J. Biochem. 38,* 325.
Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7,* 1661.
Wampler, D. E., Takahashi, M., and Westhead, E. W. (1970), *Biochemistry 9,* 4210.
Werber, M. M., Oplatka, A., and Danchin, A. (1974), *Biochemistry 13,* 2683.

The Effect of Mg(II) on the Spectral Properties of Co(II) Alkaline Phosphatase†

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ABSTRACT: Alkaline phosphatase of *Escherichia coli,* isolated by procedures which do not alter its intrinsic metal content, contains 1.3 ± 0.3 g-atom(s) of magnesium and 4.0 ± 0.2 g-atoms of zinc per mol of molecular weight 89 000 (Bosron et al., 1975). Substitution of Co(II) for Zn(II) and/or Mg(II) results in spectral properties which can be correlated with enzymatic activity. Magnesium does not activate the apoenzyme but augments the activity of the 2-Co(II) enzyme almost 3-fold and that of the 4-Co(II) enzyme 1.3-fold. The magnesium-induced increase in activity of the 2-Co(II) enzyme is accompanied by spectral changes which are consistent with an alteration from largely octahedral-like to pentacoordinate-like coordination geometry. Magnesium increases the intensity of the absorption and magnetic circular dichroism (MCD) signals of the 4-Co(II) enzyme but without evidence of changes in coordination geometry. Cobalt when bound to the magnesium sites results in octahedral-like EPR spectra, unperturbed by phosphate which significantly affects cobalt at the pentacoordinate-like sites. In the absence of magnesium, 6 g-atoms of cobalt are required to maximize the spectral properties, but activity does not increase further after the addition of only 4 g-atoms. In the presence of excess magnesium, the enzyme binds only 4 g-atoms of cobalt, while activity is optimal with only 2 g-atoms of cobalt. Hydrogen–tritium exchange measurements indicate that magnesium also stabilizes the dynamic structural properties of the apo- and 2-Co(II) enzymes but has little effect on the structure of 4-Co(II) phosphatase. The response to magnesium of both the spectral properties and enzymatic activities of cobalt alkaline phosphatase demonstrates that magnesium regulates cobalt (and zinc) binding and modulates the activity of the resultant products.

The spectral properties and chemical reactivities of chromophoric metalloenzymes differ greatly from those of the bidentate complex ions of the corresponding metals. Such metal complexes can be characterized by their electronic and magnetic properties as revealed by their absorption, natural and magnetic circular dichroic, electron paramagnetic, and other spectra (Vallee and Williams, 1968a,b).

Zinc metalloenzymes cannot be studied by spectral means, but substitution of chromophoric, paramagnetic metal ions permits examination of the nature of their metal coordination. Cobalt(II) has proved particularly suitable in this regard entailing both enzymatic activity and characteristic spectra to virtually all zinc enzymes examined thus far including alkaline phosphatase (Vallee and Williams, 1968a,b; Vallee, 1974).

Alkaline phosphatase isolated from *E. coli* is a dimer containing 4 g-atoms of zinc per molecular weight of 89 000 (Simpson et al., 1968). One pair of zinc atoms has been postulated to be involved primarily in catalysis while the other is thought to serve in structure stabilization. As isolated, native alkaline phosphatase also contains 1 to 2 g-atoms of magnesium (Plocke et al., 1962; Simpson et al., 1968) whose regulatory role has been recognized only recently (Bosron et al., 1975; Anderson et al., 1975).

The present data demonstrate that magnesium significantly affects the manner in which zinc and cobalt participate in catalysis and structure stabilization of alkaline phosphatase. The characteristics of cobalt and zinc–cobalt hybrid phosphatases have been studied in the presence and absence of magnesium by absorption, magnetic circular dichroic, and electron paramagnetic resonance spectroscopy. In the absence of magne-

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