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Localization of the Zinc Binding Site of Aspartate Transcarbamoylase in the Regulatory Subunit

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ABSTRACT **Aspartate transcarbamoylase (EC 2.1.3.2) from *Escherichia coli* contains six zinc ions per molecule. Renaturation studies of this allosteric enzyme and its isolated subunits show that the metal binding site is in the regulatory polypeptide chain. Ultraviolet difference spectra of the cadmium and zinc derivatives have been used to show that the sites in the isolated subunit and the full enzyme are similar. Results with an apo derivative of the regulatory subunit suggest that the metal is not required for the binding of the feedback inhibitor, but that it is involved in the structural stability of the protein. A close relationship between the reactivity of the cysteine residues in the regulatory subunit and the metal ion has been found.**

Carbamoylphosphate:L-aspartate carbamoyltransferase [aspartate transcarbamoylase] (EC 2.1.3.2) from *Escherichia coli* has been shown to contain tightly bound zinc ions (1, 2). The stoichiometry of the metal ions is in agreement with that of the polypeptide chains: one enzyme molecule contains six catalytic and six regulatory chains, as well as six zinc ions. It has been argued on the basis of crystallographic (3) and chemical (1, 4) data that the polypeptide chains are arranged around one three-fold and three two-fold axes of symmetry.* If all six metal ions are situated in spatially equivalent positions, they must be localized either in the catalytic or in the regulatory chains or at the subunit contacts. This report describes renaturation experiments that allow one to localize the binding sites in the regulatory subunits.

MATERIALS AND METHODS

Aspartate transcarbamoylase was purified as described by Gerhart and Holoubek (5). Cadmium enzyme was prepared *in vivo* by substitution of $ZnSO_4$ by $CdCl_2$ in the growth medium. Purified Zn and Cd enzymes were interconverted *in vitro* by successive denaturation, replacement of the metal, and renaturation. Enzyme was denatured in 6 M guanidine·HCl, 0.1 M β-mercaptoethanol, and 1 mM EDTA. After 4 hr of incubation at 20°C, the protein was subjected to gel filtration on Sephadex G-25 in 6 M urea–20 mM β-mercaptoethanol–0.1 M Tris·HCl, pH 7.8. Zn or Cd ions were added to the pooled protein fractions in an amount equimolar to the concentration of metal binding sites. Renaturation was achieved by dilution into buffer solution (1). Dissociation of the enzyme into its subunits by cleavage with *p*MB (*p*-hydroxy mercuribenzoic acid; ref. 5) or by heat treatment (1) has been described in detail.

Metal derivatives of the regulatory subunit were prepared as follows. 250 ml of enzyme solution [2 mg/ml in 4 mM potassium phosphate (pH 7.0)–0.4 mM EDTA–2 mM β-mercaptoethanol] was heated to 64°C for 8 min, then rapidly cooled in ice and centrifuged. The supernatant contained 60–70% of the theoretical amount of C subunit, no R chains (as judged by acrylamide gel electrophoresis in sodium dodecyl sulfate), and Zn ions in quantitative amounts. The pellet consisted of R subunit and the remaining fraction of C subunits. All further experiments with isolated R subunit were performed in 0.2 M Tris acetate at pH 8.2 (buffer A). The pellet was dissolved in 4 ml of buffer A–8 M guanidine·HCl–0.13 M β-mercaptoethanol–1.3 mM EDTA. After a 4-hr incubation, gel filtration in urea was performed as described above. Protein fractions were pooled, and Zn or Cd was added in a 1.5-molar excess of the metal binding sites. R subunits were renatured by dripping the protein into eleven volumes of buffer A–0.1 M β-mercaptoethanol, followed by a 4-hr incubation at 4°C. After concentration by ultrafiltration, EDTA was added (1 mM), and the protein was subjected to gel filtration in buffer A–2 mM β-mercaptoethanol. R subunit was separated by zone centrifugation from a small amount of the complete enzyme. The total recovery was 50%. Apo R subunit was prepared by an identical procedure, except that no metal ions were added before renaturation. This derivative still contained about 20% of the original complement of zinc ions.

R subunit, prepared by *p*MB cleavage of enzyme (5), was used as an alternative starting material to obtain metal derivatives: 5–10 mg/ml of this subunit in buffer A was made 0.1 M in β-mercaptoethanol, and then 6 M in guanidine·HCl. After gel filtration in 6 M buffered urea–20 mM β-mercaptoethanol, reactivation was obtained as described above.

Metal analyses were performed by atomic absorption spectrophotometry on a Perkin-Elmer 303 instrument. Before analysis, protein solutions were routinely made 1 mM in EDTA, and then immediately subjected to gel filtration in buffer A.

Abbreviations: *p*MB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); C and R, catalytic and regulatory subunits (chains) of the enzyme.

* Conclusive proof for D_3 symmetry has been obtained recently from further crystallographic studies. Wiley, D. C., C. H. McMurray, D. R. Evans, S. G. Warren, and W. N. Lipscomb (to be published).

TABLE 1. *Metal content of aspartate transcarbamoylase and its subunits*

Protein	Derivative	Metal content* Zn^{++}	Metal content* Cd^{++}
Enzyme (RC)-pair	Zn	0.95 ± 0.04	<0.03
	Cd	0.03	0.95 ± 0.04
	dissociated†	<0.03	<0.03
C subunit‡	—	<0.01	<0.01
R subunit	Zn	0.82 ± 0.12	<0.01
	Cd	0.01	0.89 ± 0.11
	Apo	0.20	<0.01
	CM§	<0.01	<0.01
	Competition¶	0.54	0.30
	Exchange‖	0.60	<0.01
	Chelation**: Zn–R	0.44	0.01
	Cd–R	0.01	0.19

* Expressed in g-atoms per protomeric (RC)-pair (molecular weight 50,500) in the enzyme, and per polypeptide chain in the subunits (C chain 33,500; R chain 17,000)(1).

† Enzyme after dissociation by *p*MB, neohydrin, or guanidine·HCl, and gel filtration.

‡ Similar values were obtained for the C subunit prepared by heat treatment, and for different preparations of reactivated C subunit.

§ After carboxymethylation with iodoacetate.

¶ Determined after reactivation of R subunits in the presence of both zinc and cadmium ions, each in amounts equimolar to the concentration of potential binding sites.

‖ The extent of metal exchange was examined after dialysis and incubation of cadmium-containing R subunit: a 4-hr dialysis against a 100-times larger volume of buffer A containing 2 mM β-mercaptoethanol and $ZnSO_4$, in amounts equimolar to the concentration of metal binding sites inside the dialysis bag, was followed by a 20- to 170-hr incubation at 4°C.

** The initial metal concentrations were 0.85 g atoms of Zn and 0.92 g atoms of Cd, respectively. The proteins were dialyzed against 1 mM EDTA (in buffer A that contained 2 mM β-mercaptoethanol) for 24 hr at 4°C. Metal analysis was performed after gel filtration. In control experiments, R subunit was subjected to gel filtration without prior treatment with EDTA, or after a 24-hr dialysis against 0.2 M EDTA. In the first control, no metal ions were displaced, whereas, in the second control, no protein-bound metal could be detected.

Treatment of proteins with *p*MB, 3-chloromercury-2-methoxypropylurea (neohydrin), or $AgNO_3$ was performed at a concentration 1.5-fold higher than that of the sulfhydryl groups present. Proteins were freed from thiol reagents by gel filtration. Regulatory subunit was alkylated by incubating the metal-containing subunit (2–10 mg/ml) with iodoacetic acid in buffer A–2 mM β-mercaptoethanol. The reagent was added in an amount 40 times that of all reactive sulfhydryl groups present. After a 10-hr incubation at 20°C, the reaction was terminated by the addition of β-mercaptoethanol and the solution was subjected to gel filtration. The specificity and the stoichiometry of the reaction were checked using [^{14}C]iodoacetate (Amersham/Searle, 21 Ci/mol). Amino acid analysis was performed to identify the radioactively labeled amino acids.

Binding of CTP to the R subunit was studied by the gel filtration procedure of Hummel and Dreyer (6). Protein solutions (0.4 ml, containing 1 mg of protein) were made 5% in sucrose and layered under the solvent onto the top of the gel (Sephadex G-25; 25-ml bed volume), which had been equilibrated with buffer A containing concentrations of CTP between 1.25×10^{-6} and 5×10^{-5} M. Column buffer and sample contained sufficient [^{14}C]CTP (New England Nuclear Corp., 345 Ci/mol) to give approximately 2000 cpm/ml. Protein was determined by amino acid analysis. All other procedures used and sources of chemicals have been reported (1).

RESULTS

Metal binding

The transcarbamoylase isolated from *E. coli* grown in the presence of Zn or Cd salts contains six cations of either metal per molecule (300,000 daltons) (Table 1). The ions are not available to chelating agents (EDTA, *o*-phenanthroline, hydroxyquinoline sulfonic acid, nitrilotriacetic acid, or Chelex). Exchange of ions cannot be observed either with ions of the same species, as determined† by incubation with ^{65}Zn, or with the alternate ion (conditions described in Table 1). The Zn and Cd derivatives of the enzyme are identical in most physical, chemical, and kinetic characteristics (1). They do, however, differ in their optical properties. The Cd enzyme has a strong ultraviolet absorption band in the region between 240 and 280 nm that is absent in the zinc derivative. The two spectra, as well as the difference spectrum, are shown in Fig. 1*a*. The difference spectrum is characterized by a maximum at 246 nm, with $\Delta\epsilon^{M}_{246nm} = 1.78 \times 10^{4}\ M^{-1}\ cm^{-1}$ (expressed per mole of bound metal, corresponding to 50,500 g of protein). The following experiments demonstrate that the higher ultraviolet absorptivity of the Cd enzyme is the result of a specific metal–protein interaction. (*a*) Denaturation of either metal derivative in guanidine HCl yields preparations with identical spectra. Unfolding is accompanied by release of the metal ion as judged by gel filtration in urea (Table 1). When the resulting polypeptide chain mixture is subsequently renatured in the presence of either divalent cation, the added ions are incorporated into the enzyme. The spectrum of the reactivated derivative is that predicted on the basis of its metal content. (*b*) Cleavage of the enzyme into its subunits by organic mercurials releases the metal ions from the protein (Table 1). Both derivatives can be interconverted by metal exchange after dissociation into subunits, followed by reconstitution to the full enzyme (1). Interconversion is accompanied by the predicted changes of the spectral properties. (*c*) The difference spectrum disappears when both enzymes are dissociated with neohydrin. Since mercaptide bonds formed by this reagent do not absorb at wavelengths greater than 240 nm (7), a direct spectral analysis is possible.

We have shown previously that C subunit, prepared by dissociation of the transcarbamoylase with *p*MB, followed by reactivation with β-mercaptoethanol, or by heat treatment, is free of zinc ions and shows full enzymatic activity (1). Furthermore, renaturation after exposure to guanidine·HCl occurs with high yield whether or not a strongly chelating agent is present. If renaturation is performed in the presence of zinc or cadmium ions, the resulting C subunit does not contain metal ions (Table 1), and has physical, chemical,

† We thank Dr. F. Quiocho, Dept. of Chemistry, Harvard University, for allowing us to quote this result.

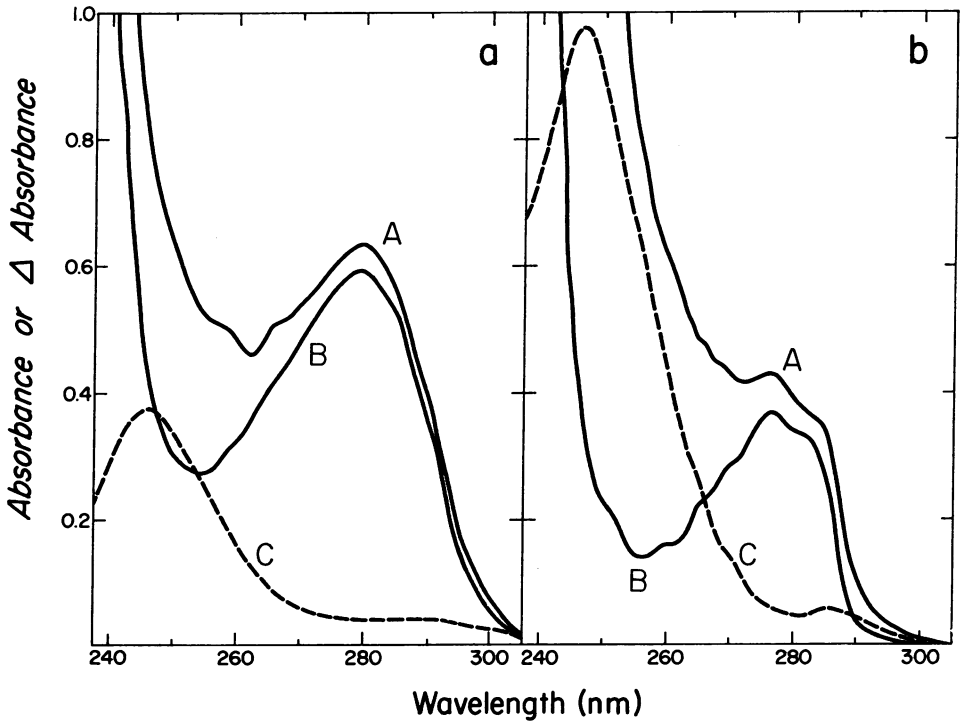

FIG. 1. Ultraviolet absorption spectra of the Cd (*curve A*) and Zn (*curve B*) derivatives of aspartate transcarbamoylase (*panel a*) and the regulatory subunit (*panel b*). Spectra were recorded on a Cary 11 spectrophotometer in quartz cells of 1-cm path length at a protein concentration of 1 mg/ml. The scales for absorbance and difference absorbance are identical. Zn and Cd derivatives of the enzyme and of the isolated R subunits show very similar difference spectra (*curve C*), with $\Delta\epsilon^{M}_{246\ nm} = 1.78 \times 10^4\ M^{-1}\ cm^{-1}$ for the complete enzyme and $1.68 \times 10^4\ M^{-1}\ cm^{-1}$ for the R subunit.

spectral, and kinetic characteristics identical to those of the C subunit obtained by the other procedures. These results rule out the participation of metal ions in enzymatic activity or the folding of the polypeptide chains, and make it unlikely that the C subunit provides the metal binding site.

R subunits, renatured from guanidine·HCl in the presence of Zn or Cd ions, contain 0.8–0.9 g atoms of metal ions per molecular weight 17,000 (Table 1). That these two divalent metals occupy an identical site in the protein was shown by competition and exchange experiments. Fully denatured R chains were renatured in the presence of both metal ions, each at a concentration equimolar to that of potential metal binding sites in the protein. Analysis revealed that the sum of the incorporated ions is identical to the amount of metal bound if R subunits were renatured in the presence of either metal ion alone (Table 1, *competition analysis*). To test whether the two ions can exchange, the Cd derivative was incubated in the presence of Zn ions. Table 1 (*exchange analysis*) shows that all of the initially bound Cd ions were displaced.

Protein-bound Zn and Cd are available to added chelating agents. From the data, shown in Table 1 (*chelation analysis*), it is possible to estimate that the apparent association constant of Zn and Cd to the R subunit is approximately of the same order of magnitude as that of the metal–EDTA complexes (11).

The function of the metal

To demonstrate that Zn- and Cd-R subunits are functional, we have studied the binding of the feedback inhibitor CTP and their association with C subunits. CTP binding data of the metallo R derivatives are presented in the Scatchard plot of Fig. 2, and compared with those obtained with R subunits, freshly prepared by *p*MB cleavage (5), and compared also with the apo R derivative, prepared as described in

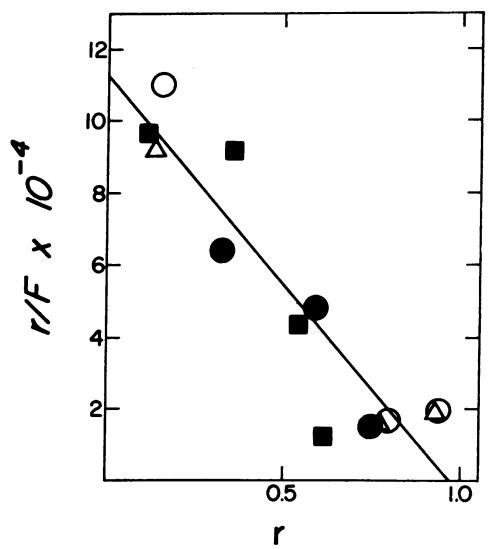

FIG. 2. Scatchard plot for the binding of CTP to different derivatives of the regulatory subunit. The Zn and Cd derivatives are represented by *open circles* and *triangles*, respectively. Symbols for the conventionally obtained R subunit and the apo derivative are *closed circles* and *squares*, respectively. The *solid line* represents the least-squares fit to all experimental points obtained. The dissociation constant of the inhibitor–protein complex is of the order of 10^{-5} M.

Methods. The result of one binding site per R polypeptide chain for the metallo R derivatives is in good agreement with that obtained independently by Hammes *et al.* (8), who used R subunits prepared by *p*MB treatment only. From Fig. 2, the dissociation constant of the CTP-protein complex is estimated to be 10^{-5} M. Both Zn- and Cd-R subunits thus show unimpaired CTP binding, while the apo derivative yields a slightly lower CTP binding (0.8 binding sites per 17,000 daltons). This figure is considerably higher than the value of 0.2 expected if only that fraction of R subunits that contains metal ions (20%) could bind CTP. The zinc contamination, therefore, cannot account for this result.

The metal derivatives of the R subunit recombine with stoichiometric amounts of C subunit to form complete enzyme. Sedimentation analysis revealed that both the Zn and Cd derivative have sedimentation coefficients similar to that obtained for the conventionally prepared R subunit (2.6–2.8S in 0.2 M Tris buffer versus $s_{20,w} = 2.9$, see ref. 1) and that reconstitution with C subunits to the full enzyme occurred in $\geqslant$ 90% yield. Separation of the reconstituted enzyme from a small amount of higher aggregates by zone centrifugation yields enzyme preparations with kinetic properties qualitatively very similar to those of untreated enzyme‡. The apo derivative, however, has a sedimentation coefficient of only 2.2S, and attempts to recombine it with catalytic subunits have thus far failed§. Furthermore, the apo derivative is unstable. This instability can be followed by an increase in its absorbance (within hours) in the spectral region, 250–270 nm, in which disulfide bonds are known to absorb (9). Titration of the sulfhydryl groups with DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] yielded values between one and two cysteine residues reactive both in guanidine·HCl and in buffer solution, a value substantially lower than the more constant values (3.4-SH/17,000 daltons; see below) obtained for the metallo R derivatives.

Spectral properties of the Zn- and Cd-R subunit

The ultraviolet absorption spectra of the two metallo derivatives of the R subunit are shown in Fig. 1*b*. Their difference spectrum exhibits a maximum at 246 nm with $\Delta\epsilon^{M}_{246nm} = 1.68 \times 10^{4}$ (expressed per mole of bound metal corresponding to 17,000 g of protein). The higher absorptivity of the Cd subunit can be abolished by denaturation in guanidine·HCl, by treatment with neohydrin, or by carboxymethylation (see below). Identical spectra of the Cd- and Zn-R subunit are obtained after these treatments. Full enzymes, reconstituted *in vitro* from catalytic and Zn- or Cd-R subunits, have spectra equivalent to those of Zn- or Cd-transcarbamoylase prepared *in vivo*. Spectra calculated from the absorptivities of either metallo R subunits and the C subunit (according to their mass ratios) are in agreement with those of the full enzyme derivatives. These results demonstrate that the difference spectrum found for Zn- and Cd-transcarbamoylase can be attributed to the spectral properties of the isolated Zn- and Cd-R subunits.

The reactivity of sulfhydryl groups

Nonmetallic sulfhydryl reagents (DTNB, iodoacetic acid), as well as *p*MB and $AgNO_3$, were used in this study. Cysteine residues of the isolated R subunit can be titrated both with DTNB and [^{14}C]iodoacetate, while the same residues in the native enzyme cannot react with either reagent (10, 11)¶. In the R subunit, 3.5 SH per 17,000 daltons react with DTNB, in agreement with the value obtained in guanidine·HCl (3.3 SH/17,000 daltons), and close to the value of four expected from the amino acid sequence of the R subunit (4). Substitution of cysteine residues in buffer solution with [^{14}C]iodoacetate yielded 3.5 mol of carboxymethylcysteine per mol of R chain, and only trace amounts of side products. Metal analysis demonstrated that upon substitution of the sulfhydryl groups, Zn and Cd ions were quantitatively released from the protein (Table 1). The absorption spectrum of the alkylated derivative was very similar to that expected from the amino acid composition (4) and to that obtained from the denatured subunit‖. The release of metal ions upon substitution of cysteine residues with organic mercurials and silver ions was also followed by spectral analysis. The reactions are fast and are essentially completed within less than 10 seconds, whereas the reaction of *p*MB with native transcarbamoylase has a rate constant (7) of 5×10^{-3} sec^{-1}. After these reactions, the R subunit is free of metals, as judged from gel-filtered material.

DISCUSSION

Metal analyses of native transcarbamoylase and its subunits, renatured from guanidine·HCl, show that the metal binding sites are located in the regulatory polypeptide chains. The ultraviolet difference spectra of the Cd and Zn derivatives, of both the native enzyme and the R subunit, are very similar in shape and maximum difference extinction coefficient. Thus, the metal binding site in the regulatory subunit must resemble that in the native enzyme.

Zn- or Cd-containing R subunits show similar functional properties as R subunit obtained by the conventional (5) *p*MB cleavage of the enzyme. They bind CTP and reassociate with C subunits to form enzyme. However, conventionally prepared R subunit neither contains zinc ions, nor does it take up stoichiometric amounts of Zn or Cd without prior denaturation. It has optical properties (2, 7, 11) that indicate the presence of a chromogenic group originating from residual binding of mercury or organic mercurial. Thus, only the Zn- or Cd-containing R subunits can be considered natural counterparts of the R subunit in the native enzyme; we have therefore restricted our studies to those derivatives. Renatura-

‡ The specific activity and feedback inhibition were reduced by 20 and 40%, respectively, and the Hill coefficient was approximately 2.6. It should be noted that reactivation of previously dissociated enzyme always leads to somewhat reduced kinetic parameters.

§ In a preparation of apo R subunit (zinc content 0.2 g-atom) 10–20% was reconstituted to enzyme after the addition of excess C subunit. Renaturation of a zinc-free subunit mixture from guanidine·HCl yielded an enzyme preparation (sedimentation coefficient 12 S)that contained only 0.2–1.0 g-atom of zinc per molecular weight 300,000. This derivative, which is subject to an "aging" process, is presently under investigation.

¶ Under certain conditions, the cysteine residues of the C polypeptide chains can be reacted selectively in the full enzyme. This derivative does not dissociate and retains the zinc ions (personal communication, Wiley, D. C., C. H. McMurray, D. R. Evans, S. G. Warren, and W. N. Lipscomb).

‖ A fully alkylated derivative, free of Group IIB metal ions, and with identical absorption spectrum, can also be obtained from the conventionally prepared R subunit.

tion of R subunit in the absence of Zn or Cd ions yields an unstable product that still contains residual amounts of zinc. Although these characteristics complicate the study of the function of the metal ions, it is possible to demonstrate that fresh preparations have a CTP-binding capacity only slightly lower than that expected if metal ions are not required for inhibitor binding ($n = 1.0$). The actual value obtained ($n = 0.8$) can be considered a minimum estimate, since aged preparations bind less CTP. Therefore, we conclude tentatively that the metal ion is not required for CTP binding, but that it is important for the stability of the three-dimensional structure of the R subunit. Such a function is also indicated in the full enzyme by the result that *E. coli*, grown in the absence of Zn ions, contains decreased amounts of full enzyme and increased amounts of catalytic subunit (2, 11).

Zn and Cd are more accessible in the isolated R subunit than in the native enzyme. This may be due to a conformational change on association of the different subunits, or to direct shielding of the metal binding site by the C subunit. The latter possibility could imply a localization of the metal ions close to the subunit contacts. This possibility is especially interesting in an allosteric enzyme, in which subunit contacts are necessary for the transfer of conformational message. Both mechanisms can also explain the greatly increased reactivity of the sulfhydryl groups in the isolated R subunit. The reactivity of the metal ions and of the sulfhydryl groups closely parallel each other in that they exhibit high reactivity in the R subunit and low reactivity in the full enzyme. This parallel reactivity could imply a direct interaction between zinc and cysteine residues. The possibility of a Zn- or Cd-cysteine bond has also to be discussed in view of the strong difference absorption band ($\Delta\epsilon^{M}_{246nm} = 1.7 \times 10^4\ M^{-1}\ cm^{-1}$) obtained from the Cd and Zn derivatives of the enzyme and its R subunit. For conalbumin, which binds Zn or Cd ions by tyrosine ligands, a much weaker difference spectrum ($\Delta\epsilon^{M} < 10^3$) at 260 nm has been reported (12). Alkaline phosphatase, in which histidine residues have been identified as metal ligands (13), shows no noticeable spectral difference for its Zn and Cd derivatives (J. Coleman**, personal communication). Metallothionein, however, which has been shown to bind Zn and Cd ions by cysteine residues, and certain model complexes of Zn and Cd ions with sulfhydryl compounds, shows difference spectra strikingly similar to those described here for the transcarbamoylase and the R subunit: an absorption band with $\Delta\epsilon^{M}_{246nm}$ of $1.4\text{–}1.7 \times 10^4\ M^{-1}\ cm^{-1}$ has been reported by Kägi and Vallee (14). Furthermore, the sulfhydryl groups in metallothionine and in isolated R subunits show similar reactivities. Reaction of either protein with *p*MB or silver ions causes the immediate release of the metal ions, and alkylation of cysteine residues prevents metal binding. These observations cannot be interpreted as a proof for a direct metal–sulfur interaction in the R subunit and the enzyme, and do not rule out the participation of other donor groups as metal ligands. However, none of the evidence accumulated so far is in contradiction to such possible interaction. For an unambiguous identification of the ligands, however, more experimental evidence, especially high-resolution x-ray analysis, will be needed. The methods for renaturation of the enzyme from its polypeptide chains reported here should allow the construction of aspartate transcarbamoylase derivatives in which specific residues of either polypeptide chain have been modified before reconstitution. Studies with such derivatives should give information about the residues involved in metal binding and in allosteric transformation.

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