The Enthalpy of Binding of Various Transition Metal Ions to Bovine Apocarbonic Anhydrase*

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ABSTRACT: Reaction calorimetry has been employed to determine the enthalpy changes accompanying the binding of Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+} to the apoproteins derived from bovine carbonic anhydrases A and B.

Small *increases* in enthalpy, ranging from 3 to 10 kcal $mole^{-1}$, were observed. Combination of the data for zinc with the equilibrium measurements of Lindskog and Malmström (Lindskog, S., and Malström, B. G. (1962), *J. Biol. Chem. 237*, 1129) leads to thermodynamic parameters for the binding process at 25° and pH 7.0 as follows: $\Delta G' = -16.4$ kcal $mole^{-1}$ (standard state for hydrogen ions at activity of 10^{-7} M), $\Delta S' = +88.0$ cal deg^{-1} $mole^{-1}$, and $\Delta H = 9.8$ kcal $mole^{-1}$. Comparison of these thermodynamic parameters with those which have been reported for a variety of small ligands suggests that carboxylate ions may contribute significantly to the binding of zinc in the enzyme. It appears that the crevice (Fridborg, K., Kannan, K. K., Liljas, A., Lundin, J., Strandberg, B., Strandberg, R., Tilander, B., and Wiren, G. (1967), *J. Mol. Biol. 25*, 505) in which the zinc is bound is polar in character in the apoenzyme and becomes relatively nonpolar on addition of the zinc ion.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme containing one atom of very tightly bound zinc(II) per molecule of 30,000 molecular weight. Lindskog and Malmström (1962) and Lindskog (1963) have shown that under appropriate conditions the zinc atom can be removed, with complete loss by the enzyme of its catalytic properties, and that fully active enzyme, apparently native in all respects, can be regenerated by the addition of 1 equiv of Zn^{2+}.

Lindskog and Malmström (1962) estimated the equilibrium constants for the interaction of Zn^{2+} with the apoenzyme derived from bovine carbonic anhydrase over a range of pH by means of equilibrium dialysis of the apoenzyme against a solution of Zn^{2+} buffered by 1,10-phenanthroline. On the assumption that the chelating agent has no effect on the enzyme–Zn^{2+} equilibrium, they found binding constants in the range 10^9–10^{12} M^{-1} at 25°.

In this paper we report measurements by direct reaction calorimetry of the enthalpy changes in the binding of Zn^{2+} and various other divalent metal ions by the apoenzymes derived from the A and B forms of bovine carbonic anhydrase. Comparison of the thermodynamics of the enzyme reactions with that observed in reactions of the metal ions with various small ligands is pertinent to the problem of the mode of binding of the metal ions to the proteins.

Experimental Section

Bovine carbonic anhydrases A and B were prepared from commercial samples of the enzyme by chromatography on DEAE-cellulose, according to the method of Lindskog (1960). Atomic absorption measurements with a Perkin-Elmer Model 303 spectrophotometer showed that the preparations contained 1.00 ± 0.05 atom of zinc/molecule, assuming an absorptivity at 280 mμ of 5.7×10^4 M^{-1} cm^{-1} for the enzyme (Lindskog and Nyman, 1964). This same value for the absorptivity was also used for the apoenzymes, since it has been found (Henkens, 1967) that the absorptivity of the apoenzyme B is only 3×10^2 M^{-1} cm^{-1} greater than that of the native carbonic anhydrase B. The zinc-free apoenzymes were prepared by prolonged dialysis against 1,10-phenanthroline in 0.1 M acetate buffer (pH 5.0) in the cold room (Lindskog and Malmström, 1962). Atomic absorption and activity measurements, using both CO_2 and *p*-nitrophenyl acetate as substrates, agreed in indicating that the removal of zinc was more than 98% complete in all cases. The apoenzymes were fully reactivatable with exactly 1 equiv of Zn^{2+}, even after storage at pH 7 in the cold room for as long as 12 months.

The bulk of the calorimetric measurements was made with a Beckman Model 190 microcalorimeter. These experiments are designated by the term "Batch" in Table I. In the reaction cell of the calorimeter 0.1–0.2 ml of a metal ion solution in water was added to 15 ml of buffered apoprotein solution. In some experiments a

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TABLE I: Enthalpy Changes in the Binding of Metal Ions to Apocarbonic Anhydrase at 25°.

Type of Expt	Metal Ion	pH	No. of Expt	ΔH (kcal mole^{-1})	Av Dev (kcal mol^{-1})
		Apocarbonic Anhydrase A			
Batch	Zn^{2+}	7.0	3	8.69	±0.12
Batch	Zn^{2+}	7.0	1	8.48	
Flow	Zn^{2+}	7.0	4	8.3	0.6
Batch	Zn^{2+}	5.5	3	5.65	0.20
Batch	Co^{2+}	7.0	4	9.37	0.50
Flow	Co^{2+}	7.0	3	8.3	0.2
Batch	Co^{2+}	5.5	3	4.33	0.18
Flow	Cu^{2+}	7.0	3	3.4	0.2
Flow	Cd^{2+}	7.0	2	4.4	0.3
Flow	Ni^{2+}	7.0	2	3.2	0.3
		Apocarbonic Anhydrase B			
Batch	Zn^{2+}	7.0	3	10.26	0.32
Batch	Zn^{2+}	7.0	1	9.41	
Batch	Zn^{2+}	5.5	3	3.50	0.31
Batch	Zn^{2+}	5.5	2	4.22	0.31
Batch	Co^{2+}	7.0	1	8.13	

small amount of HCl was added to the metal ion solution to prevent hydrolysis; in such cases appropriate corrections were applied for reaction of the acid with the buffer in the protein solution. In the tare cell the same volume of metal ion solution was added to 15 ml of water. Water rather than buffer was used in the tare cell in order to avoid heat effects due to metal ion hydrolysis or complexation with buffer anions. It was ascertained that the heats of dilution of the proteins, which were used in concentrations of 3–16 × 10^{-5} M, are of negligible magnitude. In each experiment the metal ion was present in limiting amount to avoid contributions from nonspecific binding (Henkens, 1967), and also because the concentration of the metal ion was known more accurately than that of the protein. In a few supplementary experiments with Zn^{2+} and apocarbonic anhydrase B it was shown that addition of excess metal ion to the reaction product led to heat effects below the level of detectability.

The sets of experiments in Table I designated by "Flow" were performed in a flow modification of the Beckman Model 190 microcalorimeter. This modification has been briefly described (Lyons and Sturtevant, 1969). Although in cases, such as the present one, of very small heat effects the flow calorimeter is not capable of quite such high accuracy as the batch calorimeter, its use is nevertheless attractive because of the greatly increased convenience and rapidity of operation compared with the batch calorimeter. (It may be noted here that in cases of larger heat effects the flow calorimeter can lead to an accuracy as much as ten times better than that achievable in the batch apparatus.)

All experiments at pH 5.5 were performed in 0.1 M acetate buffer, and those at pH 7.0 in 0.1 M phosphate buffer. The cation present, in addition to the bivalent metal ions, was Na^{+}. The metal ions were added in the form of their chlorides except for Co^{2+}, which was added as the perchlorate. No other salts were present.

Results and Discussion

The results of the calorimetric experiments are summarized in Table I. Where two sets of experiments under a given set of conditions are indicated, the sets were performed at widely separated times and with different apoenzyme preparations. At pH 5.5 and 7.0, 1.0–1.5 protons are liberated per Zn^{2+} ion bound (Lindskog, 1963; Henkens, 1967), and we have assumed that the same is true with the other metal ions. This necessitates inclusion of corrections for buffer ionization heats; taking the proton liberation to be 1.0, these corrections are −0.09 kcal mole^{-1} (Edsall and Wyman, 1958) for the experiments in acetate buffer and +1.13 kcal mole^{-1} (G. D. Watt, 1968, unpublished measurements) for those in phosphate buffer. These corrections have been included in the ΔH values listed in the table.

Some interesting differences in the enthalpy of binding of the various metal ions to apocarbonic anhydrase ap-

TABLE II: Thermodynamic Parameters for the Specific Binding of Zn^{2+} by Bovine Apocarbonic Anhydrase B at 25°.

pH	pK	$\Delta G'$ (kcal mole^{-1})	ΔH (kcal mole^{-1})	$\Delta S'$ (cal deg^{-1} mole^{-1})
5.5	−10.5	−14.3	3.86	60.9
7.0	−12.0	−16.4	9.84	88.0

TABLE III: Thermodynamic Parameters for the Binding of Zn^{2+} by Small Ligands at 20–25°.

Ligand	Complex Formed	$\Delta G°$[b]	ΔH[b]	$\Delta S°$[c]	Reference
Glycinate ion	ZnL_2	−12.8	−6.3	+22.1	Stack and Skinner (1967)
Histidinate ion	ZnL_2	−16.0	−11.7	+14.6	Stack and Skinner (1967)
2,2′-Bipyridyl	ZnL_2^{+}	−13.4	−10.5	+8.0	Atkinson and Bauman (1962)
Diethylenetriamine	ZnL^{2+}	−12.0	−6.45	−18.5	Ciampolini *et al.* (1961)
Triethylenetetramine	ZnL^{2+}	−16.4	−8.9	+25.0	Sacconi *et al.* (1961)
EDTA[a]	ZnL^{2-}	−22.4	−4.85	+59.0	Anderegg (1963)
CDTA[a]	ZnL^{2-}	−25.9	−1.94	+81.8	Anderegg (1963)

[a] Temperature is 20°. [b] In kcal $mole^{-1}$. [c] In cal deg^{-1} $mole^{-1}$.

pear in Table I. The two ions, Zn^{2+} and Co^{2+}, which form enzymically active complexes are bound to the apoenzyme A at pH 7 with an enthalpy increase of about 8.5 kcal $mole^{-1}$ while those which form inactive complexes lead to an increase of only 3–4 kcal $mole^{-1}$. In the case of the Zn^{2+} ion, the addition to the apoenzyme A appears to be significantly less endothermic at pH 7, and more endothermic at pH 5.5, than the addition to the apoenzyme B. These differences are rather small, however, compared with the total free-energy changes involved.

In all cases the binding is endothermic, although very strongly exergonic. The unfavorable enthalpy is thus overwhelmed by a very large entropy increase. The equilibrium data reported by Lindskog and Malmström (1962) for the reaction of Zn^{2+} with apocarbonic anhydrase B are combined with our calorimetric data to give the thermodynamic parameters listed in Table II. The standard states for apoenzyme, metal ion, and reconstituted enzyme are 1 M, and for H^+ the pH of the experiment. Unity activity coefficients are assumed, and the usually very small variation of ΔH values with concentration is neglected. We are unable to suggest interpretations of the differences in enthalpy and entropy which are large compared to the difference in free energy, at pH 5.5 and 7.0, in terms either of ionizations of groups at the zinc binding site or on the zinc atom itself (Riepe and Wang, 1968), or of the views on the nature of the binding process discussed below.

Stack and Skinner (1967) have recently determined calorimetrically the enthalpy changes in the complexing of various transition metal ions with amino acids and have given a convenient summary of the thermodynamic data available for these reactions. Since our interest is mainly in Zn–carbonic anhydrase, and since no additional clarification appears to result from considering the data for the other metal ions, the ensuing discussion is limited to the binding of Zn^{2+} ions.

The data given by Stack and Skinner (1967) for the formation of ZnL_2 complexes with glycine and histidine at high pH are given in Table III. The glycine complex is probably tetrahedral. In the histidine complex (Kretzinger *et al.*, 1963; Harding and Cole, 1963) the Zn atom is bonded to four N atoms in distorted tetrahedral geometry, with two oxygen atoms weakly bonded in octahedral positions. Stack and Skinner (1967) and Sharma and Mathur (1965) have concluded that large positive entropies of binding for these and related complexes result primarily from charge neutralization in the interaction of carboxylate ions with Zn^{2+}, while large negative enthalpies of binding are primarily due to interaction of the metal with amino groups or other nitrogen groups. Stack and Skinner note that the difference between the enthalpy changes in the histidine and glycine cases is closely equal to one-half the enthalpy of formation of the complex formed with 2,2′-bipyridyl (*cf.* Table III). This suggests that the enthalpy contribution for an imidazole N is approximately the same as for a pyridine N.

The stability of any complex which involves the binding of amino groups to a metal ion will be strongly dependent upon pH because of the large values of $\Delta G°$ associated with the ionization of such groups (Edsall and Wyman, 1958). The same is true to a lesser extent with complexes involving imidazole groups. For example, if we use the ionization parameters listed by Edsall and Wyman (1958) we obtain for the reaction

$$2\overset{+}{N}H_3CH_2COO^- + Zn^{2+} = (NH_2CH_2COO^-)_2Zn^{2+} + 2H^+ \quad (1)$$

$\Delta G° = +13.9$ kcal $mole^{-1}$, so that $\Delta G' = -5.2$ kcal $mole^{-1}$ at pH 7.0 and -1.1 kcal $mole^{-1}$ at pH 5.5. Even though there is a cratic entropy term, amounting to -2.4 kcal $mole^{-1}$ in $\Delta G'$, favoring the binding of Zn^{2+} to the apoenzyme as compared with its binding to two molecules of glycine, and one or more amino groups at the binding site of the apoenzyme could have effective pK values not far removed from pH 7, it appears that the glycine–Zn^{2+} complex is not an adequate model for the binding of Zn^{2+} in carbonic anhydrase. In similar manner, it is difficult to reconcile the thermodynamic parameters for the complexing of Zn^{2+} to histidine, bipyridyl, diethylenetriamine, or triethylenetetramine with those observed for the enzyme.

The association of large entropy increases with complexation by ligands containing carboxylate ions is par-

TABLE IV: The Effect of pH on the Reaction of CDTA with Zn^{2+} at 20°.

Reaction	pH	$\Delta G°$ or $\Delta G'$ (kcal mole^{-1})	ΔH (kcal mole^{-1})	$\Delta S°$ or $\Delta S'$ (cal deg^{-1} mole^{-1})
$HL^{3-} + Zn^{2+} = H^+ + ZnL^{2-}$	0.0	−9.4	+4.7	+47.9
$H_2L^{2-} + Zn^{2+} = 2H^+ + ZnL^{2-}$	0.0	−1.2	+6.8	+26.8
Actual reaction	5.5	−16.6	+6.5	+78.8
Actual reaction	7.0	−19.0	+4.7	+80.9

ticularly prominent in the cases of EDTA and 1,2-cyclohexylenediaminetetraacetate (CDTA) studied by Anderegg (1963). The data for these ligands are listed in Table III.

At pH 5.5 and 7.0 a ligand such as CDTA would not carry four negative charges. From the protonation data given by Anderegg (1963) one obtains the thermodynamic data given in Table IV. As before, $\Delta G°$ and $\Delta S°$ refer to reactions with the activity of hydrogen ions equal to 1 M, and $\Delta G'$ and $\Delta S'$ to reactions with other hydrogen ion activities as specified by the pH. The values derived for the actual reactions at pH 5.5 and 7.0, particularly at the latter pH, are intriguingly similar to those observed with the apoenzyme; it is seen from the small values for ΔH that the quantities will not be significantly different 25°, at provided that ΔC_p for the reaction is not unexpectedly large.

The large entropy increase observed with a ligand such as CDTA has been attributed to charge neutralization on formation of the complex, with attendant liberation of water. The similarly large entropy increase found in the enzymic case can be qualitatively accounted for by the presence in the apoenzyme of solvated negatively charged groups in the cavity in which the zinc is bound (Fridborg *et al.*, 1967). The only groups carrying a negative charge at a pH as low as 5.5 are carboxylate ions.

According to this view, the cavity would be polar in character in the apoenzyme and would become markedly less polar as the result of charge neutralization by the zinc ion. There are several independent experimental observations which are consistent with this picture. (a) Henkens and Sturtevant (1968) reported a difference spectrum between the native enzyme and the apoenzyme with small peaks at about 300 and 285 mμ and a larger peak at about 235 mμ. The difference spectrum shown in Figure 1 (solid line) was taken under conditions of higher resolution than those used by Henkens and Sturtevant (1968), and shows more structure. It is similar, though shifted to longer wavelengths, to those produced when a tyrosine derivative (dotted curve, Figure 1) or a tryptophan derivative (dashed curve, Figure 1) is transferred from water to a less polar medium. This similarity suggests that certain tyrosyl and tryptophanyl groups are in a more polar environment in the apoenzyme than in the zinc enzyme. A somewhat similar difference spectrum was reported by Riddiford (1964) for human carbonic anhydrase B at pH 13 with the enzyme at pH 7 as reference. (b) Chen and Kernohan (1967) concluded on the basis of the enhancement of the fluorescence of dansylsulfonamide when it is specifically bound to the active site of the native enzyme that the active site is highly nonpolar in character. (c) Riepe and Wang (1968) have interpreted the infrared absorption spectrum of carbon dioxide bound to carbonic anhydrase to indicate that the binding takes place at a hydrophobic site.

The difference spectrum alluded to above suggests that consideration should be given to the possibility that one or more tyrosyl groups play a role in binding the zinc atom. There are at present no enthalpy or entropy data available for the formation of zinc complexes with phenolic compounds which can be compared with the thermodynamic parameters for the enzymic binding process. Some of the proton liberation (Lindskog, 1963)

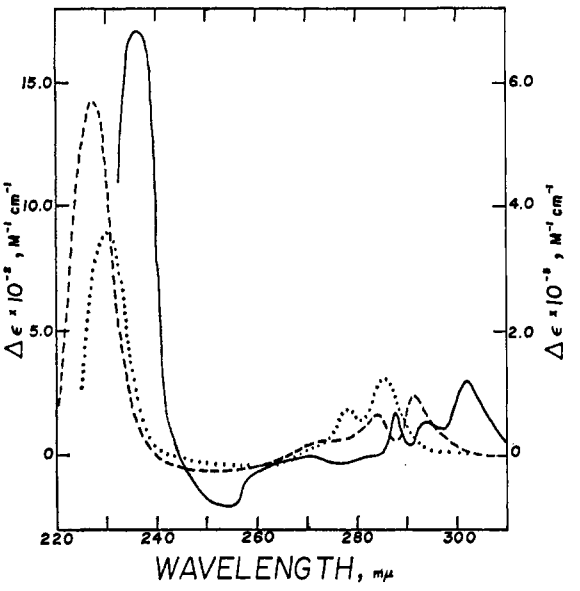

FIGURE 1: The difference spectrum of regenerated zinc carbonic anhydrase relative to the apoenzyme compared with those produced when *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tryptophan ethyl ester are transferred from water to 50% (v/v) ethylene glycol–water. Solid curve, left ordinate, ε (native enzyme) − ε (apoenzyme), pH 6.9, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer. Dotted curve, left ordinate, ε (glycol–H_2O) − ε (H_2O) for *N*-acetyl-L-tyrosine ethyl ester (pH ~3). Dashed curve, right ordinate, ε (glycol–H_2O) − ε (H_2O) for *N*-acetyl-L-tryptophan ethyl ester. The difference spectrum for the enzyme is essentially independent of pH in the range 5.5–8.0. All spectra were observed at 25°.

which accompanies zinc binding might be due to a previously uncharged tyrosyl group which becomes a zinc ligand.

The thermodynamic data for zinc binding certainly do not rule out contribution of imidazole or amino groups to the binding. The enzyme contains no reactive sulfhydryl groups (Lindskog and Malmström, 1962), so that no consideration need be given to the possibility that such groups are involved in metal binding.

It is probable that one coordination position of the zinc ion in the enzyme at low pH is occupied by a water molecule (Riepe and Wang, 1968), which loses a proton with an apparent pK of approximately 7 to generate active enzyme. Correspondingly, the effect of ionic strength on the rate of binding of zinc (Henkens, 1967; Henkens and Sturtevant, 1968) indicates a decrease of positive charge in the pH range 5.7–8.4. Thus there must be at least one charge present in the cavity of the zinc enzyme either at low pH or at high pH even though the cavity appears to be nonpolar in character.

In summary, the thermodynamics of binding of transition metal ions to various small ligands shows that complexation by amino and imidazole nitrogen atoms leads to large enthalpy decreases with little entropy change, whereas complexation by carboxylate ions is accompanied by small enthalpy changes but large increases in entropy, presumably because of charge neutralization. The small enthalpy and large entropy increases observed in the binding of zinc to apocarbonic anhydrase thus lead to the suggestion that carboxylate ions may contribute importantly to the binding. However, in attempting to draw conclusions concerning the nature of a macromolecular process on the basis of thermodynamic comparisons with small molecules as model systems, it must be recognized that there is as yet no past experience of success which gives a basis for confidence in such efforts.

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