Reactivation *in vitro* of Zinc-Requiring Apo-Enzymes by Rat Liver Zinc-Thionein

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The ability of rat liver zinc-thionein to donate its metal to the apo-enzymes of the zinc enzymes horse liver alcohol dehydrogenase, yeast aldolase, thermolysin, *Escherichia coli* alkaline phosphatase and bovine erythrocyte carbonic anhydrase was investigated. Zinc-thionein was as good as, or better than, ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ or $\text{Zn}(\text{NO}_3)_2$ in donating its zinc to these apo-enzymes. Apo-(alcohol dehydrogenase) could not be reactivated by zinc salts or by zinc-thionein. Incubation of the other apo-enzymes with near-saturating amounts of zinc as ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, $\text{Zn}(\text{NO}_3)_2$, or zinc-thionein resulted in reactivation of the apo-enzymes. With apo-aldolase zinc-thionein gave 100% reactivation within 30 min. Reactivation by ZnSO_4 and $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ was complete and instantaneous. Zinc-thionein was somewhat better than $\text{Zn}(\text{NO}_3)_2$ in completely reactivating apo-thermolysin. With apo-(alkaline phosphatase) 43% reactivation was obtained with $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ and 18% with zinc-thionein. With apo-(carbonic anhydrase) zinc-thionein was better than ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ or $\text{Zn}(\text{NO}_3)_2$, with a maximal reactivation of 54%. That zinc was really being transferred from zinc-thionein to apo-(carbonic anhydrase) was shown by the fact that 2,6-pyridine dicarboxylic acid and 1,10-phenanthroline had minimal effects on the reactivation of apo-(carbonic anhydrase) when added after the incubation {[apo-(carbonic anhydrase) + zinc thionein] + chelator}, but inhibited reactivation when added before the incubation {apo-(carbonic anhydrase) + [zinc-thionein + chelator]}. These observations support the idea that zinc-thionein can function in zinc homeostasis as a reservoir of zinc, releasing the metal to zinc-requiring metalloenzymes according to need.

Metallothionein, a low molecular weight, cysteine-rich, metal-binding protein, was first recognized in equine renal cortex by Margoshes and Vallee (1957); subsequently the protein was isolated and characterized by Kägi and Vallee (1960, 1961) from equine kidney and by Pulido *et al.* (1966) from human kidney. Metallothioneins have been identified in many other animal species (Nordberg *et al.*, 1972; Winge & Rajagopalan, 1972; Cousins *et al.*, 1973). The proteins thus far isolated from different species contain varying amounts of zinc, cadmium and copper up to a total of 6–7 g-atom of metal/mol of protein (Kägi *et al.*, 1974). Each metal ion is bound by three cysteinyl residues, producing a negatively charged mercaptide complex (Kojima *et al.*, 1976). Metallothioneins can be induced by administration to animals of $\text{Zn}(\text{II})$, $\text{Cd}(\text{II})$, $\text{Cu}(\text{II})$, $\text{Hg}(\text{II})$, and $\text{Ag}(\text{II})$ (Winge *et al.*, 1975; Day *et al.*,

1978). Horse liver (Kägi *et al.*, 1974) and liver from other mammals (Bühler & Kägi, 1974) have been shown to contain zinc as the naturally occurring metal bound to metallothionein.

Due to its structure, metal-binding properties, and its induction by various metals, metallothionein has been thought to function in metal metabolism. It has also been postulated to be a detoxifying agent for heavy metals (Ito *et al.*, 1966; Nordberg, 1972; Weser *et al.*, 1973), performing a protective function against cadmium toxicity. A more encompassing role has been suggested by Kojima & Kägi (1978), whereby metallothionein functions homeostatically as a reservoir, able to sequester and/or donate its metal, e.g. zinc or copper, intracellularly to metal-requiring metalloproteins. This is supported by other reports (Bremner & Davies, 1975; Richards & Cousins, 1975a,b) showing a definite interrelationship between tissue zinc levels and zinc thionein.

Abbreviation used: Cbz-Gly-Phe-NH₂, *N*-benzyloxy-carbonylglycylphenylalanylamide.

The metal binding properties of metallothionein have been demonstrated unequivocally by different laboratories (Shaikh & Lucis, 1970; Nordberg *et al.*, 1972; Davies *et al.*, 1973; Richards & Cousins, 1975b; Squibb & Cousins, 1977), who have shown that exogenously administered zinc is recovered as zinc-thionein. In this paper we report the ability of zinc-thionein to donate its metal to zinc-requiring apo-enzymes. This is the first demonstration *in vitro* supporting a direct role for zinc-thionein in zinc metabolism.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300g) were purchased from Sasco Inc., Omaha, NE, U.S.A., and were maintained on Purina rat chow and tap water *ad libitum*.

Chemicals

Chelex resin and Biogel were purchased from Bio-Rad, Richmond, CA, U.S.A. CHCl_3 , CCl_4 , ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CP}_2)_2$ and $\text{Zn}(\text{NO}_3)_2$ were purchased from Fisher Scientific Co., St. Louis, MO, U.S.A. All other reagents were analytical grade and were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., as were Sephadex and DEAE-cellulose. Budweiser yeast was obtained from a local bakery.

Preparation of metallothionein

Metallothionein was prepared according to established procedures from zinc-induced rat liver (Winge & Rajagopalan, 1972; Kägi *et al.*, 1974; Winge *et al.*, 1975). This involved induction with 10mg of $\text{Zn}(\text{II})/\text{kg}$ body weight (intraperitoneally), as ZnSO_4 , killing of the animals 18h after injection, removal of their livers, homogenization, high-speed centrifugation, chromatography of the cytosol on a column of Sephadex G-75, and chromatography of the pooled metallothionein fraction on a column of DEAE-cellulose. Electrophoretically homogeneous zinc-thionein II was used in these studies.

Enzyme assays and preparation of apoenzymes

All buffers used with the apo-enzymes were extracted with dithizone in CCl_4 . All glassware was soaked in 2M-nitric acid for at least 24h and was rinsed with metal-free water before use (Vallee & Gibson, 1948). Dialysis bags were prepared by boiling them three times in 1.0mM-EDTA and were stored in the same solution. The bags were rinsed with de-ionized water before use (Hughes & Klotz, 1956).

Bovine erythrocyte carbonic anhydrase B, horse-liver alcohol dehydrogenase, thermolysin, and

Escherichia coli alkaline phosphatase were all purchased from Sigma Chemical Co. Rabbit muscle glycerol 3-phosphate dehydrogenase/triose phosphate isomerase mixture was purchased from Boehringer-Mannheim. Yeast aldolase was purified and assayed according to the method of Rutter & Hunsley (1966), except that their last crystallization step was replaced by a chromatography step on a column (2.5 cm $\times$ 100 cm) of Sephadex G-75, previously equilibrated with 0.2M-glycylglycine/0.2M-potassium acetate/0.05M 2-mercaptoethanol/1.0mM-phenylmethylsulphonyl fluoride, pH 7.5 at 4°C (Kobes *et al.*, 1969).

E. coli alkaline phosphate (lot 127C-0486) had a specific activity of 34 $\mu\text{mol}/\text{min}$ per mg of protein. The homogeneous enzyme was reported to have a specific activity of 24.8 (Malawy & Horecker, 1966). Alkaline phosphatase activity was assayed by the method of Garen & Levinthal (1960), by monitoring the hydrolysis of *p*-nitrophenyl phosphate (1mM) in 1M-Tris/HCl, pH 8.0 at 25°C, at 415nm. Apo-(alkaline phosphatase) was prepared by the Chelex slurry method of Csopak (1969). Apo-(alkaline phosphatase) thus prepared had 5.4% of catalytic activity and 7.9% of zinc, as compared to the holo-enzyme.

Thermolysin, protease type X (lot 57C-0116) had a specific activity of 0.12 $\mu\text{mol}/\text{min}$ per mg of protein with the substrate Cbz-Gly-Phe-NH₂. The homogeneous enzyme was reported to have a specific activity of 3.0 towards this substrate (Matsubara, 1970), when assayed by the ninhydrin method (Moore & Stein, 1954; Moore, 1968). Apo-thermolysin was prepared according to the 1,10-phenanthroline method of Holmquist & Vallee (1974). Apo-thermolysin, thus prepared, had 40% of catalytic activity and 11.1% of zinc as compared to the holo-enzyme.

Yeast aldolase had a specific activity of 82.6 μmol of fructose 1,6-bisphosphate cleaved/min per mg of protein. Kobes *et al.* (1969) reported a specific activity of 85–105 for the homogeneous enzyme. Aldolase was assayed according to the method of Rutter & Hunsley (1966). Apo-aldolase was prepared according to the Chelex slurry method of Csopak (1969), as modified by Kobes *et al.*, (1969) using 8-hydroxyquinoline 5-sulphonic acid. Apo-aldolase, thus prepared, had zero catalytic activity and 1.1% zinc as compared to the holo-enzyme.

Bovine erythrocyte carbonic anhydrase B (lot 125C-8081) had a specific activity of 2630 Wilbur-Anderson units/mg of protein. A specific activity of 2200 has been reported for the homogeneous enzyme (Roughton & Booth, 1946). Carbonic anhydrase was assayed according to the method of Parker & Stone (1967). Apo-(carbonic anhydrase) was prepared according to the dipicolinic acid method of Hunt *et al.* (1977). Apo-(carbonic anhydrase) thus

prepared had 7.6% of catalytic activity and 5.0% of zinc, as compared to the holo-enzyme.

Horse liver alcohol dehydrogenase (lot 116C-8090) had a specific activity of $1.85 \mu\text{mol/min}$ per mg of protein. A specific activity of 2.6 has been reported for the homogeneous enzyme (Hayes & Velick, 1954). Alcohol dehydrogenase activity was assayed according to the method of Hayes & Velick (1954). Apo-(alcohol dehydrogenase) was prepared by a modification of the succinate method of Oppenheimer *et al.* (1967). Apo-(alcohol dehydrogenase) thus prepared had zero catalytic activity and 4.0% of zinc as compared to the holo-enzyme.

Protein concentrations were determined by the method of Lowry *et al.*, (1951). Zinc concentrations were determined by flame atomic absorption spectroscopy, using a Perkin-Elmer model 303 spectrometer. Absorbance measurements and spectrophotometric assays were performed with a thermostatted Cary 118C spectrometer, equipped with a multiple sample changer.

Reactivation of apoenzymes

Apo-(alkaline phosphatase) ($100 \mu\text{g}$) in 1.0 ml of 1 M-Tris/HCl, pH 8.0, was mixed with near-saturating levels of zinc as ZnSO_4 , $\text{Zn}(\text{NO}_3)_2$, $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, or zinc-thionein in an acid-washed test tube. The mixtures were incubated at 25°C in a rotary shaker. At appropriate times, portions ($50 \mu\text{l}$) of each mixture were transferred to assay cuvettes, containing 1.0 mM-*p*-nitrophenyl phosphate in 1 M-Tris/HCl, pH 8.0. The hydrolysis of *p*-nitrophenyl phosphate at 25°C was monitored for 2 min at 415 nm. The amount of zinc added in each case was 152 ng, equivalent to 2 zinc atoms/protein dimer of mol.wt. 86 000.

Apo-aldolase ($50 \mu\text{g}$) in 1.0 ml of 0.05 M-Tris/HCl, Tris/HCl/0.01 M- CaCl_2 (pH 7.5) was mixed with increasing concentrations of zinc, as $\text{Zn}(\text{NO}_3)_2$ or zinc-thionein, in 0.5 ml of an assay mixture containing 5 mM-Cbz-Gly-Phe-NH₂. The assays were incubated for 10 min at 40°C . The remainder of the assay was as described above for thermolysin.

Apo-aldolase ($50 \mu\text{g}$) in 1.0 ml of 0.05 M-Tris/HCl pH 8.0, was mixed with near-saturating levels of zinc, as ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, or zinc-thionein, in 0.1 M-Tris/HCl, pH 7.5. The mixtures were incubated at 25°C in a rotary shaker. At appropriate times portions ($50 \mu\text{l}$) of each mixture were transferred to assay cuvettes, and the oxidation of NADH was monitored at 340 nm. The amount of zinc added in each case was 41 ng, equivalent to one zinc atom/protein molecule of mol.wt. 80 000.

Three types of reactivation experiments were performed with apo-(carbonic anhydrase): an incubation experiment, a zinc-concentration-dependence experiment, and an incubation plus chelator experiment. In the incubation experiment $100 \mu\text{g}$ of

apo-(carbonic anhydrase) in 1.0 ml of 0.2 M-potassium phosphate buffer, pH 7.0, was mixed with near saturating amounts of zinc, as ZnSO_4 , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, $\text{Zn}(\text{NO}_3)_2$, or zinc-thionein. These mixtures were incubated on a rotary shaker at 37°C . At appropriate times portions ($50 \mu\text{l}$) of each mixture were removed and transferred to assay cuvettes. The hydrolysis of *p*-nitrophenyl phosphate was monitored at 400 nm. The amount of zinc added in each case was 211 ng, equivalent to one zinc atom/enzyme molecule of mol.wt. 31 000. In the zinc-concentration-dependence experiment, increasing concentrations of zinc, as ZnSO_4 , $\text{Zn}(\text{NO}_3)_2$, $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, or zinc-thionein, were included in an assay mixture in a cuvette. The reactions were started by the addition of $10 \mu\text{g}$ of apo-enzyme, and the assays were completed within 2 min after mixing. Finally, in the incubation plus chelator experiment, apo-(carbonic anhydrase) was incubated with zinc-thionein, as above, for 40 min at 37°C before assay, or the zinc-thionein was incubated with various levels of chelators (dipicolinic acid or 1,10-phenanthroline) for 10 min at 37°C before mixing the solution with apo-(carbonic anhydrase) and incubating it for 40 min at 37°C before assay.

Apo-(alcohol dehydrogenase) ($10 \mu\text{g}$) in 1.0 ml of 0.05 M-Tris/HCl, pH 8.8, was mixed with near-saturating levels of zinc, as ZnSO_4 , $\text{Zn}(\text{NO}_3)_2$, $\text{Zn}(\text{CH}_3\text{CO}_2)_2$, or zinc-thionein. At appropriate times, portions ($50 \mu\text{l}$) of each mixture were transferred to assay cuvettes, and the reduction of NAD^+ was monitored at 340 nm. The amount of zinc added in each case was 31.5 ng, equivalent to 4 zinc atoms/enzyme molecule of mol.wt. 83 000. Alternatively, apo-(alcohol dehydrogenase) was mixed with increasing concentrations of zinc, as $\text{Zn}(\text{NO}_3)_2$ or zinc-thionein, in an assay mixture at 25°C . These assays were completed within 3 min after mixing.

All reactivation experiments were performed at least three times, except for the reactivation of apo-(carbonic anhydrase) in the presence of chelators which was done twice. The data in each Figure and in the Table are representative of each group of experiments, although there was some variation due to the use of different preparations of each of the apo-enzymes during the course of this work.

Results

Reactivation of apo-(alkaline phosphatase)

As shown in Fig. 1, up to 1 h of incubation $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ was best in donating Zn^{2+} ions to apo-(alkaline phosphatase), followed by zinc-thionein, ZnSO_4 and $\text{Zn}(\text{NO}_3)_2$. After 20 h of incubation $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ had reactivated the apoenzyme to 43% of control activity, $\text{Zn}(\text{NO}_3)_2$ to 26%, zinc-thionein to 20%, and ZnSO_4 to 16%.

Fig. 1. *Reactivation of apo-(alkaline phosphatase)* See the Materials and Methods section for details. Δ , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$; $\square$, ZnSO_4 ; $\blacktriangle$, $\text{Zn}(\text{NO}_3)_2$; $\bullet$, zinc thionein. Holo-enzyme activity = $1100 \Delta A_{415} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

Fig. 2. *Reactivation of apo-thermolysin* See the Materials and Methods section for details. $\bullet$, Zinc-thionein; $\blacktriangle$, $\text{Zn}(\text{NO}_3)_2$. Holo-enzyme activity = 10 nmol of Phe-NH₂ produced/10 min.

Reactivation of apo-thermolysin

Fig. 2 shows that zinc-thionein restored the activity of apo-thermolysin in a concentration-dependent manner to 93% of control activity, comparable to the reactivation by $\text{Zn}(\text{NO}_3)_2$, which reached 86%. The 40% catalytic activity of the apo-enzyme may be due to the relative impurity of the thermolysin available from Sigma, such that other non-zinc-requiring proteases may be present, or to contamination of the substrate Cbz-Gly-Phe-NH₂ with zinc; this could not be extracted with dithizone in CCl_4 because it is precipitated below 40°C.

Reactivation of apo-aldolase

Under the conditions of incubation ZnSO_4 and $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ instantaneously and completely reactivated apo-aldolase (Fig. 3). The activity of the

Fig. 3. *Reactivation of apo-aldolase* See the Materials and Methods section for details. $\square$, ZnSO_4 ; Δ , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$; $\bullet$, zinc-thionein. Holo-enzyme activity = $560 \Delta A_{340} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

Fig. 4. *Effects of incubation on the reactivation of apo-(carbonic anhydrase)* See the Materials and Methods section for details. $\square$, ZnSO_4 ; Δ , $\text{Zn}(\text{CH}_3\text{CO}_2)_2$; $\blacktriangle$, $\text{Zn}(\text{NO}_3)_2$; $\bullet$, zinc-thionein. Holo-enzyme activity = $2500 \Delta A_{400} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

$\text{Zn}(\text{CH}_3\text{CO}_2)_2$ -reconstituted enzyme decreased with time from 100% to 3%, probably due to a time-dependent precipitation that was observed in the incubation mixture. Zinc-thionein completely reactivated yeast apo-aldolase within the first 30 min of incubation.

Reactivation of apo-(carbonic anhydrase)

With a reactivation to 54% of control activity zinc-thionein was better than any of the zinc salts in donating its zinc to apo-(carbonic anhydrase) (Fig. 4) under the conditions of incubation. ZnSO_4 reactivated the enzyme to 43%, while $\text{Zn}(\text{NO}_3)_2$ and $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ reactivated to 35%. The dependence of reactivation on the concentration of Zn^{2+} under

assay conditions is shown in Fig. 5. ZnSO_4 reactivated apo-(carbonic anhydrase) to 52% of the holo-enzyme activity. At the highest concentration of ZnSO_4 examined the apoenzyme was just saturated with Zn^{2+} . $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ and $\text{Zn}(\text{NO}_3)_2$ gave identical results of 38%. Zinc-thionein showed only a slight stimulatory effect (to 30%), less than that seen under the incubation conditions of Fig. 4.

The mechanism of zinc transfer from zinc-thionein to apo-(carbonic anhydrase) was investigated further, using the chelators dipicolinic acid and

Fig. 5. Zinc-concentration-dependence of the reactivation of apo-(carbonic anhydrase)

See the Materials and Methods section for details. $\square$, ZnSO_4 ; $\triangle$, $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ or $\text{Zn}(\text{NO}_3)_2$; $\bullet$, zinc-thionein. Holoenzyme activity = $2500 \Delta A_{400} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$.

1,10-phenanthroline. The rationale was that if metal transfer involved liberation of the free metal ions, chelating agents should be able to interfere with Zn^{2+} exchange from zinc-thionein to apo-(carbonic anhydrase). Table 1 presents the results of experiments in which the chelators were incubated with zinc-thionein before or after its incubation with apo-(carbonic anhydrase). As can be seen, only when the chelators were present in the incubation mixtures before the addition of apo-(carbonic anhydrase) was there significant inhibition of reactivation. If they were added after the incubation of zinc-thionein with apo-(carbonic anhydrase), little or no inhibition of reactivation was seen.

Reactivation of apo-(alcohol dehydrogenase)

All attempts to reconstitute apo-(alcohol dehydrogenase) using zinc salts or zinc-thionein were unsuccessful. Neither incubation of the apo-enzyme with zinc salts or zinc-thionein before assay, nor assay of the apo-enzyme in the presence of increasing amounts of zinc salts or zinc-thionein yielded any signs of reactivation.

Discussion

The results presented in this study provide the first direct evidence *in vitro* consistent with a physiological function for zinc-thionein in zinc homeostasis. The ability of zinc-thionein to provide Zn^{2+} for the reactivation of apo-enzymes prepared from *E. coli* alkaline phosphatase, thermolysin, yeast

Table 1. Effects of chelators on zinc-thionein activation of apo-(carbonic anhydrase)

In incubation 2, saturating amounts of zinc, as zinc-thionein, were mixed with $10 \mu\text{g}$ of apo-(carbonic anhydrase), and the mixture was incubated for 40 min before assay. In incubations 3 and 4, saturating amounts of zinc, as zinc-thionein, were mixed with the chelators for 10 min prior to the addition of apo-(carbonic anhydrase). These mixtures were then incubated for 40 min before assay. Holoenzyme activity = $2500 \Delta A_{400} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ (=100%).

Incubations	Chelator concn. in assay (mM)		Activity	
	Dipicolinic acid	1,10-Phenanthroline	$\Delta A_{400} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$	Percentage of holoenzyme activity
1. Apo-(carbonic anhydrase)	0	0	108	4.3
2. Apo-(carbonic anhydrase) + zinc-thionein	0	0	1000	40.0
	0.5	0	1000	40.0
	1	0	1128	45.1
	3	0	1050	42.0
	5	0	1050	42.0
	0	0.3	1050	42.0
	0	0.5	925	37.0
3. Dipicolinic acid + zinc-thionein + apo-(carbonic anhydrase)	3	0	692	27.7
	5	0	675	27.0
	10	0	408	16.3
4. 1,10-Phenanthroline + zinc-thionein + apo-(carbonic anhydrase)	0	0.3	267	10.7
	0	0.5	229	9.2
	0	1	110	4.4

aldolase and bovine erythrocyte carbonic anhydrase supports the proposal by many investigators that zinc-thionein can function *in vivo* as a reservoir for the storage of zinc and/or as a conduit for zinc, which can be made available as a cofactor for newly synthesized zinc-requiring apo-enzymes.

The experimental difficulties of the preparation of apo-enzymes from metalloenzymes and of the reactivation of these apo-enzymes are well known to workers in these fields. The variety of responses we report in the ability of zinc-thionein and zinc salts to reactivate five different apo-enzymes was therefore not unexpected. A comparison with previous work involving reactivation of these zinc-requiring apo-enzymes is useful for judging the relative meaningfulness of the work reported here with zinc-thionein as a zinc donor. Bock & Kowalsky (1978) and Anderson *et al.* (1975) reported less than 50% reactivation of apo-(alkaline phosphatase) with the addition of two zinc atoms/mol of enzyme. A reactivation by $\text{Zn}(\text{CH}_3\text{CO}_2)_2$ to 43% of control value after 120 min, as reported here, compares favourably with their results. Reactivation by zinc-thionein to a level of 20% is moderately successful. Thermolysin is rapidly inactivated by chelating agents, and the apo-enzyme is easily reactivated with complete restoration of activity by the addition of zinc (Riordan & Vallee, 1976). We obtained similar results using zinc-thionein or $\text{Zn}(\text{NO}_3)_2$. Csopak (1969) and Kobes *et al.*, (1969) were able completely to reactivate apo-aldolase using one zinc atom/mol of enzyme. Similar results were obtained by us with ZnSO_4 or $\text{Zn}(\text{CH}_3\text{CO}_2)_2$. Zinc-thionein restored activity to 100% after 30 min of incubation. Lindskog (1963) and Lindskog & Malmström (1962) instantaneously and completely reactivated bovine apo-(carbonic anhydrase) by incubation with a 100-fold excess of Zn^{2+} at pH 5.0; reactivation with Co^{2+} was time-dependent and reached 45% (Lindskog & Malmström, 1962) and 75% (Hunt *et al.*, 1977). Our observations of reactivation by ZnSO_4 to 53% under assay conditions and by zinc-thionein to 54% under incubation conditions compare favourably with these results, since we used stoichiometric levels of zinc in our studies. Our inability to reactivate horse-liver apo-(alcohol dehydrogenase) under incubation or assay conditions was not unexpected. Rudolph *et al.* (1978) reported only a 35% reactivation after dissociation of the apoenzyme into subunits and denaturation of the subunits in 6 M-guanidinium chloride. Reactivation of apo-(alcohol dehydrogenase) under conditions comparable to ours has not been reported.

Kägi & Vallee (1960) reported that the apparent association constant for the binding of zinc to equine renal cadmium/zinc-thionein was 10^{14} . Kägi (1970) reported that the K_a for equine hepatic zinc-thionein

was 10^{11} . The apparent association constants for the binding of zinc by the apo-enzymes used in this study have been reported to be 10^{12} at pH 7–8 for carbonic anhydrase (Lindskog & Malmström, 1962), 4.2×10^{12} at pH 7.2 with Ca^{2+} present for thermolysin (Feder *et al.*, 1971), 6.0×10^{11} at pH 8.0 for alkaline phosphatase (Csopak, 1969), and 5.3×10^6 (calculated) at pH 7.5 for aldolase (Mildvan *et al.*, 1971).

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