REVERSIBLE DISSOCIATION OF ZINC IN BOVINE CARBONIC ANHYDRASE

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Keilin and Mann (1940) demonstrated that carbonic anhydrase (CA) is a zinc metalloenzyme. The zinc content reported from various laboratories varies within a wide range (see Keller and Peters, 1959) but recent analytical work (Lindskog, 1960) on a homogeneous preparation of the protein has revealed the presence of one atom of Zn per molecule of enzyme. The metal is firmly attached to the protein since extensive electrodialysis does not remove it (Scott and Mendive, 1941), and there is no exchange with radioactive Zn at neutral pH even during a period of a month (Tupper, Watts and Wormall, 1951). Removal of the metal has only been possible on destruction of the protein at low pH (see Keller and Peters, 1959). We have, however, now found it possible to dissociate the metal reversibly from the enzyme at pH 5 in the presence of chelating agents, such as 1,10-phenanthroline (OP) or citrate. The removal of Zn causes a loss of activity, which, however, can be fully regained by addition of Zn.

Experimental

Bovine erythrocyte CA was prepared by the method of Lindskog (1960). The protein was freed of extraneous metals (Cu, Fe) by dialysis against OP at neutral pH, which causes no loss of zinc (see Lindskog, 1960). The enzyme activity was estimated by the colorimetric procedure described previously (Lindskog, 1960)

except that a two-channel stopped-flow apparatus, similar to that of Beers (1956), was employed. The equilibrium dialysis technique used for the radioactive exchange measurements has been described earlier (Malmström, 1953). The concentration of Zn^{65} was estimated with scintillation counter (Baird-Atomic) by comparing the γ-ray activity of 1-ml. samples with a series of Zn^{65} solutions of known concentration, prepared by dilution of the same stock solution as used in the exchange experiments.

Results and Discussion

The loss of activity when a solution of CA is dialyzed at 4° against acetate buffer, pH 5.0, containing 10^{-3} M OP is shown in Fig. 1. At given intervals, aliquots were removed and the CA activity measured. Since CA loses activity slowly when standing in solution at pH 5, a control dialysis against buffer without OP was included, and the activities have been expressed as per cent of the control activity. When Zn^{++}, in a final concentration of $2 \cdot 10^{-3}$ M, was added to the inactivated CA samples containing OP, the enzyme was in all cases reactivated to the same activity as the control (Fig. 1). Other metal ions (Co^{+2}, Ni^{+2}, Cd^{+2}) added failed to reactivate the enzyme under the same conditions, so that a simple removal of OP, acting as an inhibitor, by the metal cannot be involved.

While the loss of activity in the experiment just described could be due to factors other than removal of Zn, the reactivation is a strong indication that a loss of the metal is involved. In addition, exchange studies with Zn^{65}, summarized in Table I, give direct evidence that a reversible removal of the metal is taking place in the presence of chelating agents. In these experiments, extraneous Zn^{65} was added to exchange with the Zn in the enzyme, details of concentrations being given in the table. An

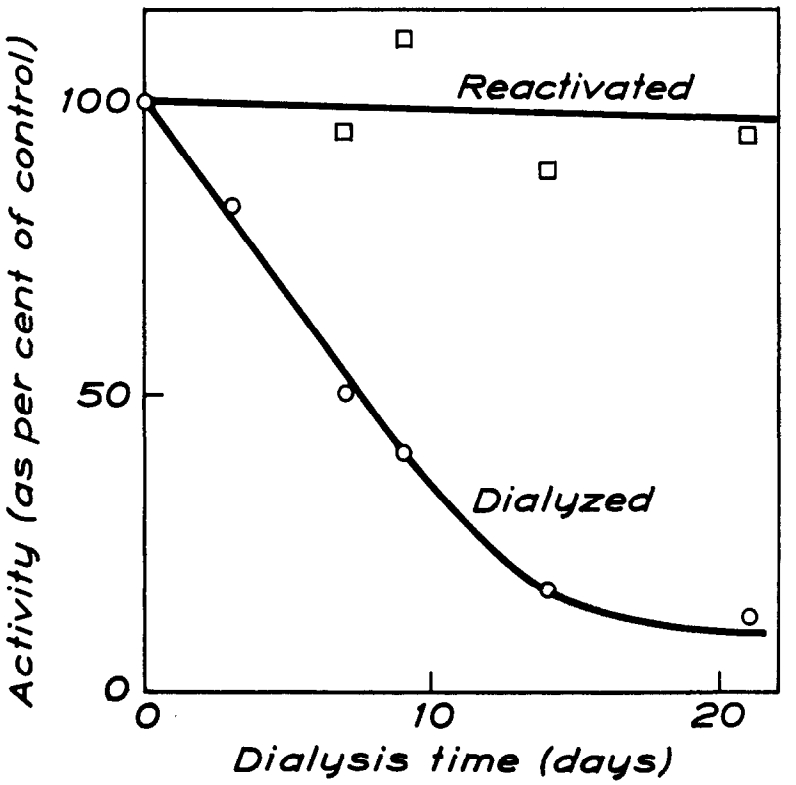

Fig. 1. Loss and regain of CA activity during dialysis against OP. Three ml. of a 0.07 % CA solution dialyzed at 4° against 100 ml. of 0.05 M acetate buffer, pH 5.0, containing 10^{-3} M OP, with change of dialysis liquid every 24 hrs. The lower curve (dialyzed): activity as a function of dialysis time. The upper curve (reactivated): activity after addition of Zn^{++} in a final concentration of $2 \cdot 10^{-3}$ M.

uptake of radioactive Zn was observed in the protein-containing compartments of the dialysis cells. This uptake continued for about 2 weeks while dialysis equilibrium is established in 48 hrs. The concentrations at equilibrium given in Table I are based on the radioactivity of the extraneous Zn added and are thus not corrected for dilution from the non-radioactive Zn in the enzyme. However, Zn analyses showed that such a dilution had taken place, thus demonstrating exchange, and a correction has been applied

in calculating the average number ($\bar{\nu}$) of Zn^{++} bound per molecule of enzyme; a complete equilibration between intrinsic and extraneous Zn was assumed. For comparison, Zn^{++} binding to a number of other proteins of varying zinc affinity, namely bovine serum albumin (non-specific binding; Gurd and Goodman, 1952), enolase (metal-enzyme complex; Malmström, 1953) and carboxypeptidase (metalloenzyme), was measured under the same conditions. No binding could be detected to albumin and enolase while carboxypeptidase (CP) showed a degree of binding very similar to that of CA, in accordance with the observations of Vallee, Rupley, Coombs and Neurath (1958). However, in contrast to the case with CA, equilibrium was established as fast as the dialysis equilibrium.

Table I

Equilibrium dialysis of CA in the presence of chelating agents at pH 5.0 and 4°

Dialysis medium	Enzyme conc. (molar)	Extraneous Zn^{++} conc. (molar)		$\bar{\nu}$
		Protein comp.	Non-protein comp.	
0.1 M nitrate buffer, 1 M NaCl	$10 \cdot 10^{-5}$	$5.5 \cdot 10^{-5}$	$2.5 \cdot 10^{-5}$	0.68
0.05 M acetate buffer, $2.5 \cdot 10^{-4}$ M OP	$6.9 \cdot 10^{-5}$	$9.4 \cdot 10^{-5}$	$7.2 \cdot 10^{-5}$	0.45

The experiments described demonstrate that, under certain conditions, Zn can be reversibly dissociated from CA, and that removal of the metal results in a loss of activity. Such a reversible dissociation of Zn in a metalloenzyme has previously

been accomplished by Vallee et al. (1958) with CP. While the binding strength in the two enzymes is very similar, the Zn^{++} in CP appears more "available" than in CA, as evidenced by the differences in the rate of equilibration and in the inhibiting effects of various chelating agents.

The studies are being extended and a complete report will be published elsewhere.

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