# THE ROLE OF ZINC IN CARBONIC ANHYDRASE

H.Keller, P.Gottwald and N.Wendling

Institut für Physiologische Chemie und Physikochemie
der Universität Kiel

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Recently Lindskog and Malmström (1960) demonstrated that carbonic anhydrase (CAH) could be inactivated by dialysis over a period of days against a solution of o-phenanthroline (OP). Addition of zinc then serves to reactivate the enzyme. The authors concluded from these data that the inactivation was caused by a loss of zinc.

We have conducted a series of experiments concerning the binding action of zinc in CAH. We have concluded that zinc is present in CAH as a zinc cysteine-complex, whose binding constant, by nature of the tertiary structure of the protein molecule, is greater than the binding constant of the known zinc-cysteinyl-1:2-complex (Keller 1960 a). We have shown earlier (Keller et al. 1959 b) that the zinc cannot be removed from the enzyme-zinc-complex by the addition of OP, dithizone, ethylenediaminetetraacetic acid, or 8-hydroxychinoline. If the pH is less than 5 the 1:2-complex as well as the enzyme is no longer stable. If the enzyme is irreversibly denaturated by a longer incubation in an excess of protons one can find two SH-groups per molecule and if the enzyme is reversible denaturated one can find only one SH-group.

## Experimental and Results

The enzyme was prepared according to the method B of Keilin and Mann (1940). The determination of the enzyme activity was performed manometrically by a method previously described (Keller et al. 1950 a) and calculated according to Mitchell et al. (1945) in Mitchell-units (MU). Zinc was determined polarographically (Keller et al. 1960 c). SH-groups were amperometrically titrated in 8 molar urea according to Benesch and Lardy (1955). The concentration of $Zn^{65}$ was de-

termined by a Geiger-Müller-counter.

$Zn^{65}$-containing CAH

An enzyme which has been inactivated by an excess of protons can not be reactivated by an increase in pH if the enzyme is treated in the acidic state with zinc chelating agents e.g. OP, dithizone, etc., or if cadmium ions are introduced in concentrations equalling those of the enzyme-zinc in the solution. We interpret these results to mean that an increase in proton concentration brings about an unfolding of the protein with a concomitant reduction of the zinc-protein binding constant. Thus an equilibrium between protein and chelating compounds exists and a zinc-cadmium exchange is possible. In view of the above results it was expected that the non-radioactive enzyme-zinc could be replaced by the radioactive isotope (Keller 1960 b).

A 0.1% CAH solution was incubated with $Zn^{65}$ at 20° C for 5 minutes at pH 2.9. The pH of the solution was then changed to 8.5 and the solution was allowed to stand for one hour in order to restore the enzyme activity. The solution was then fractionated with ammonium sulfate (AS) at 0° C. We observed that the irreversibly denaturated protein could be precipitated by half-saturation with AS whereas the reactivated part is precipitated in a fully saturated solution. The latter precipitate was then washed with AS-solution at 0° C until no further radioactivity was apparent. The resultant precipitate was dissolved in distilled water and dialysed with stirring against flowing distilled water whose pH was held at 7.8 by bubbling ammonia through the water. The total zinc content, the radioactive zinc, and the enzymatic activity of the resultant solution were determined. The solution contained 0.106 $\mu$g/$Zn^{65}$ MU and a total zinc content of 0.147 $\mu$g/MU. The exchange under these conditions was approximately the theoretical value. The lower limit of the zinc content is given by the known ratio: enzyme activity/zinc content (0.137 $\mu$g/MU), the upper limit which is more important for these experiments is given by the following: the total zinc content must be smaller than the lower limit plus the content of $Zn^{65}$ which is, for the added amount, theoretically exchangeable. If the original zinc which was present in the enzyme and the excess $Zn^{65}$ which was not exchanged for the original Zn are not washed out of the solution, then it is impos-

sible to differentiate the specifically bound and the unspecific adsorbed zinc. This must be done by zinc-analysis. Further, the outlined method of incorporation of $Zn^{65}$ enabled us to prepare a solid radioactive enzyme preparation by lyophilisation.

## Determination of end-groups

We have found earlier (Keller et al. 1959 b) that $10^{-3}$M 2:4-dinitrofluorbenzene (DNFB) does not inhibit CAH if allowed only short periods of incubation. However, with longer periods of incubation with DNFB at 20° C the enzymatic activity was decreased. As the activity decreases the optical density at 355 mu increases. The relationship of activity decrease to the optical density increase is linear for the first four hours. We have also observed, that the optical density of an acid-denaturated enzyme, which has been incubated with DNFB, is greater than the optical density of an enzyme which has not been treated with acid (or solutions with smaller amounts of denaturated protein). The acid-inactivated enzyme which has been treated for 30 minutes at 30° C with DNFB was hydrolysed and chromatographed. In the same manner the solution of native enzyme treated with DNFB was hydrolysed. Both preparations had glutamic acid as end-groups, whereas the denaturated product had additional cystine/cysteine as end-groups. The greater the loss of activity before the DNFB-treatment, the greater is the content of cystine/cysteine after hydrolysis. The relation between the enzymatic activity and the content of free cystine/cysteine is also a linear reciprocal function within certain limits. However, it is probable that the proton inactivation liberates cystine/cysteine end-groups.

## Titration of SH-groups

The amperometric titration of SH-groups of the native CAH showed that no free SH-groups were present. These results are similar to those reported by Scott and Mendive (1941). If the enzyme was irreversibly denaturated by 4 hours incubation at pH 3 we found two free SH-groups. If this treatment was for 10 minutes only, denaturation was reversible and only 1 SH-group could be titrated after reactivation for 30 minutes at

pH 7.5. When reactivation was allowed for 2 hours the number of titrable SH-groups was below 1.0.

## Conclusions

From the above results we have concluded that the zinc of CAH forms a type of cysteine-2:1-complex. This complex is surrounded by the protein molecule, which is stabilized within itself by-S-S-linkages. It is probable that the metal as a chelate is the active site of the enzyme. In the future we hope to determine whether the coordination number is 4 or 6. In the latter case two salt-bridges would be possible, one of which could arise from the carboxyl group of the terminal glutamic acid. The other could arise from the carbonic acid.

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