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Fluorescence Studies of Zinc Binding to Beef Liver Glutamate Dehydrogenase*,**

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By fluorometric measurements the binding between zinc and glutamate dehydrogenase from beef liver has been investigated. The dissociation constant of the enzyme-zinc complex was found to be 0.1 μM and the equivalent weight per zinc binding site to be 15 000.

Zinc ions increase the fluorescence intensity of the binary GluDH-NADH complex without further shift of the emission maximum.[1] In addition zinc salts are relatively strong inhibitors of the reaction catalyzed by GluDH from beef liver especially of the reductive amination of the α-ketoglutaric acid.[2,3] The enhancement of the fluorescence intensity following addition of zinc sulfate was used as a method for quantitative estimation of the binding between zinc and beef liver GluDH. The method for the investigation of enzyme zinc binding proved to be sensitive and time-saving.

EXPERIMENTAL

The fluorometric observations were carried out in the spectrofluorophotometer previously described by Theorell and Winer.[4] All measurements were made at 20°C in sodium phosphate buffer (μ = 0.1), pH 7.5. The activation wavelength was 350 mμ; 440 mμ was used for recording the fluorescence intensity. A final volume of 2 ml in 1 × 1 cm quartz cuvettes was used for all reported experiments, and Carlsberg pipettes (5, 10, and 50 μl) were employed for the additions of GluDH, NADH, and $ZnSO_4$ solutions. The additions were made on a glass rod with rapid stirring. Measurements of pH were made in the cuvettes at 19 to 21°C with a Beckman model G pH meter employing micro-electrodes. A NADH solution (about 10 μM in 0.01 M tris solution, pH about 10) was used as a standard.

* Studies of glutamate dehydrogenase, part III. Part II: H. Sund and Å. Åkeson, *Biochem. Z.* **340** (1964) 421.

** Abbreviations: GluDH, glutamate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; E, enzyme; tris, tris(hydroxymethyl)aminomethane.

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The enzyme preparations used were the same as previously investigated.[1,5]* For the fluorometric experiments the enzyme was dialyzed at 2 to 4°C for two days against sodium phosphate buffer (μ = 0.1), pH 7.5. The buffer was changed after 24 h. No inactivation was observed during dialysis. NADH was obtained from the Sigma Chemical Company, St. Louis, Missouri, USA. It was 87 % pure indicated by measurements of the absorption at 340 mμ and 83 % by enzymatic assay with catalytic amounts of yeast alcohol dehydrogenase taking the absorbancy index of NADH at 340 mμ as 6.22 $\times 10^6$ cm^2 per mole.[6] $ZnSO_4 \cdot 7\ H_2O$, a gift from Dr. E. Abrahamczik, Ludwigshafen, Germany, highly purified, was separated from heavy metal impurities by extraction with 8-hydroxyquinoline and acetylacetone.

RESULTS

Analogous to the titration of a pyridine nucleotide dependent dehydrogenase with the reduced coenzyme a titration curve is obtained after successive addition of zinc sulfate solution to a solution of the GluDH-NADH complex (Fig. 1). From this curve it is possible to calculate the number of zinc

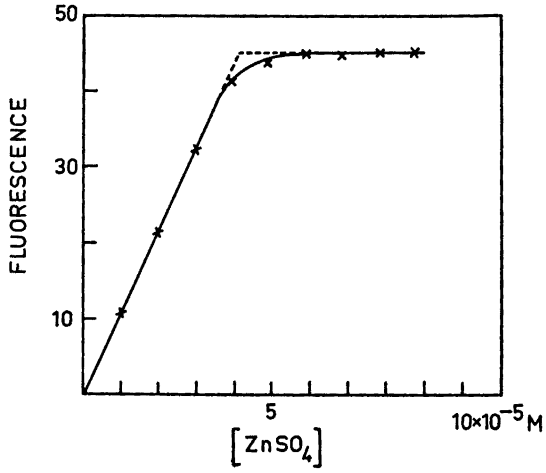

Fig. 1. Titration of beef liver GluDH with $ZnSO_4$. Measurements in sodium phosphate buffer (μ = 0.1) pH 7.5 at 20°C. GluDH, 0.59 mg/ml. NADH, 1.02 μM. Ordinate: Fluorescence in mm. Recording at 440 mμ, excitation at 350 mμ. The curve was calculated with $K_{E,Zn}$ = 0.1 μM and with an equivalent weight of 15 000. ×, experimental points.

binding sites as well as the dissociation constant $K_{E,Zn}$ of the enzyme-zinc complex (Eqn. 1, E denotes the free enzyme as well as the enzyme-coenzyme complex).

$$K_{E,Zn} = \frac{[E]\ [Zn]}{[EZn]} \quad (1)$$

Under the conditions of the experiment the equivalent weight per zinc binding site is 15 000 and the dissociation constant about 0.1 μM. The calculated titration curve agrees with the experimental values (Fig. 1). However, the accuracy of the data does not allow any conclusions whether all the binding sites are

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really equal, but if differences exist the dissociation constants in any case must all be low.

The concentrations of enzyme and NADH (ratio of the concentration of the NADH binding sites to the concentration of NADH about 10) were so chosen that all the coenzyme was bound to the enzyme. Therefore the increase of the fluorescence intensity was not the result of a decrease of the dissociation constant of the binary GluDH-NADH complex in the presence of zinc whereby additional coenzyme could be bound to the enzyme if free coenzyme would be present in solution. In addition the agreement between the calculated and experimental values suggests that the binding of zinc to the free enzyme is similar to and indistinguishable from its binding to the enzyme-coenzyme complex. Zinc ions have almost no effect on the fluorescence of either enzyme or NADH when investigated separately.

DISCUSSION

The low dissociation constant of the GluDH-zinc complex of 0.1 μM agrees with the observations [2,3] that zinc ions inhibit very strongly the reaction catalyzed by GluDH. From the inhibition of enzyme catalyzed reductive amination of the α-ketoglutaric acid the inhibitor constant is calculated to about 0.5 μM [3] which is reasonably close to the value obtained fluorometrically.

The intensification of the fluorescence intensity of an enzyme-NADH complex by addition of metal ions makes it possible and relatively simple to estimate quantitatively and with high sensitivity the binding between metal ions and pyridine nucleotide dependent dehydrogenases.

The concentration of zinc in the liver varies from 1 mM to 0.1 mM.[7,8] Part of it is bound by complex formation to the different components of the cell especially to the various metabolites. In addition the zinc is probably not distributed equally in the cells. In spite of these restrictions it is justifiable to assume that the concentration of free, not complex bound zinc ions *in vivo* is large enough to inhibit the enzymic activity of the beef liver GluDH. Therefore it is possible that the inhibition of the GluDH reaction by zinc ions may be an essential factor in the regulation of the metabolism of proteins and carbohydrates.

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