BINDING OF ETHIDIUM BROMIDE TO LIVER GLUTAMATE DEHYDROGENASE

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Summary

Liver glutamate dehydrogenase forms a complex with ethidium bromide in solution; binding parameters of this complex depend on pH and enzyme concentration, but are independent of the ionic strength of solution. Binding of ethidium bromide occurs outside the coenzyme binding site, but appears to be closely related to the oligomer association sites of the enzyme.

Introduction

Liver glutamate dehydrogenase undergoes a concentration dependent association-dissociation equillibrium in solution. In neutral solutions, at low enzyme concentrations, predominantly an enzymatically active hexamer of mol. wt.336000 is found (1). At higher enzyme concentrations oligomer associates into the particles with higher molecular weight. Binding of different natural and synthetic ligands to the enzyme may depend on its state of polymetization. In this communication we report the binding of ethidium bromide to glutamate dehydrogenase. Binding of ethidium bromide was studied by the fluorescence titration technique; binding parameters were calculated from the quenching of enzyme fluorescence by ethidium bromide, assuming that the fractional saturation of the enzyme was proportional to the quenching of its fluorescence by the ligand.

Materials and Methods

Liver glutamate dehydrogenase (EC 1.4.1.3) was purchased from Boehringer G.m.b.H., Mannheim, and recrystallized from ammonium sulfate for each experiment; its concentration was determined spectrophotometrically, using the extinction coefficient, $A_{1\%} = 9.7$ (2). Fluorimetric titrations were performed in a selfrecording spectrophotofluorimeter Aminco-Bowman, at 27°C. A constant volume of an enzyme or a tryptophan derivative solution (2.5 ml) was titrated stepwise with small aliquots (10-50 µl) of ethidium bromide solution. Fluorescence emission at 350 nm (exctitation at 303 nm) was corrected for the increase in volume, and for inner filter effects, due to the presence of dye, using a molar extinction coefficient for ethidium bromide, $\epsilon_{303} = 21600\ l.mol^{-1}.cm^{-1}$, at pH 7.3 - 9.6. Binding parameters were calculated from corrected fluorescence, according to Scatchard (3). Activity of glutamate dehydrogenase was tested spectrophotometrically at 340 nm in a test mixture of the following composition: 0.1 M sodium pyrophosphate buffer, pH 9.3, semicarbazide 38 mM, glutamate 76 mM, EDTA 0.2 mM, and 8 - 75 µM $NADP^+$.

Results and Discussion

Fluorescence of N-acetyl tryptophan and N-acetyl tryptophan ethyl ester at 350 nm (excitation at 303 nm) was not quenched by ethidium bromide. 3.4 µM solution of N-acetyl tryptophan or N-acetyl tryptophan ethyl ester in 0.1 M sodium phosphate buffer, pH 7.0, was titrated with ethidium bromide solution (final concentration 8.3 µM), with no significant quenching of indole fluorescence observed. Under similar conditions, the fluorescence of glutamate dehydrogenase was quenched by ethidium bromide, which indicates the complex formation between the enzyme and the dye. Protein fluorescence at 350 nm was completely quenched by ethidium bromide (except at high enzyme concentrations), as estimated by a linear extrapolation of the reciprocal of corrected protein fluorescence ($1/\Delta F$) vs. the reciprocal of the ethidium bromide concentration ($1/[\text{ethidium bromide}]$), in a double reciprocal plot.

Glutamate dehydrogenase has three specific kinds of binding areas, which are responsible for different functions of the enzyme: (a) catalytic site, (b) two identical sites for the association of the

oligomer, and (c) regulatory site (or sites). Binding of ethidium bromide was investigated with respect to these specific binding areas of the enzyme.

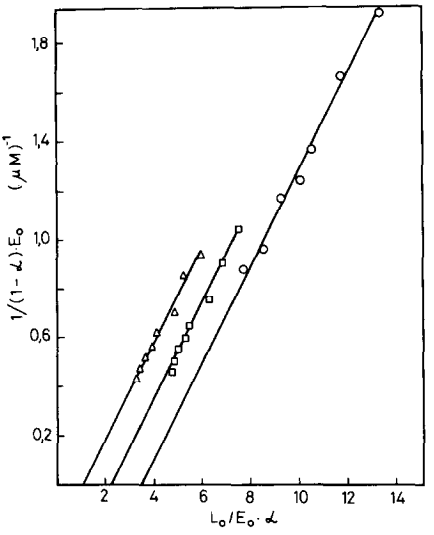

Fig. 1. Scatchard (3) plot of ethidium bromide binding to liver glutamate dehydrogenase. Glutamate dehydrogenase (subunit concentration 1.26 μM, 2.57 μM and 2.83 μM, mol. wt. 56000) was titrated in 0.1 M sodium phosphate buffer, pH 7.3 (0.5 mM EDTA) (o), in 0.1 M sodium pyrophosphate buffer, pH 9.3 (0.5 mM EDTA) (□), and 0.1 M sodium carbonate + sodium bicarbonate buffer, pH 9.6 (0.5 mM EDTA) (▲) with ethidium bromide (final concentrations 9.9 μM, 12.1 μM and 10.6 μM). E_o denotes subunit concentration of enzymes in μM, L_o concentration of ethidium bromide in μM, and α fractional saturation of enzymes with the dye (4).

Fig. 1 indicates that the ethidium bromide-binding capacity of glutamate dehydrogenase depends on pH. At pH values 7.3, 9.3 and 9.6, ethidium bromide binds to the enzyme with a stoichiometry of 3.5, 2.3 and 1.1 molecule/subunit (mol. wt. 56000), respectively. Dissociation constant for the enzyme-ethidium bromide complex (5 μM) remains unchanged between pH 7.3 and 9.6. This dissociation constant is a true dissociation constant of the binary complex enzyme-ethidium bromide, provided the fractional saturation of the enzyme by the ligand is linearly related to

the quenching of its fluorescence (4); that this condition is satisfied is indicated by the linearity of Scatchard plots on Fig. 1, and by the absence of enhancement of ethidium bromide fluorescence at 590 nm (excitation at 480 nm), when the dye was bound to the enzyme.

Table I indicates that the dye binding parameters, K_{diss} and ethidium bromide-binding capacity of the enzyme, depend on protein concentration. At low enzyme concentration (0.05 mg/ml), protein fluorescence at 350 nm is fully quenched at saturating concentrations of ethidium bromide, and at high enzyme concentration (1 mg/ml) it is quenched only by 47%, at saturating dye concentrations. This influence of enzyme concentration upon binding parameters indicates that the binding of ethidium bromide occurs at the sites responsible for the association of oligomers; self-association of oligomers at high enzyme concentrations reduces its capacity to bind ethidium bromide, and weakens its binding.

TABLE I

Binding of ethidium bromide to liver glutamate dehydrogenase at different protein concentrations

Protein concentration (mg/ml)	Binding capacity (mols of ethidium bromide bound/56000 g of enzyme)	Dissociation constant (μM)	Average mol. wt. of enzyme (taken from Markau et al. (1))
0.05	4.1	4.9	400.000
0.1	3.5	5.0	470.000
1	0.5	41.7	1.000.000

Fluorescence titrations were performed in 0.067 M sodium phosphate buffer, pH 7.6 (0.5 mM EDTA), as described in the Methods section.

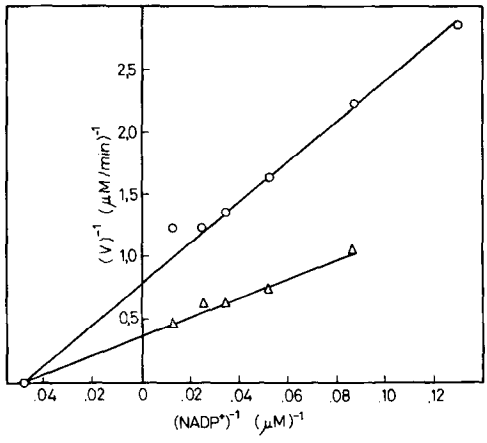

Fig. 2. Double reciprocal plot of glutamate dehydrogenase Michaelian kinetics in the absence (Δ) and in the presence (o) of ethidium bromide (77 μM). Enzymic test is described in the Methods section.

Fig. 2 indicates the relation of ethidium bromide binding to the catalytic site of enzyme. Ethidium bromide is a non-competitive inhibitor of glutamate dehydrogenase at pH 9.3, with respect to $NADP^+$, which indicates that, at this pH, it is bound outside the coenzyme binding site of the enzyme. Large difference between the inhibitor constant for ethidium bromide (68 μM), determined kinetically (Fig. 2), and the dissociation constant of the dye-enzyme complex (5 μM), determined fluorimetrically (Fig. 1), also indicates a binding outside the coenzyme binding site.

Results presented in this communication indicate that, at the neutral and slightly alkaline pH, ethidium bromide is bound outside the coenzyme binding site, and in or near the association sites of oligomer. Ethidium bromide binding parameters are independent of ionic strength at neutral pH, at low portein concentration (o.2 mg/ml); both K_{diss} and the binding capacity are identical in 0.01 M, 0.1 M and 1 M sodium phosphate buffer, pH 7.3. Detailed investigation of the particle

size indicates that the enzyme polymerizes at high protein concentrations in neutral pH (1); this polymerization is responsible for weaker binding and the decrease in ethidium bromide-binding capacity of the enzyme. Decrease in binding capacity for ethidium bromide with increasing pH may indicate that an association-dissociation equillibrium is shifted at alkaline pH. Studies in the ultracentrifuge indicate that glutamate dehydrogenase dissociates into subunits at alkaline pH; the extent of dissociation depends on time; pH and ionic strength (5). Decrease in binding capacity with pH, and unchanged K_{diss} for the enzyme-ethidium bromide complex between pH 7.3 and 9.6, indicate that the pH-dependent changes are complex. Full understanding of the detailed mechanism of ethidium bromide binding to glutamate dehydrogenase at alkaline pH requires the investigation of binding parameters under various conditions of pH, ionic strength and protein concentrations.

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