Interaction of Zn^{2+} and Eu^{3+} with bovine liver glutamate dehydrogenase

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Bovine liver glutamate dehydrogenase is potently inhibited by Zn^{2+} ions. At pH 7.0 a kinetic dissociation constant for Zn^{2+} of $18 \mu\text{M}$ is obtained. The fluorescent lanthanide Eu^{3+} competes for the Zn^{2+} -binding site and relieves the Zn^{2+} -induced inhibition, but does not cause inhibition. Studies on the effects of Zn^{2+} or Eu^{3+} on the tertiary and quaternary structure of the enzyme by the use of protein fluorescence, heat-stability and re-activation after guanidinium chloride denaturation indicate that, whereas Zn^{2+} affects both tertiary and quaternary structure, Eu^{3+} does not affect either, consistent with its lack of effect on enzymic properties. Eu^{3+} fluorescence had a strong excitation peak at 395 nm with emission at 456 nm. In the presence of glutamate dehydrogenase the fluorescence emission is shifted to 501 nm. Eu^{3+} , with high-affinity binding site and distinctive fluorescence properties after binding, would appear to be an ideal fluorophore for use in conformational studies or resonance-energy-transfer studies.

INTRODUCTION

Bovine liver glutamate dehydrogenase is a hexameric enzyme [1] containing chemically identical subunits [2,3]. The enzyme is known to show complex initial-rate kinetics in both the oxidative deamination reaction [4,5] and the reductive amination reaction [6,7], which have been variously attributed to complex subunit interactions [4,5,8–10] or the involvement of a second regulatory coenzyme-binding site for NADH [11,12]. Although two coenzyme sites per subunit are known to exist [13–15], the affinity of the 'regulatory' site for NAD^+/NADH is much greater than for $\text{NADP}^+/\text{NADPH}$. This observation, combined with the similar effects of NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$ in kinetic and ligand-binding studies [4,5,8–10], suggests that subunit interactions are responsible for much of the enzyme complexity, although second-site binding of NADH is clearly important in a number of circumstances [6,7].

Glutamate dehydrogenase was originally considered to be a 'zinc metallodehydrogenase' [16]; however, subsequent work [17] showed that, rather than being a zinc enzyme, the protein was fully active in the absence of Zn^{2+} . Zn^{2+} was shown to have a single binding site per polypeptide chain, and it causes inhibition of the enzyme. The latter observations were consistent with a number of reports [18–20] indicating that the activity of the enzyme was decreased in the presence of Zn^{2+} . Subsequent studies [21] showed that Zn^{2+} , like GTP, induced a conformation in the protein where a second NADH-binding site could be spectrally observed in c.d. studies.

Although a variety of work [22–25] has investigated anion effects on glutamate dehydrogenase, few studies have examined cation effects, although Ca^{2+} [26] and Mg^{2+} [27] have been reported to interact with the liver enzyme, producing effects on protein stability.

In the present study we have examined the effects of Zn^{2+} on various kinetic and structural properties of glutamate dehydrogenase, and have shown that the

fluorescent lanthanide Eu^{3+} is a competitive inhibitor with respect to Zn^{2+} but upon binding to the enzyme produces no detectable perturbations in the enzyme's properties. Since the Eu^{3+} fluorescence is markedly shifted and enhanced upon binding to glutamate dehydrogenase, Eu^{3+} is an ideal fluorophore to be used for a variety of studies with this enzyme.

MATERIALS AND METHODS

Bovine liver glutamate dehydrogenase used in these studies was obtained as a glycerol solution from Sigma Chemical Co., and was thoroughly dialysed against 0.1 M-sodium phosphate buffer, pH 7.0, containing $10 \mu\text{M}$ -EDTA, before use. Enzyme concentrations were measured spectrophotometrically, by using an absorption coefficient at 280 nm of 0.93 cm^{-1} for a 1 mg/ml solution [28]. Concentrations of enzyme were calculated by using a subunit M_r value of 55700. All substrates, coenzymes and buffer salts were obtained from Sigma Chemical Co. ZnCl_2 and EuCl_3 were obtained from Alfa Inorganics. All solutions were made up with 18M Ω distilled deionized water from a four-bowl Milli-Q system. Metal ion solutions were made up in the appropriate phosphate buffer, and the pH was adjusted as required before addition to enzyme solutions.

Rate measurements were made by monitoring absorbance changes at 350 nm and calculating the rate of product formation by using a millimolar absorption coefficient for NADH of $6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [29]. Results given are the averages of triplicate determinations.

Fluorescence measurements were made with a Fluorolog-212 system photon-counting fluorimeter (Spex Industries). In all spectral measurements 1 nm slits on both emission and excitation sets of monochromators were used. Results were collected at a 1 s integration time at the wavelength intervals indicated in the appropriate Figure legends.

Three-dimensional protein fluorescence spectra were recorded with the use of a grid of excitation and emission

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Fig. 1. Effects of Zn²⁺ and Eu³⁺ on initial-rate kinetic studies of glutamate dehydrogenase

(a) Zn²⁺ (▲) or Eu³⁺ (▲) concentrations were varied, with 50 mM-glutamate and 250 μM-NAD⁺ at pH 7.0. Reaction was initiated by the addition of enzyme (14 nM). (b) Eu³⁺ concentrations were varied in the absence of Zn²⁺ (△) or in the presence of Zn²⁺ at 25 μM (□), 50 μM (▲), 75 μM (■) and 200 μM (●). Other conditions were as in (a) except the reaction was initiated by the addition of enzyme at 292 nM.

wavelengths. The emission wavelengths (on the x-axis) are monitored from 300 to 400 nm. The excitation wavelengths (on the y-axis) were varied between 270 and 295 nm. For representational purposes the z-axis is offset by 1.5 nm along the x-axis for each excitation-wavelength increment.

Guanidinium chloride renaturation experiments were conducted as described previously [10]. Heat denaturation profiles were obtained by heating the enzyme at 50 °C and withdrawing samples at the indicated time intervals, which were immediately assayed in a standard assay mix containing 20 mM-glutamate and 250 μM-NAD⁺ in 0.1 M-sodium phosphate buffer, pH 7.0.

RESULTS

Initial studies examined the effects of Zn²⁺ on the oxidative deamination of L-glutamate at pH 7.0. With fixed concentrations of NAD⁺ (250 μM) and glutamate (20 mM), Zn²⁺ is a potent inhibitor, with maximal inhibition reached at 200 μM-Zn²⁺, as shown in Fig. 1(a). Also shown in Fig. 1(a) is the complete lack of effect of Eu³⁺ over the same concentration range. Least-squares analysis of the fit of the data for Zn²⁺ to the equation:

$$\Delta v = \frac{\Delta V_{\max} \cdot [\text{Zn}^{2+}]}{[\text{Zn}^{2+}] + K_D^{\text{Zn}}}$$

where ΔV is the difference in rate measured in the absence of Zn²⁺ and at any particular Zn²⁺, $\Delta V_{\max}$ is the value of Δv at saturating Zn²⁺ concentrations and K_D^{Zn} is the kinetic dissociation constant for Zn²⁺ [34], gives a

value of 18 μM for K_D^{Zn} . When Eu³⁺ concentrations are varied at a series of fixed Zn²⁺ concentrations, the inhibition caused by the Zn²⁺ is relieved, as indicated by the data in Fig. 1(b). Clearly Eu³⁺, although it causes no inhibition, can effectively compete with and relieve the inhibitory effects of Zn²⁺.

In light of our desire to use Eu³⁺ as a fluorescent probe for glutamate dehydrogenase, we have examined the inhibition caused by Zn²⁺ in more detail so that the nature of the inhibition is better characterized. Initial-rate studies, in the form of Lineweaver-Burk plots with either NAD⁺ or glutamate as the substrate whose concentration was varied, are given in Fig. 2 in the absence and in the presence of various Zn²⁺ concentrations.

Since, as discussed below, Eu³⁺ clearly binds to glutamate dehydrogenase and displaces Zn²⁺ (which is a potent inhibitor), it was decided to examine the effects of Zn²⁺ on the enzyme further, with particular emphasis on whether ADP or leucine can reverse the Zn²⁺-induced inhibition. Experiments with the concentration of either leucine or ADP varied in the presence and in the absence of 50 μM-Zn²⁺ were performed. Although ADP reverses the Zn²⁺-induced inhibition (results not shown), leucine cannot.

In addition to the initial-rate kinetic studies described here, we have examined the effects of Zn²⁺ and Eu³⁺ on a number of properties of the enzyme that can be related to its tertiary and quaternary structure.

When glutamate dehydrogenase is incubated at 50 °C a rapid irreversible inactivation is observed. When Eu³⁺ is included in the incubation mixture, the time-dependence of the heat-inactivation is unchanged.

Fig. 2. Uncompetitive inhibitory effects of Zn^{2+} on glutamate dehydrogenase

Initial-rate studies of the oxidative deamination reaction were performed with (a) glutamate as the varied-concentration substrate at a fixed NAD^+ concentration of $250 \mu\text{M}$ and (b) with NAD^+ as the varied-concentration substrate at 20 mM -glutamate. Results were obtained in the absence of Zn^{2+} ($\blacktriangle$) and in the presence of $100 \mu\text{M-Zn}^{2+}$ ($\blacksquare$). All studies were at pH 7.0 in 0.1 M-phosphate buffer.

However, when 0.2 mM-Zn^{2+} is included in the incubation mixture, the inactivation is significantly faster. Rate constants of heat-inactivation of glutamate dehydrogenase of 0.35 min^{-1} , 0.35 min^{-1} and 0.61 min^{-1} were determined in the absence of metal ions, in the presence of Eu^{3+} and in the presence of Zn^{2+} respectively.

Glutamate dehydrogenase, at 1 mg/ml final concentration, was incubated with various guanidinium chloride concentrations between 0 and 3 M at pH 7.0 for 60 min. At the end of the incubation, samples of the enzyme were renatured by addition to reaction mixture and recovery of enzyme activity was calculated. As shown in Fig. 3, the presence of Eu^{3+} with the guanidinium chloride had no significant effect on the renaturation curve. When Zn^{2+} was included, however, the denaturation-renaturation curve was shifted to markedly lower guanidinium chloride concentrations.

The effects of Zn^{2+} and Eu^{3+} on the overall conformation of the protein, as judged by three-dimensional protein fluorescence spectra, were monitored. The results, given in Fig. 4, indicate that, whereas Eu^{3+} has no effect on the three-dimensional protein fluorescence spectrum, Zn^{2+} causes considerable changes in the observed contours.

Eu^{3+} fluorescence spectra, in the absence of glutamate dehydrogenase, have a strong excitation peak at 395 nm when emission is monitored at 456 nm . Fig. 5 shows Eu^{3+} emission spectra (with 395 nm excitation) of Eu^{3+} free and in the presence of glutamate dehydrogenase. As clearly shown, the bound Eu^{3+} has a shifted emission maximum at 501 nm . When Eu^{3+} was added to glutamate dehydrogenase in the presence of a saturating Zn^{2+} concentration of $200 \mu\text{M}$, no significant difference in

Fig. 3. Guanidinium chloride denaturation-renaturation of glutamate dehydrogenase

Enzyme, at a final concentration of 1 mg/ml , was incubated at the indicated guanidinium chloride concentrations for 60 min before 300-fold dilution into standard assay mixture for activity determinations. Incubations with guanidinium chloride were performed in the absence of added metal ions ($\square$), in the presence of 0.5 mM-Eu^{3+} ($\triangle$) and in the presence of 0.05 mM-Zn^{2+} ($\circ$).

the Eu^{3+} fluorescence, compared to that of free Eu^{3+} in solution, was observed.

DISCUSSION

A variety of reports have shown that Zn^{2+} is a potent inhibitor of glutamate dehydrogenase [20–23], although

Fig. 4. Three-dimensional protein fluorescence spectra

Three-dimensional protein fluorescence spectra were recorded by using 1 nm intervals for monitoring emission intensity (*y*-axis) at various wavelengths (*x*-axis). Spectra of native protein (*a*) and in the presence of 0.1 mM-Eu³⁺ were identical. Spectra in the presence of 0.1 mM-Zn²⁺ are given in (*b*). Intensities are expressed in photons·s⁻¹.

Fig. 5. Eu³⁺ fluorescence emission spectra

Emission spectra of 0.1 mM-Eu³⁺ were determined in the absence of protein (-----) and in the presence of 12 μM-glutamate dehydrogenase (—). Each spectrum was recorded in 0.1 M-phosphate buffer at pH 7.0. Appropriate blank spectra (either buffer alone or enzyme + buffer) were subtracted.

the nature of the inhibition has not been totally characterized. In the current work the potent inhibition of the oxidative deamination reaction catalysed by glutamate dehydrogenase at pH 7.0 has been re-examined and the ability of Eu³⁺ to replace Zn²⁺ in its binding (but produce no inhibition) is established. Fig. 1 clearly indicates that Eu³⁺, although itself non-inhibitory, can displace Zn²⁺ in terms of reversing its inhibitory effects. The Lineweaver-Burk plots in Fig. 2 show that, as reported for GTP [30], Zn²⁺ is an uncompetitive inhibitor with respect to both glutamate and NAD⁺, but does not appear to affect the discontinuities in Lineweaver-Burk plots with NAD⁺ as the substrate whose concentration is varied (Fig. 2*b*).

The Zn²⁺-induced inhibition of glutamate dehydrogenase also resembles GTP inhibition in terms of the way ADP and leucine affect the Zn²⁺-inhibited enzyme; ADP (although itself inhibitory at pH 7) reverses Zn²⁺ inhibition. Leucine activates the Zn²⁺-inhibited enzyme, but, as has been shown for the GTP-inhibited enzyme (E. T. Bell, A. M. Stilwell & J. E. Bell, unpublished work), leucine does not reverse Zn²⁺ inhibition. In addition, the slight substrate inhibition of high glutamate concentrations (Fig. 2*a*) in the presence of Zn²⁺ is similar to that induced by GTP, which is attributed to increased stability of an enzyme-NAD(P)H-glutamate abortive complex [6,7].

Whatever effects Zn²⁺ is inducing in glutamate dehydrogenase, they resemble those of GTP, and it might be speculated that GTP and Zn²⁺ bind at the same site on the protein to produce their inhibitory effects.

Eu³⁺, as indicated by its kinetic effects already discussed, and as shown by its fluorescence properties free in solution and in the presence of glutamate dehydrogenase (Fig. 5), binds to the enzyme and appears to displace Zn²⁺. Zn²⁺, when bound to the enzyme, prevents Eu³⁺ binding, as judged by Eu³⁺ fluorescence. Zn²⁺ and Eu³⁺, in addition to their differences in ability to inhibit glutamate dehydrogenase, behave quite differently in a number of experiments designed to compare their effects on the overall conformation of the protein. In heat-stability studies Zn²⁺ considerably destabilizes the enzyme, with the rate constant for inactivation increasing by 70% to a value of 0.061 min⁻¹. Eu³⁺ has no significant effects on the stability of the protein. Renaturation from guanidinium chloride has been shown [10] to be a function of the ability of the hexamer to dissociate first to the trimer level (from which renaturation is possible) and subsequently to the monomer level (from which renaturation does not occur). In the experiments illustrated in Fig. 3, when Zn²⁺ is included with the guanidinium chloride the ability to re-activate the enzyme is shifted to lower concentrations, suggesting that Zn²⁺ affects the stability of the trimer adversely. Eu³⁺ does not have this effect.

The native fluorescence of the protein, monitored as three-dimensional protein fluorescence spectra, is unchanged by the presence of Eu³⁺. Zn²⁺, however, causes considerable changes in the contour structure of these spectra (Fig. 4), suggesting that it markedly alters the environment of the three tryptophan residues per polypeptide chain.

The results presented here show that Eu³⁺ binds to glutamate dehydrogenase, presumably at the single Zn²⁺-binding site per polypeptide chain [17], but does not cause inhibitory effects and, as far as can be judged by

the use of a variety of conformational probes, produces no conformational change in the protein as a result of binding. In light of these findings, Eu^{3+} fluorescence may be ideally suited in this enzyme for use in further probes of conformational changes elicited by other regulatory ligands, or for use in resonance-energy-transfer studies.

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