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A Novel Mechanism of V Type Zinc Inhibition of Glutamate Dehydrogenase Results from Disruption of Subunit Interactions Necessary for Efficient Catalysis

Jaclyn Bailey¹, Lakeila Powell², Leander Sinanan³, Jacob Neal³, Ming Li⁴, Thomas Smith⁴, and Ellis Bell³

¹Gustavus Adolphus College, St Peter, Minnesota, 56082

²Virginia State University, Petersburg, VA, 23806

³Department of Chemistry, University of Richmond, Richmond, Virginia, 23173

⁴Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132

SUMMARY

Bovine Glutamate Dehydrogenase is potently inhibited by zinc and the major impact is on V_{max} suggesting a V-type effect on catalysis or product release. Zinc inhibition decreases as glutamate concentrations decrease suggesting a role for subunit interactions. With the monocarboxylic amino acid, norvaline, which gives no evidence of subunit interactions zinc does not inhibit. Zinc significantly decreases the size of the pre-steady state burst in the reaction but does not affect NADPH binding in the Enzyme-NADPH-glutamate complex that governs the steady state turn-over again suggesting that zinc disrupts subunit interactions required for catalytic competence. While differential scanning calorimetry suggests zinc binds and induces a slightly conformationally more rigid state of the protein, limited proteolysis indicate regions in the vicinity of the antennae regions and the trimer-trimer interface become more flexible. The structures of GDH bound with zinc and europium show that zinc binds between the three dimers of subunits in the hexamer, a region shown to bind novel inhibitors that block catalytic turnover and is consistent with the above findings. In contrast, europium binds to the base of the antenna region and appears to abrogate the inhibitory effect of zinc. Structures of various states of the enzyme have shown that both regions are heavily involved in the conformational changes associated with catalytic turnover.

These results suggest that the V-type inhibition produced with glutamate as the substrate results from disruption of subunit interactions necessary for efficient catalysis rather than by a direct effect on the active site conformation.

Introduction

Bovine liver glutamate dehydrogenase [E.C. 1.4.1.3, GDH] catalyzes the oxidative deamination of L-glutamate and various monocarboxylic acid substrates (1). The enzyme also shows the unique ability, among mammalian dehydrogenases, of being able to utilize either NAD^+ or $NADP^+$ as cofactor in the reaction with near equal affinity, although $NAD(H)$ has an additional binding site per subunit (2). The enzyme, which is a hexamer of chemically identical polypeptide chains (3,4), exhibits negative cooperativity (5,6) resulting from coenzyme induced conformational changes (7–9). More recent work has shown that

this coenzyme induced conformational change requires a dicarboxylic acid substrate or analog with a 2-position substituent (10). A variety of previous studies have shown the importance of two appropriately positioned carbonyl groups for strong interaction of substrates or analogs with the enzyme (11–13) and for synergistic binding of substrate [or analog] with either oxidized (14,15) or reduced cofactor (2). With alternative amino acid substrates such as norvaline, the manifestations of cooperative interactions between the subunits of the enzyme are absent (5,16). Since it has been shown that the entire hexamer is required to give optimal activity of the enzyme (17) with glutamate as substrate, it is likely that the cooperative interactions between subunits in the hexamer are required for maximal activity. Our recent work has shown the importance of conformational flexibility (18) and the strength of subunit interactions (19) in glutamate promoted cooperativity that is absent with norvaline. This is consistent with the fact that the overall rate of oxidative deamination is very much lower with alternative amino acid substrates.

Glutamate dehydrogenase from mammalian sources is highly regulated by a diverse array of small molecules, with ADP, GTP, Leucine, and the combination of malate and palmitoyl CoA being the most effective regulators of the activity (20–22). The enzyme was originally considered to be a zinc metalloenzyme (23), however subsequent work (24) showed that the enzyme demonstrates full activity in the absence of any bound zinc, and that zinc is in fact a potent inhibitor of the enzyme. Our own more recent studies (25) showed that the trivalent europium ion could displace zinc from the enzyme and relieve the zinc-induced inhibition. Like the allosteric inhibitor GTP, zinc induces the presence of a second, inhibitory NADH site on the enzyme which, unlike the active site, shows a considerable preference for NAD(H) over NADP(H) (2). The physiological importance of possible zinc inhibition of glutamate dehydrogenase is not clear although zinc poisoning (26) shares some similar symptoms as Reye's syndrome which has previously been shown to involve alterations in the regulation of glutamate dehydrogenase (27) and elevated zinc levels have been associated with neurological disease (28). Under normal circumstances in vivo zinc concentrations have been estimated to be in the range 25–100 μ M (29).

Although the crystal structure of both bovine and human forms of the enzyme are now available (30–32) and have led to considerable insight into the structural basis for subunit interactions in this enzyme and the mechanism of regulation by purine nucleotides, the structures have not revealed either the nature of the zinc binding site or the basis for zinc inhibition.

In the current study, in addition to further defining the nature of the interaction of zinc with glutamate dehydrogenase, we have thoroughly investigated the effects that variation of the amino acid substrate concentration has on the ability of zinc to inhibit the activity of this enzyme. The major zinc binding site is located in the GTP binding site and likely inhibits the enzyme in a similar manner as GTP. Europium binds in the core of the antenna region where it alleviates zinc inhibition. This is entirely consistent with previous studies demonstrating that the antenna is necessary for GTP inhibition (33) and from naturally occurring mutations in the antenna region that results in the loss of GTP inhibition (34). These results demonstrate that the ability of zinc to inhibit the enzyme is intimately tied to the ability of the hexamer to exhibit subunit interactions necessary for efficient catalysis.

RESULTS

At saturating concentrations of substrates in either the forward, oxidative deamination reaction, or reverse, reductive amination reaction, catalyzed by glutamate dehydrogenase, zinc is a potent inhibitor.

Initial Rate Studies

The dependence of this inhibition on the concentration of the substrate glutamate was examined. In these experiments a fixed concentration of NADP⁺ of 250 μM was used and the K_i for zinc was determined at a series of fixed glutamate concentrations between 0.25 mM and 50 mM. As is shown in Figure 1, and summarized in Table 1, there is a marked decrease in the affinity of the enzyme for zinc as the glutamate concentration decreases below 5 mM, but very little difference between 50 mM and 5 mM. Similar results were obtained using NAD⁺ or NADP⁺ as cofactor at either pH 7.0 or pH 8.0. Control experiments - data not shown - showed that the presence of magnesium had no effect on the activity of the enzyme or the inhibition by zinc, consistent with previous observations (35) suggesting that magnesium had little effect on the activity of glutamate dehydrogenase in the absence of ATP or GTP.

We have examined the effects of the ability of zinc to inhibit when the enzyme is using the monocarboxylic acid substrate, norvaline. Since previous work has shown that initial rate measurements with norvaline require a higher pH, these studies were conducted at both pH 8.0 (Figure 2) and pH 9.0 (data not shown), allowing a significantly higher concentration of norvaline (200 mM) to be used to give a reasonable saturation of the enzyme with norvaline. In these experiments, at pH 8.0, no significant inhibition by zinc was detected when norvaline was used as substrate. In control experiments, using glutamate as substrate at pH 8.0, zinc produced effective inhibition at this pH. The K_i for zinc calculated for this data indicates however that the affinity for zinc does decrease slightly as the pH is raised.

Stopped Flow Studies

Using glutamate as the substrate, the effects of zinc on the pre-steady state phase of the reaction were studied. In both cases there is a clear pre-steady state phase and when the steady state region (from 4–8 seconds) is subtracted the resultant pre-steady state phase shows the expected rise to a maximum (Figure 3). allowing both an amplitude and rate constant for the pre-steady state phase to be calculated. The parameters for both the steady state phase and the pre-steady state phase are given in Table 2.

Effects of Zinc on Cofactor Binding

The effects of zinc on the binding of the reduced cofactor, NADPH to the enzyme, at pH 8.0, in the presence or absence of glutamate were examined using fluorescence titrations, making use of the enhanced fluorescence of the NADPH on binding to the enzyme. Titrations of enzyme alone and enzyme in the presence of 20 mM glutamate, together with equivalent titrations in the presence of 100 μM zinc. and control titrations of NADPH in the absence of enzyme. allowed plots of ΔF versus NADPH concentration to be constructed (Figure 4) to determine the dissociation constant for cofactor binding. Similar titrations were conducted in the presence of norvaline with NADPH. The data obtained is summarized in Table 3. Each condition was also used for titrations with NADH (data not shown), and similar effects were observed.

Effects of Zinc on the Stability of the Enzyme

The thermal stability of the enzyme was determined using differential scanning calorimetry in the presence and absence of zinc under a variety of conditions. The T_m values obtained are summarized in Table 4.

Effects of Zinc on Limited Proteolysis of the Enzyme

In limited proteolysis experiments three peaks show in the first 15 minutes of digestion (Table 5) in the absence or presence of zinc, one at 34,645 (corresponding to residues 144–

459), one at 3,446 (corresponding to residues 114–146) and one at 4089 (corresponding to residues 1–35). Based upon the relative amounts of the peaks, in the presence of zinc the 3,446 and 4,089 peak appear significantly faster in the digestion while the peak at 34,645 which appears a little faster than in the absence of zinc. The various cleavage sites that yield these fragments are illustrated in Figure 5, with two clusters seen, one around the base of the antennae region and the other near the subunit interfaces within each trimer. Residue 144 is near the trimer-trimer interface.

Locations of the Zn²⁺ and Eu³⁺ binding sites

Using the previously determined structure of GDH complexed with NADPH+GTP +glutamate (26), the structure refinement of GDH complexed with the metals quickly converged (refinement statistics shown in Table 6). The average B value for protein atoms for the zinc and the europium structures are slightly lower than the GDH•NADH•GTP•Glu structure. The RMSD values between the original abortive complex structures for a particular subunit were 0.66Å, and 0.59Å, for the europium and zinc structures, respectively. When comparing the metal binding sites, there were only limited conformational changes in some of the ligating residues. Therefore, there were no large effects in the overall structure of GDH due to metal binding. The deleterious effect of europium on diffraction resolution is mostly likely due to it essentially removing GTP from its binding site. These R factors are higher than the original structure of the NADPH abortive complex, and is most likely due to the negative impact that the metals had on diffraction.

In the case of the zinc complexes, the electron density is entirely unambiguous and difference electron density maps (Fo-Fc) showed strong (>6 σ) peaks at two locations (Figures 6 and 7) suggesting the location of two zinc sites. As a control, the crystals were also soaked in the 0.1 M triethanolamine-HCl (pH 7.0) buffer in the presence of 5 mM EDTA. These two strong peaks disappeared under these conditions (data not shown), lending support to the contention that these two peaks represent bound zinc.

One of the bound zinc atoms is found at the interface between the bound GTP and the enzyme (Figure 7A). The B value for this bound zinc is the same as the surrounding protein atoms and therefore bound very tightly. Initially, there was concern that the GTP and zinc might be binding as a complex to GDH. However, when GTP was removed during the metal soaking process, the GTP density weakened while the zinc density did not (data not shown). This could only be done to a limited degree since there was decay in the diffraction when GTP was entirely removed from the synthetic mother liquor. This is akin to the disruption of the crystals by europium as it strips away the bound GTP. The Zn²⁺ ion binds to two histidine residues (His209 and His450) and to one phosphate oxygen atom in GTP. It is also important to note that His450 is on the pivot helix and His209 is on the loop connecting the NAD binding domain to the glutamate-binding domain. Both regions are part of the ~18° movement of the NAD binding domain during catalysis (27,38). Therefore, it is possible that zinc, binding here, could mimic the effects of GTP binding to this location.

The other bound zinc atom also lies near a dynamic region of the enzyme (Figure 7B). His57 and Glu151 from a two-fold related subunit make clear interactions with the bound zinc. The B value for this zinc atom is approximately twice that of the surrounding protein atoms. Therefore, zinc is apparently not bound here as tightly as the one near the GTP site. There is an additional histidine residue (His94) very close to the bound zinc, but the electron density for the side chain suggests that it might not be directly involved in binding. As noted in this figure, this binding site is near the loop containing the trypsin cleavage site (Arg35). In addition to the data presented above, previous MALDI studies demonstrated that the motility of this loop is diminished when the enzyme is locked into an abortive complex (27) and the α-helix immediately upstream from this loop moves as the catalytic cleft opens and closes

(27). Recent studies have shown that chymotrypsin cleavage in this region removes this helical region, resulting in an activated form of the enzyme (34). Finally, we recently determined the structures of two different drug/GDH complexes these potent inhibitors were found to bind in the immediate vicinity of this zinc-binding site. It was proposed that the drugs act by affecting protein dynamics necessary for catalysis and it seems likely that zinc does the same (36,37).

The addition of Eu^{3+} to the GDH crystals had a deleterious effect on diffraction yielding a resolution of $\sim 3.3\text{\AA}$ with a final R factor of 26% ($R_{\text{free}} = 31\%$). This seems likely due to interactions between Eu^{3+} and GTP. When Eu^{3+} was added, the density for GTP was extremely weak and not clearly apparent in difference ($F_o - F_c$) maps. Therefore it seems likely that the europium interacted with GTP and decreased its effective free concentration. This loss of GTP binding is likely to be the cause of the damage to the crystals incurred upon the addition of Eu^{3+} . Nevertheless, there was a very large peak ($>6\sigma$) inside the base of the antenna region (Figure 8). The refined B value for this metal is about twice that of the surrounding protein atoms. Particularly since the addition of Eu^{3+} damaged the diffraction of these crystals, this metal is apparently well bound to this site. Three glutamate residues (Glu402) of three ascending helices in the trimeric antenna form the binding site for the Eu^{3+} ion. In this model, the OE1 oxygens from the three glutamates are $\sim 3.0\text{--}3.3\text{\AA}$ away from the Eu^{3+} and the OE2 oxygens are $\sim 3.5\text{--}3.9\text{\AA}$ away. As shown in this diagram, this site does not overlap with the Zn^{2+} site or the GTP binding pocket (Figures 6 and 8), but is not far removed from the latter. However, akin to the motility observed in the zinc binding sites, the three ascending helices of the trimer that form the Eu^{3+} binding site rotate about each other as the active site opens and closes during catalysis (35). Also shown in figure 8 is the same region in the Zn^{2+} complex. Compared to the Eu^{3+} complex, the three acid side chains (E402) are shifted away from the core of the antenna and the base of the antenna appears to be slightly expanded. It is important to note that previous studies demonstrated that Eu^{3+} abrogates Zn^{2+} inhibition when glutamate is used as substrate, but does not compete directly with its binding (25).

DISCUSSION

Zinc has long been known to be a potent inhibitor of glutamate dehydrogenase, and as we (25), and others (24), have shown inhibits the reaction with high affinity. The ability of zinc to inhibit the enzyme was reversed by the trivalent metal ion Eu^{3+} , although Eu^{3+} itself had no effect on the activity of the enzyme. This observation has been extended to include a number of other metal ions.

Our observations of the effects of decreasing glutamate concentrations on the apparent affinity of the enzyme for zinc, Figure 1 and Table 1, clearly indicate that zinc is a less effective inhibitor under conditions where there is a low degree of saturation with glutamate. In light of previous work showing that the kinetic manifestations of subunit cooperativity in this enzyme require at least half saturation of the system with glutamate and cofactor, it is tempting to speculate that, under conditions of low glutamate concentration, where subunit cooperativity does not occur, zinc binds to the enzyme, but has no effect what-so-ever on the activity. This raises the possibility that zinc exerts its inhibitory effect by interfering with subunit cooperation in the hexamer that is required for the full activity of the enzyme under normal circumstances. The experiments shown in Figure 2 clearly support this notion. When the enzyme utilizes the alternate, monocarboxylic amino acid norvaline as substrate there is no subunit cooperation. Under these conditions zinc exerts no effect on the catalytic activity of the enzyme.

The pre-steady state effects of zinc show that while the rate constant for the pre-steady state rate is not affected, the amplitude of that phase is significantly reduced, suggesting that less of the enzyme is involved in productive enzyme-NADPH complexes involved in the overall rate limiting step of the reaction.

The effects of zinc on the binding of reduced cofactor to the enzyme, Table 3 and Figure 4, show that while the major effect of zinc is on V_{max} , there is little or no effect on the affinity for reduced cofactor in the enzyme-glutamate-reduced cofactor complexes. In the absence of glutamate zinc appears to significantly tighten NADPH binding. Interestingly in the presence of norvaline, zinc has little effect on the binding of NADPH.

The major conclusion that can be drawn from these experiments is that zinc inhibits glutamate dehydrogenase by interfering with a glutamate dependent subunit cooperativity necessary for effective enzyme action rather than by interfering with ligand binding or directly with catalytic efficiency.

Our previous work (24) demonstrated that the presence of zinc caused a significant change in the three-dimensional fluorescence spectrum of the protein suggesting that a conformational change had occurred. The differential scanning calorimetry and limited proteolysis experiments described here shed further insight on the conformational states of glutamate dehydrogenase and how substrates (glutamate or norvaline) and zinc impact the overall stability and local flexibility of the enzyme. As shown in Table 4, the addition of zinc to enzyme alone causes a small increase in thermal stability which when glutamate is present is largely negated by the small increase in stability caused by glutamate. Norvaline, to a much greater effect, stabilizes the protein and again zinc has a minimal effect. Although zinc does not cause large effects on the T_m of the protein, the differential scanning calorimetry experiments clearly demonstrate that zinc binds to the enzyme in the absence of other ligands or in the presence of glutamate or norvaline - the lack of inhibition of the norvaline dependent reduction of $NAD(P)^+$ is clearly not due to a lack of zinc binding, again supporting the concept that zinc inhibits by interfering with cooperative interactions in the enzyme that are not supported by norvaline.

The limited proteolysis experiments demonstrate that zinc does indeed cause changes in local flexibility, and it is interesting that all of the zinc induced changes are regions located either at the base of the antennae region of the molecule or at subunit interfaces; the general locations of the zinc binding sites. This suggests that zinc causes conformational effects that interfere with the normal transmission of subunit interactions within the hexamer. Specifically, these crucial 'flex points' appear to be at the back of the glutamate-binding domain near residue 35 and within the GTP binding site. Again, the loop that contains residue 35 was observed to be less sensitive to proteolysis in the presence of the NADH+Glu abortive complex and His450 and His209 are intimately involved in GTP inhibition (31). In contrast, Eu^{3+} binds to the internal base of the antenna and abrogates the inhibition by zinc without affecting zinc binding. This is clearly a classic case of allostery where the two metals cause opposing effects on the enzyme without directly competing for binding. It may be that zinc binding to one or both of the observed locations makes it harder for the enzyme to undergo the conformational changes during catalysis while europium may be facilitating such motion by drawing the three Glu402 residues closer together. Perhaps Eu^{3+} accomplishes this by facilitating the observed rotation of the three ascending helices about each other as the catalytic cleft opens (30, 31, 39, 42,).

In summary, the work presented here demonstrates a novel basis for the potent inhibition of glutamate dehydrogenase by zinc; interference with a glutamate induced conformational change that appears to be required for maximal activity of the enzyme, thus resulting in a

potent inhibition of the overall maximum rate of the oxidative deamination of L-glutamate. This further emphasizes the vital role that subunit-subunit interactions play in the normal catalytic cycle of this complex enzyme, and suggests that a previously unseen mode of regulation of the enzyme occurs, one that involves interference with subunit-subunit interactions. In the case of glutamate dehydrogenase such subunit interactions appear to involve a reciprocating subunit type effect where glutamate induced changes on one subunit are necessary for maximal overall catalysis on another subunit. Such a mechanism lends itself to potent V-type inhibition by interference with the subunit communications.

MATERIALS AND METHODS

Bovine liver GDH was obtained as a glycerol solution from Sigma Chemical Co. All other chemicals were also purchased from Sigma. Enzyme solutions were prepared as described previously (16), using 0.1 M phosphate buffer at pH 7, containing 10 μ M EDTA. All solutions were made up with 18 megohm deionized water from a 4 bowl Milli Q system. Enzyme concentrations were determined spectrophotometrically by absorbance at 280 nm, using an extinction coefficient of 0.98 for a 1 mg/ml solution (35). Coenzyme concentrations were also determined spectrophotometrically using absorbance measurements at 260nm and a millimolar extinction for NAD(P)⁺ at 260nm of 15.9 $\text{cm}^{-1}\text{mM}^{-1}$. The enzyme concentrations reported here are the concentrations of subunits, using a subunit molecular weight of 55,700.

Initial rate kinetic measurements were made for the oxidative deamination reaction by monitoring absorbance changes (using a Thermospectronic UV1 Spectrophotometer) due to the production of NAD(P)H at 340 nm, using a millimolar extinction coefficient of 6.22 $\text{mM}^{-1}\text{cm}^{-1}$. All rate measurements were performed in triplicate and the results shown are the averages of the experimental values obtained. In the graphs shown, all data is presented either as % activity, with the activity in the absence of zinc defined as 100%.

Dissociation constants for zinc binding, K_i , were calculated from the data using the equation:

$$V_0 - V_i = \{V_m [Zn^{2+}] / ([Zn^{2+}] + K_i)\}$$

where V_0 and V_i are the % rates in the absence or presence of various zinc concentrations, and V_m is the maximum extent of zinc inhibition. From plots of $V_0 - V_i$ vs $[Zn^{2+}]$, values for K_i and for standard deviations were obtained by non-linear curve fitting using Sigmaplot.

Stopped flow measurements were made with a rapid mixing chamber attached to a Thermospectronic Aminco-Bowman Spectrofluorimeter. with a deadtime of 1ms using fluorescence detection [excitation at 340nm and emission at 450nm] Data was collected every millisecond for a total of 8 seconds with the steady state rate being reached by 4 seconds. The steady state rate was subtracted from the overall trace and the pre-steady state phase was fit to:

$$\text{Fluorescence} = A(1 - e^{-k_1 t})$$

allowing the rate constant for the pre-steady state phase, k_1 , and the amplitude of the burst phase, A , to be calculated

Fluorescence measurements were made using an Thermospectronic Aminco-Bowman Spectrofluorimeter. Reduced cofactor binding was studied using fluorescence titrations of

fixed concentrations of enzyme [0.88mg/mL] with reduced cofactor over a range up to 22 μ M, in 0.1 M Phosphate buffer at the indicated pH values. Titrations, using an excitation wavelength of 340 nm and an emission wavelength of 450nm, were performed in the presence of various combinations of 100 μ M zinc acetate and 20 mM Glutamate as well as in the absence of other coligands. Reference titrations were performed in the absence of enzyme and the incremental fluorescence, ΔF at each NADH concentration calculated where:

$$\Delta F = \text{Fluorescence in the Presence of Enzyme} - \text{Fluorescence in the Absence of Enzyme}$$

The dissociation constant for NAD(P)H binding in the appropriate complex was determined by fitting the data to the equation:

$$1/\Delta F = 1/\Delta F_{\max} + \{K_d/\Delta F_{\max}\} \times 1/[NADH]$$

No attempt was made to estimate the stoichiometry of ligand binding since the experiments were conducted at near stoichiometric amounts of enzyme and cofactor and were designed to investigate the effects of zinc on cofactor affinity.

Differential Scanning Calorimetry

Calorimetric curves were obtained using a Microcal differential scanning calorimeter. Glutamate dehydrogenase was dialyzed a minimum of 2 times for 12 hours using a 500 fold excess of 0.1 M phosphate buffer, pH 7.0, containing the appropriate ligand. Samples were exhaustively degassed and then injected into the calorimetric cell. A baseline scan was completed with 0.1 M Phosphate buffer, pH 7.0 (with ligand as appropriate) in both reference and sample cells. For the sample run, glutamate dehydrogenase (2 mg/ml) was used in sample cell, with 3 atm of pressure and a temperature range of 25°C – 85°C. Data were analyzed by using a sigmoidal curve through CPCalc software.

Limited Proteolysis

To perform limited proteolysis, glutamate dehydrogenase was incubated at a concentration of 2 mg/mL (0.1M Phosphate Buffer, pH 8.0) with immobilized trypsin. Preliminary experiments established a suitable ration of GDH to protease to give limited proteolysis over a one-hour time course. The digestion was “limited” by removing, at time = 0, 5minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes and 60 minutes a sample from the digestion mix and centrifuging for 1 minute to remove the immobilized protease. Upon completion of limited proteolysis, identification of cleavage sites, through the use of MALDI-TOF, revealed molecular level detail in terms of exposed peptide bonds for the degradation of GDH with no ligands present or in the presence of zinc. Control experiments with azo case in showed zinc at the concentrations used did not effect the immobilized protease. Low molecular weight masses were calculated using ACTH (2464.199 daltons) as an internal calibrant. High molecular weight fragments were characterized using BSA (66,429.09) as an external calibrant). For the low molecular mass fragments identified, quantitation was achieved using peak intensities relative to that of ACTH as either the internal or external calibrant. For the high molecular mass fragment relative quantitation was achieved using the ratio of the height of the emerging peak to that of the undigested glutamate dehydrogenase. For MALDI-TOF calibration purposes, bovine serum albumin (BSA) was used as a standard and was diluted from 2 mg/mL to 0.5mg/mL using 6 M guanidine hydrochloride.

The cleavage sites were analyzed using Protein Prospector; a program made available by University of California, San Francisco. The program determines the sequence of the

cleavages, by finding all theoretical sites, and determining the masses of potential fragments. By comparing the two results, the most probable location of cleavage can be determined.

Structure Determination

Crystallization of GDH was performed using the hanging-drop vapor diffusion method at room temperature. Crystallization drops were formed using a 1:1 mix of protein and reservoir solutions. The reservoir solution contained 0.1 M sodium phosphate (pH 7.0), 0.15–0.2 M sodium chloride, and 11–13% (w/v) polyethylene glycol 8000. Protein stock solution contained 4 mg/ml GDH, 2 mM NADPH, 2 mM GTP, and 20 mM sodium glutamate.

All complex crystals were transferred stepwise into synthetic cryoprotectant mother liquor solutions saturated with either zinc acetate ($\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$) or europium (III) chloride (EuCl_3) and progressively higher concentrations of glycerol (3–20%). The synthetic solutions consisted of 8% polyethylene glycol 8,000, 0.15 M NaCl, 5% methylpentandiol, 0.1 M triethanolamine-HCl (pH 7.0), 50 mM monosodium glutamate, 2mM GTP, and 2 mM NADPH. X-ray data were collected using an Oxford Cryosystem at 100°K N₂ stream and a Proteum R Smart 6000 CCD detector attached to a Bruker-Nonius FR591 rotating anode generator. The diffraction maxima were integrated and scaled using Proteum software package (Bruker AXS, Inc.).

The structure of GDH complexed with the NADPH abortive complex (GDH + GTP + NADPH + glutamate; PDBID 1HYZ; (30)) was used as an initial model for molecular replacement. Phenix (37) was used for refinement and COOT (38) was used for model building. The initial locations and positions of the metals were identified as peaks in difference maps (Fo-Fc) with maximum values greater than 6 σ . For refinement using Phenix, six-fold non-crystallographic (NCS) restraints were applied to four sections of the protein: 10–208, 209–392, 393–444, and 445–489. These segments correspond to the glutamate binding domain, the NAD binding domain, the antenna, and the pivot helix, respectively. These restraints greatly improved the geometry of the model and yielded superior results compared to using the entire subunit as a single segment for NCS restraints. Final refinement statistics are shown in Table 6.

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Abbreviations

NADP⁺ or NADPH	Nicotinamide Adenine Dinucleotide Phosphate, oxidized or reduced form
NAD⁺ or NADH	Nicotinamide Adenine Dinucleotide: oxidized or reduced form
ADP	Adenosine Diphosphate
GTP	Guanosine Triphosphate
CoA	Coenzyme A
GDH	Glutamate Dehydrogenase
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry

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Figure 1.

The Effects of Glutamate Concentration on Zinc Inhibition at pH 7.0. Experiments were conducted in 0.1M Phosphate buffer at pH 7.0 with 0.5mM NADP⁺ as cofactor and the indicated glutamate concentrations, ranging from 0.25mM (closed circles) to 50mM (open diamonds)

Figure 2. Effects of Zinc on the Oxidative Deamination of NorValine by Glutamate Dehydrogenase. Zinc acetate concentrations were varied up to 120 μM , at pH 8.0 in the presence of 200mM NorValine (closed circles) or 20mM Glutamate (open circles), and 0.5mM NADP⁺. Other conditions as in Figure 1.

Figure 3.

Stopped Flow Kinetics of Glutamate Dehydrogenase: the Effects of Zinc. The pre-steady state phase was obtained by subtracting fluorescence intensity of the steady state phase (4–8 seconds) from that of the pre-steady state phase (0–4 seconds) to give Δ Fluorescence for the pre-steady state phase. Fluorescence, excitation at 340nm was monitored at 450nm in the presence or absence of zinc. Other conditions: 9 μ M Enzyme, 0.1M Phosphate Buffer, pH 7.0, 0.5mM NADP⁺, 20mM Glutamate.

Figure 4. Fluorescence Titrations of Glutamate Dehydrogenase with NADPH: The Effects of Zinc Saturation Curves for NADPH binding in the absence (open circles) or presence (closed circles) of zinc acetate were obtained from titrations in the presence and absence of protein to give Δ Fluorescence
Other conditions: Fluorescence excitation at 340nm, emission at 450nm, 0.1M Phosphate Buffer, pH 8.0, 9 μM Active Sites.

Figure 5. Cartoon diagram of bGDH subunit (gray cartoon) from bGDH hexamer (inset, one subunit removed for clarity) shows sites of trypsin cleavage. Label color indicates residue environment: Cyan, dimer interface; red, active site; none, solvent exposed. R35 is near the trimer interface but may also be accessed by solvent.

Figure 6.

Overview of the locations of the bound Zn^{2+} and Eu^{3+} atoms. This stereo ribbon diagram is colored in a similar manner as figure 6 with the exception that the bound Zn^{2+} and Eu^{3+} atoms are represented by cyan and orange spheres, respectively. Zinc binds as a complex with GTP and near the 2-fold axes in the hexamer. Europium binds inside the base of the antenna.

Figure 7.

Binding environments of the Zn²⁺ atoms near the GTP binding site (A and C) and near the two-fold axes (B and D). A) In this stereo figure, the ribbon diagrams are colored in the same manner as figures 6 and 7 and the stick figures of the contact residues are colored according to atom type. The bound zinc atoms are represented by cyan spheres. The black mesh represents the 2Fo-Fc map contoured at 1.2σ. The mauve mesh around the zinc atom is the omit (minus the zinc atom), Fo-Fc electron density with a cutoff of 5σ. B) The color representation is the same as in (A). The only difference is that the mauve omit electron density is contoured at 4σ in this figure. C,D) These figure show details of the binding environments for these two zinc atoms.

Figure 8.

Binding environment of the Eu^{3+} ion. A) This stereo image shows the quality of the electron density of the antenna region. The black mesh shows the 2Fo-Fc electron density contoured at 1.2 σ . The orange sphere is the bound europium. The mauve mesh around the europium is the omit (minus the europium atom) Fo-Fc electron density contoured at a 5 σ cutoff. B) Details of the binding environment of the bound europium. This figure shows the 2Fo-Fc electron density of the ligating amino acids (E402) and the bound metal at a contour of 1.2 σ . C) For comparison, this figure shows the same region in the zinc•GDH complex contoured in the same manner. Note that the conformations of the E402 sidechains move up to bind europium compared to the zinc complex.

Table 1

Effects of Glutamate Concentration on Zinc “Affinity”

[Glutamate], mM	K_D^{zinc}
0.25	223 +/- 58 μ M
0.5	187 +/- 22
1.0	85 +/- 22
2.0	62 +/- 3.7
5.0	24 +/- 2
10.0	25 +/- 1.5
20	24 +/- 2.5
50	19 +/- 2

Table 2

Summary of parameters obtained from stopped flow kinetics experiments. Experiments were performed using a stopped flow with fluorescence detection [excitation at 340nm, emission at 450nm] at pH 7.0 with 1mM NAD⁺ as cofactor.

Condition	Steady State Rate $_{340F_{450}}$ /sec	Pre-Steady State Burst Rate $_{340F_{450}}$ /sec	Pre-Steady State Burst Amplitude $_{340F_{450}}$
50mM Glutamate	0.372	0.869	2.929
50mM Glutamate + 100 μ M Zinc	0.187	0.858	1.053
50mM Glutamate + 200 μ M Zinc	0.158	0.972	0.771

Table 3

Summary of The Effects of Zinc on NADPH Binding Data

Conditions	K _D , NADPH
No Additions	6.33+/-0.62μM
+ Zinc	1.04+/-0.15 μM
+20mM Glutamate	2.40+/-0.26 μM
+ Zinc	1.72+/-0.22 μM
+200mM Norvaline	7.35+/-0.65 μM
+ Zinc	9.27+/-1.27 μM

Table 4

Thermal Stability of Glutamate Dehydrogenase, Parameters from Differential Scanning Calorimetry

Conditions	TM	ΔS	ΔH
No Additions	58.3	0.518	171.8
+ Zinc	59.2	0.388	128.9
+ 20mM Glutamate	59.4	0.484	161.0
+ Zinc	59.3	0.381	126.4
+200mM Norvaline	67.8	0.554	188.7
+ Zinc	68.1	0.528	179.9

Table 5

Effects of Zinc on the Rate of Limited Proteolysis of Glutamate Dehydrogenase

Fragment	Time = 0	5 Mins	10 Mins	15 Mins
34,645 (residues 144–459) Peak Height relative to Native				
No Zinc	0	0.049	0.079	0.15
Plus Zinc	0	0.17	0.18	0.86
3446 (residues 114–146) Peak Height relative to ACTH standard				
No Zinc	0	0.05	0.19	0.99
Plus Zinc	0	0.52	0.54	0.75
4089 (residues 1–35) Peak Height relative to ACTH standard				
No Zinc	0	0	0.042	0.08
Plus Zinc	0	0	0.16	0.68

Table 6

Data and refinement statistics for the Eu^{3+} and Zn^{2+} bound structures. The numbers associated with the Bvalues of the bound metals denote the binding site. The first site for the zinc is near the hexamer 2-fold axes, the second is at the GTP site.

	Zinc	Eur
PDB Accession	3MVQ	3MVO
Data Statistics		
Wavelength (Å)	1.5418	1.5418
Space Group	P2 ₁	P2 ₁
Unit cell a, b, c (Å)	124,102, 165.6	124, 102, 165.6
β (°)	101.6	101.6
Resolution range (Å)	50–3.0 (3.14–3.0)	50–3.3 (3.45–3.3)
Unique reflections	285,421 (76,007)	138,375 (55,787)
R(I)sym (%)	10.6 (23.0)	16.3 (31.1)
Completeness (%)	96.4(62.0)	96.3 (65.0)
Redundancy	3.7(1.7)	2.5 (1.1)
I/s(I)	6.1(1.95)	4.2 (1.5)
Refinement Statistics		
Rwork (%)	21.8(27.5)	25.8 (35.6)
Rfree (%)	25.6(33.0)	30.7 (39.5)
# Protein atoms	23,370	23,268
# Ligand atoms	480	348
# Metal atoms	12	2
Average B Values (Å ²)		
Protein atoms	37.8	39.7
Ligand atoms	32.2	33.2
Metal atoms	64.8(1), 33.2(2)	65.5
RMS Deviations in Geometry		
Bond length (Å)	0.01	0.009
Bond angles (°)	1.27	1.23
Ramachandran Analysis (%)		
Most favored	88	78
Additionally allowed	11	20
Generously allowed	0.8	1.8
Disallowed	0.	0.0