

GLUTAMATE DEHYDROGENASE:

**An Investigation of Conformational Stability, Enzyme Flexibility,
and Allosteric Regulation**

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ABSTRACT

Bovine Glutamate Dehydrogenase (GDH) is subject to extensive allosteric regulation and requires substrate induced subunit interactions for maximum catalytic activity. We performed guanidine hydrochloride unfolding, heat inactivation, and differential scanning calorimetry (DSC) experiments to examine the effect of ligands on GDH conformational flexibility. The results of these experiments were correlated with previous studies on the effects of ligand induced conformational changes, cofactor binding, and overall activity. Without ligands, GDH can be thought of as poised to allow subunit interactions. Substrates which do not trigger or block cooperative interactions lead to enhanced stability, while ligands permitting efficient catalysis or enhancing catalysis were shown to increase flexibility. Particularly, studies show ADP and norvaline greatly stabilize the enzyme: ADP has previously been demonstrated to block subunit communication while norvaline is known to prevent subunit communication. Furthermore, NADPH, which only binds to the active site, makes GDH less stable than NAD(H), which has been shown to first bind the ADP binding site and then to the active site. Strength of the subunit interfaces of the GDH hexamer was investigated in GuHCl experiments; the study revealed that norvaline greatly stabilizes all interfaces while other ligands have moderate effects on either the trimer-trimer or the monomer-monomer subunit interactions. From these experiments a pattern emerges of ligands stabilizing GDH conformation, acting to block subunit interactions, and inhibiting the overall activity, or ligands increasing conformational flexibility, promoting subunit interactions, and allowing part of the binding energy to promote catalysis through subunit interactions.

INTRODUCTION

Since the introduction of allosteric regulation concepts and landmark work by Monod, Wyman & Changeux (1965) and, subsequently, Koshland et al (1966) and Dalziel & Engel (1968), ideas of allosteric regulation have been dominated by models of different protein conformations, either linked by a pre-existent equilibrium (Monod et al. 1965) or in different ligand-induced conformations. The images of the “R” and “T” states of hemoglobin, with fixed conformations of active and inactive protein were followed by demonstrations of induced conformational changes in proteins and the thermodynamic arguments of Weber et al (1972) that proteins must change conformation when a ligand binds. In the early 1970s the first direct demonstrations that proteins had a dynamic structure (Lakowicz & Weber, 1973) and that ligand-induced changes affected the active sites of other subunits in an oligomer (Bell & Dalziel, 1973) were established. Many groups documented that a variety of enzymes, receptors and other functional proteins exhibited allosteric phenomena, including the work of Boyer (1998) and Ma et al (2002) on ATP synthesis. Often aided by crystal structures of different conformational states, these studies solidified the concepts of allosteric proteins having discrete conformations: pre-existent, ligand-induced or resulting from post-translational modifications. Few studies, other than work on ATP synthesis, addressed how conformational changes were transduced in these proteins, and little is known of the detailed mechanisms of conformational change in allosteric proteins. Even in allosteric proteins as well studied as aspartate transcarbamoylase, the flexibility of the catalytic trimer is only suggested as a means of regulation (Beernink, et al 1999; Endrizzi et al 2000).

In recent years a resurgence of interest in the dynamic properties of proteins has occurred, particularly concerning those proteins associated with catalytic events that might proceed via quantum tunneling mechanisms such as hydride or proton transfer. Work by

Klinman et al (Liang et al 2004; Ceccarelli et al 2004; Knapp & Klinman, 2002; Kohen et al 1999) has focused on the experimental detection of tunneling using isotope effects and, in combination with computational approaches by Gao (Alhambra et al 2000), have provided reasonable agreement between experimental and computational analysis of the chemical mechanism for alcohol dehydrogenase. As emphasized in a recent review by Kraut, Carroll, & Herschlag (2003), one of the future challenges, in terms of understanding enzyme mechanisms, involves understanding the relationship of the dynamic properties of protein catalysis. Beyond that lies an understanding of how dynamic properties of allosteric proteins are involved with the complex events associated with homo- and heterotropic regulation, (V-type, involving rate limiting steps in the reaction, or K-type, where regulation is at the level of ligand saturation).

Bovine liver glutamate dehydrogenase (E.C. 1.4.1.3, GDH) catalyzes the oxidative deamination of L-glutamate and various monocarboxylic acid substrates. 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 (Kaplan, Ciotti, & Stolzenbach 1956), although NAD(H) may have two binding sites on each subunit. The enzyme is a hexamer of chemically identical polypeptide chains (Moon & Smith 1973; Appella & Tomkins 1966) and exhibits negative cooperativity resulting from coenzyme induced conformational changes (Dalziel & Engel 1968; Bell & Dalziel 1973; Alex & Bell 1980). More recent work showed that coenzyme induced conformational change requires a dicarboxylic acid substrate or analog with a 2-position substituent (Bell et al 1985). With alternative amino acid substrates such as norvaline, the manifestations of cooperative interactions between the subunits of the enzyme are absent (Dalziel & Engel 1968; LiMuti & Bell 1983). Since the entire hexamer is required to give optimal activity of the enzyme (Bell & Bell 1984) with glutamate as substrate, it is likely that the cooperative interactions between subunits in the hexamer are required for maximal activity. This

is consistent with GDH activity data using alternative amino acid substrates where the overall rate of oxidative deamination is much lower. 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 activity (Frieden 1965; Bailey et al 1982). The binding sites of GDH can be grouped into two categories, an active and a regulatory site. The active site is where the main reaction takes place and must have both a substrate and a cofactor present for catalysis. The regulatory site binds many small molecules, particularly ADP and GDP. NAD(H) is unique in that it can bind to both the active site, where it is utilized as a cofactor, and to the ADP regulatory site.

Although the crystal structure of both bovine and human forms of the enzyme are now available (Banerjee et al 2003; Smith et al 2002; Fang et al 2002; MacMullen et al 2001; Smith et al 2001; Peterson & Smith 1999) 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 a consistent picture of how ligand induced effects alter activity of this complex regulatory enzyme.

In the current study, we have examined the overall conformational stability of GDH using the techniques of heat inactivation, differential scanning calorimetry, and guanidine hydrochloride unfolding. The effects on hexamer stability for a wide variety of ligand structures (both substrates and allosteric regulators) are correlated with their ability to activate or to inhibit the enzyme. In this study, we equate conformational stability with a loss of protein flexibility, in that a dynamic enzyme which has been stabilized no longer has as large a range of flexible movement. Heat inactivation and differential scanning calorimetry provide insight into the global flexibility of a molecule when associated with ligands, while guanidine unfolding supplies a picture of how subunit interfaces are affected by various ligands. Through these techniques,

along with known catalytic and crystal structure data, a picture emerges where conformational flexibility is intimately tied to the ability of the glutamate dehydrogenase hexamer to exhibit subunit interactions necessary for efficient catalysis.

RESULTS

Heat inactivation of glutamate dehydrogenase with ligands

In order to investigate the global stability of glutamate dehydrogenase, we have compared the rates of inactivation of glutamate dehydrogenase (GDH) when incubated at 50°C in the absence of ligands and in the presence of various substrates, substrate analogs, cofactors and allosteric regulators. Figure 1 shows the heat inactivation of the native enzyme where a rate constant of inactivation of 0.216min^{-1} was obtained. The presence of either dicarboxylic acid substrate, L-glutamate or α -ketoglutarate had a negligible effect on the rate (Table 1). When the monocarboxylic acid alternative amino acid substrate norvaline is present, the rate of inactivation slows significantly (Figure 1) and a rate constant of 0.0148min^{-1} is obtained. Two substrate analogs, glutarate and 3,3dimethylglutarate each slowed the rate of inactivation but not as dramatically as norvaline (Table 1). Of the cofactors used, NADPH dramatically increased the rate of inactivation, resulting in a rate constant of 1.29min^{-1} (Figure 1). NADH and NADP^+ both showed smaller increases in the rate of inactivation while NAD^+ showed a slight slowing of the rate of inactivation (Table 1). While the allosteric regulator GTP had little effect, the protein is highly stabilized by 1mM ADP (rate constant of .006). The effects of these ligands on k_{inact} are shown graphically in Figure 2.

Differential scanning calorimetry (DSC) of glutamate dehydrogenase with ligands

The results obtained using differential scanning calorimetry built upon the global stability data from heat inactivation experiments, by providing a highly replicable means of determining the temperature of protein denaturation. Figure 3 shows the high level of reproducibility of DSC data: three independent runs over a two week period gave virtually identical transition temperature (T_t) values for the enzyme with ADP. Although this is an irreversible transition, the

data is very consistent and precise. The native glutamate dehydrogenase gave a T_t of 58.2°C. Similar to the heat inactivation study, NADPH, ADP, and norvaline (Figure 4) all dramatically affected T_t . ADP (T_t of 66.5 °C) and norvaline (T_t of 67.8 °C) again greatly stabilized the overall GDH hexamer, while NADPH (T_t of 51.9 °C) made the enzyme much less stable. While other ligands had smaller effects, a pattern (Table 2) consistent with heat inactivation rates was observed.

Guanidine hydrochloride unfolding of glutamate dehydrogenase with ligands

Previous work using dynamic light scattering (Tashiro et al 1982; Inoue et al 1984) has demonstrated that increasing concentrations of guanidine hydrochloride (GuHCl) result first in a hexamer-trimer transition, followed by a trimer-monomer transition and rapid, irreversible denaturation of the monomer. Figure 5 shows the representative Trp fluorescence emission spectrum of GDH incubated in various concentrations of GuHCl. When the fluorescence maximum at each GuHCl concentration is plotted against the GuHCl concentration for the native enzyme in the absence of any ligand (Figure 6) two transitions are seen. The first transition at 1M GuHCl correlates to the break down of the hexamer into two trimers, and the second transition at 1.75M is indicative of the breakdown of trimer into monomers (Tashiro et al 1982). When glutamate dehydrogenase and norvaline are incubated with increasing GuHCl concentrations, two effects on this profile are observed: there is no distinct first transition and the second transition is shifted to a higher GuHCl concentration (3.00M). Similar experiments with glutamate show a shift in the first transition to 1.75M GuHCl, while the second transition shifts to 2.25M GuHCl. The effects of other ligands used in this study are summarized in Table 3.

Circular dichroism of glutamate dehydrogenase with glutamate and norvaline

Circular dichroism data (Figure 7) was collected but showed no significant changes when the enzyme was associated with either glutamate or norvaline. This indicates there are no major changes in the α -helical structure whether there is association with ligands or the enzyme is in its apo form. This data is consistent with the idea that there is no global change in monomer subunit structure which could be detected in crystallography; instead the major effects relate to intersubunit communication or allostery.

DISCUSSION

Glutamate dehydrogenase shows complex regulation by a variety of allosteric regulators. This study indicates that the most influential ligands and regulators of the enzyme also impact the conformational flexibility of the protein. Furthermore these effects correlate to the ability of a given ligand to support or impact negative cooperativity exhibited by the enzyme. The ligands used in the present study can be grouped into the following categories, based upon structure, previous cooperativity data, and binding site:

- 1) Substrates and substrate analogs which promote the ability to exhibit negative homotropic interactions within GDH: glutamate, α -ketoglutarate and L- α -hydroxyglutarate. These ligands are structurally similar in that they all contain a substituent on the secondary carbon.
- 2) Substrate analogs that do not support negative cooperativity: glutarate and 3,3-dimethylglutarate. These ligands are similar to the other substrate analogs except that they do not have a second-carbon substituent.
- 3) Norvaline, an alternative amino acid substrate, which, when used as a substrate, shows no signs of negative cooperativity. Structurally it has a 2-position substituent, but is lacking a hydroxide group on one end of the small molecule.
- 4) Cofactors of the enzyme: NAD⁺, NADP⁺, NADH and NADPH, of which only NAD(H) appears to bind at both the active site and the ADP regulatory site
- 5) Allosteric regulators: ADP, which previous studies have shown blocks subunit interactions necessary for negative cooperativity, and GTP, which does not appear to affect subunit interactions in the hexamer

This study utilized several techniques to look at intersubunit flexibility, which we define as the ability of a dynamic enzyme to have available a large range of flexible movement, not being

confined to a unique conformation, and, thus, being less stable. Heat inactivation and differential scanning calorimetry gave insight into the overall flexibility of GDH while guanidine hydrochloride unfolding examined the flexibility in associations between subunits.

Of the substrates and substrate analogs used in this study, there is a clear division between those that support full activity and subunit interactions. Specifically, glutamate, α -ketoglutarate, and L-2-hydroxyglutarate have little impact on the overall conformational flexibility of the protein, while glutarate and 3,3-dimethylglutarate, which do not support subunit interactions, have a slight, but significant stabilizing effect as judged by the heat inactivation studies. Furthermore, guanidine hydrochloride unfolding showed increased stability for the hexamer to trimer transition with α -ketoglutarate, glutamate, and L- α -hydroxyglutarate, but not glutarate or 3,3-dimethylglutarate. The incongruity in these findings is attributed to the difference in interactions between subunits and stability of the global enzyme. It would be possible for the binding of a ligand to stabilize an individual subunit which then negatively affects the ability of that stabilized subunit to have interactions with other subunits. Interestingly, this interpretation of flexibility follows an already existing ligand classification based upon a second-position substituent and negative cooperativity. The substrates, glutamate and α -ketoglutarate, along with the substrate analog which also has a 2-position hydrogen bonding substituent—L- α -hydroxyglutarate—have little effect on the global conformational flexibility of the protein except during the hexamer-trimer transition, where it appears they prime the interface for communication. Substrate analogs glutarate and 3,3-dimethylglutarate which do not have a 2-position substituent and do not support negative cooperativity vary from the actual substrates in both overall flexibility and the ability to stabilize the hexamer-trimer interface.

The only substrate/analog which does not rigorously follow the trend designated by a 2-position substituent is norvaline, which also produced the most dramatic effect on protein

stability in each of the experimental approaches. In the heat inactivation, differential scanning calorimetry, and guanidine hydrochloride studies presented here, norvaline induced a distinct increase in the stability of GDH. This data indicates that protein bound to norvaline has fewer options for flexible conformations than does the native protein. DSC results showed that the enzyme in the presence of norvaline had nearly a ten-degree temperature increase before denaturation occurred. With guanidine hydrochloride studies the enzyme completely lost the hexamer to trimer dissociation (first transition) and appeared to dissociate immediately to a monomer at an increased concentration of 3M GuHCl. These results all demonstrate the dramatic increase in overall stability that norvaline causes in GDH.

Our findings indicate ADP, like norvaline, increased the stability of the GDH hexamer to trimer breakdown (the first transition in guanidine hydrochloride studies) and increased the overall enzyme transition temperature as determined by DSC and heat inactivation. These molecules are also similar in that, norvaline does not support negative cooperativity and ADP has been shown to disrupt negative cooperativity. This fits with information from crystallization of the bovine GDH enzyme with ADP (Banerjee et al, 2003) that suggested this molecule activates the GDH hexamer by decreasing energy associated with the catalytic cleft in its open conformation. Much like ADP, the other major allosteric regulator of the enzyme, GTP, binds to an independent regulatory site on the GDH hexamer. However, unlike ADP, GTP has been identified as a K type regulator with no influence on the enzyme's potential negative cooperativity. In these studies, GTP has no significant effect on the stability of the protein. Crystal studies indicate that two GTP molecules bind near the hinge region of the active site and only recognize the closed conformation of GDH (Peterson & Smith, 1999; Smith et al, 2002). Thus, it has been postulated that GTP only binds to the GDH subunit after substrate and cofactor are bound within the active site. If this is accurate, GTP may not be binding or may only be

binding weakly in the GDH stability studies presented here. Otherwise, these results suggest a correlation between the conformational flexibility of glutamate dehydrogenase and negative cooperativity as well as a relationship between flexibility and whether the enzyme's catalytic cleft is in the open or closed conformation.

Mammalian glutamate dehydrogenase is unique in its ability to utilize both NAD(H) and NADP(H) with near equal ability. Nevertheless, previous studies have shown that there are differences between the two cofactors, particularly in their binding. Crystal studies indicated that NAD(H) alone binds to both the ADP site and the active site, particularly at high concentrations, while NADP(H) only binds to the enzyme's active site. Furthermore, crystallization and limited proteolysis data of the enzyme with both active site ligands indicate that substrate and coenzyme binding drives the catalytic cleft into a rigid closed conformation (Peterson & Smith, 1999; Smith et al, 2002); however, the crystal structure has not been determined with only one of these ligands. Thus it is not yet discernable through crystal data whether this conformation is an effect of the ligands together or of the substrate and coenzyme individually. In our experiments, NADPH showed a large increase in flexibility through the differential scanning calorimetry and the heat inactivation studies. NADP and NADH also showed small decreases in stability through these two techniques, but the effect of NADPH was more prominent. The distinction between NADPH and NADP⁺ is attributed to tighter binding of NADPH to the active site. The second binding site of NAD was investigated by changing the concentration of the molecule. Differential scanning calorimetry data revealed no change from native protein at a low concentration of the cofactor, whereas a slight increase in stability was observed for NAD at a higher concentration. This increase in stability models the effects seen for ADP. A higher concentration of NAD also showed a slightly more stable protein in the heat inactivation experiments. None of the cofactors seemed to have an influence on hexamer-trimer transition

detected by the guanidine hydrochloride experiments. The dramatically increased conformational flexibility induced by NADPH in our studies suggests an important functional role for this cofactor which may impact how allosteric regulators help differentiate between NADH and NADPH utilization.

When the results of the various experiments described here are taken together a clear picture relating conformational stability to enzyme function emerges. Substrates necessary for full activity of the protein enhance the overall conformational flexibility of the protein, whereas substrates that do not permit subunit interactions significantly stabilize the conformation. Likewise the allosteric regulator ADP, which blocks subunit interactions, stabilizes the protein to such an extent that presumably the substrate induced conformational transitions necessary for full activity can no longer take place. GTP, a K type allosteric regulator which does not affect subunit communication, has no impact on flexibility. The protein can be thought of as having inherently sufficient conformational flexibility to allow for cofactor induced subunit interactions. Ligands that stabilize the protein block such interactions and effectively slow the overall reaction.

When discussing the overall conclusions that can be drawn from the studies reported here, it is important to note that the techniques in this study investigate different aspects of protein flexibility and it is only when they are used in conjunction with each other that a thorough picture of ligand induced changes in conformational stability can be seen. These studies suggest that in glutamate dehydrogenase there is a direct correlation between conformational flexibility and activity. Moreover, allosteric interactions require sufficient conformational flexibility of GDH so that allosteric regulation, either homotropic or heterotropic, by ADP can be mediated by alterations in conformational flexibility rather than structural transitions between relatively fixed conformational states.

MATERIALS AND METHODS

Preparation of glutamate dehydrogenase

Bovine liver GDH was obtained as a glycerol solution from Sigma Chemical Co. Enzyme solutions were prepared using 0.1 M phosphate buffer at pH 7.0, containing 10 μ M EDTA. All solutions were made up with distilled, deionized water from a 4 bowl Milli Q system. Enzyme concentrations were determined spectrophotometrically using the absorbance at 280 nm, with an extinction coefficient of 0.93 for a 1 mg/ml solution. Coenzyme concentrations were also determined spectrophotometrically using absorbance measurements at 260 nm and a millimolar extinction for NAD(P)⁺ at 260 nm of 15.9 $\text{cm}^{-1}\text{mM}^{-1}$ or at 340 nm using an extinction coefficient for NAD(P)H of 6.22 $\text{cm}^{-1}\text{mM}^{-1}$. All ligand concentrations were selected based upon binding efficiencies in order to have the protein saturated with that ligand.

Heat Inactivation studies

Measurements were made by pre-incubating buffer to 50°C and at time zero adding glutamate dehydrogenase to obtain a final concentration of 1 mg/ml: this entailed adding no more than 100 μ L of a concentrated stock solution to 3 ml of preheated buffer. An aliquot was immediately withdrawn for an enzyme assay. Further aliquots were withdrawn over a 20 minute period and assayed under standard assay conditions: 0.1 M Phosphate buffer, pH 7.0, 20 mM glutamate and 500 μ M NAD⁺ using a Thermospectronic dual beam UV 500 Spectrophotometer. The resultant data were analyzed according to:

$$\frac{enz_t}{enz_0} = e^{-k_{inact}t}$$

where enz_t is the activity at time t after introduction of the enzyme, and enz_0 is the activity at time equal zero. Replicate determinations of the rate constant for inactivation, k_{inact} , were made and averaged. When ligands were included in the inactivation incubation, they were added to the buffer at the appropriate concentration prior to the pre-incubation. Control experiments showed that inclusion of ligands in the preincubation buffer had no effect on the measured rate when an aliquot of enzyme was removed.

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 at appropriate concentration) 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.

Guanidine hydrochloride unfolding

In the experiments described here we have incubated protein (1 mg/ml) in a given concentration of guanidine hydrochloride for 1 hour before taking a fluorescence emission spectrum, with excitation at 280 nm. Data was recorded using a Thermospectronic Aminco-Bowman Spectrofluorimeter and the appropriate blanks subtracted. If a protein has several domains each contributing to the parameter being followed, a multitransitional denaturation curve will be obtained depending upon the method of following the effects of unfolding,

reflecting the variable stabilities of the different domains. If a ligand is present which stabilizes the overall structure of the protein, the denaturation curve will be shifted to higher concentrations of guanidine hydrochloride, while the opposite will be true of a ligand which destabilizes the protein.

Circular dichroism

The α -helical structures of the protein were investigated with a Jasco J-720 CD spectrometer. Glutamate dehydrogenase at a concentration of 0.2 mg/ml was exhaustively degassed and scanned at wavelengths of 280-180 nm in a 1mm quartz cuvette. Replicate scans were completed at a rate of 20 nm/min and a response time of 4 seconds. The appropriate baselines were subtracted and samples with ligands were compared to the native enzyme.

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Table 1. The effect of ligands on the rate constant of the heat denaturation of glutamate dehydrogenase as determined by heat inactivation studies.

Ligand	Rate (k)	Std Error
Native GDH	0.216	0.061
20mM glutamate	0.185	0.058
20mM α -ketoglutarate	0.187	0.026
20mM L- α -hydroxyglutaric acid	0.190	0.041
200M norvaline	0.014	0.009
20mM glutarate	0.062	0.016
3,3-dimethylglutaric acid	0.075	0.026
2mM NADP	0.382	0.022
0.2mM NADPH	1.29	0.368
2mM NAD	0.129	0.020
0.2mM NADH	0.491	0.133
1mM ADP	0.006	0.007
0.1mM GTP	0.265	0.010

Table 2. The effect of ligands on the transitional temperature of glutamate dehydrogenase as determined by differential scanning calorimetry.

Ligand	Transitional temperature (T_t , °C)	Global protein flexibility
Native GDH	58.3	----
20 mM glutamate	59.4	Slight decrease
20 mM α -ketoglutarate	61.2	Slight decrease
200mM norvaline	67.5	Dramatic decrease
2 mM NADP	57.8	Minimal effect
0.2mM NADPH	50.2	Dramatic increase
0.2 mM NAD	58.3	Minimal effect
2 mM NAD	59.8	Slight decrease
0.2 mM NADH	57.6	Minimal effect
1 mM ADP	66.5	Dramatic decrease
0.1 mM GTP	58.7	Minimal effect

Table 3. The effect of ligands on the unfolding of glutamate dehydrogenase during two transitions, hexamer to trimer and trimer to monomer as identified by a guanidine hydrochloride unfolding curve.

Ligand	Hexamer-> Trimer	Trimer-> Monomer	Interface stability
Native GDH	0.875 M	1.95 M	----
20mM glutamate	1.625 M	2.40 M	Increase, both transitions
20mM α -ketoglutarate	1.625 M	2.10 M	Increase, hexamer to trimer
20mM L- α -hydroxyglutaric acid	1.150 M	2.10 M	Increase, hexamer to trimer
200mM norvaline	----	3.00 M	Dramatic increase, both transitions
20mM glutarate	1.000 M	1.60 M	Minimal effect
20mM 3,3-dimethylglutaric acid	0.675 M	1.75 M	Minimal effect
2mM NADP	0.900 M	1.75 M	Minimal effect
0.2mM NADPH	0.875 M	1.80 M	Minimal effect
2mM NAD	0.675 M	2.00 M	Minimal effect
0.2mM NADH	0.875 M	2.00 M	Minimal effect
1mM ADP	1.500 M	2.00 M	Increase, hexamer to trimer
0.1mM GTP	0.675 M	2.00 M	Minimal effect

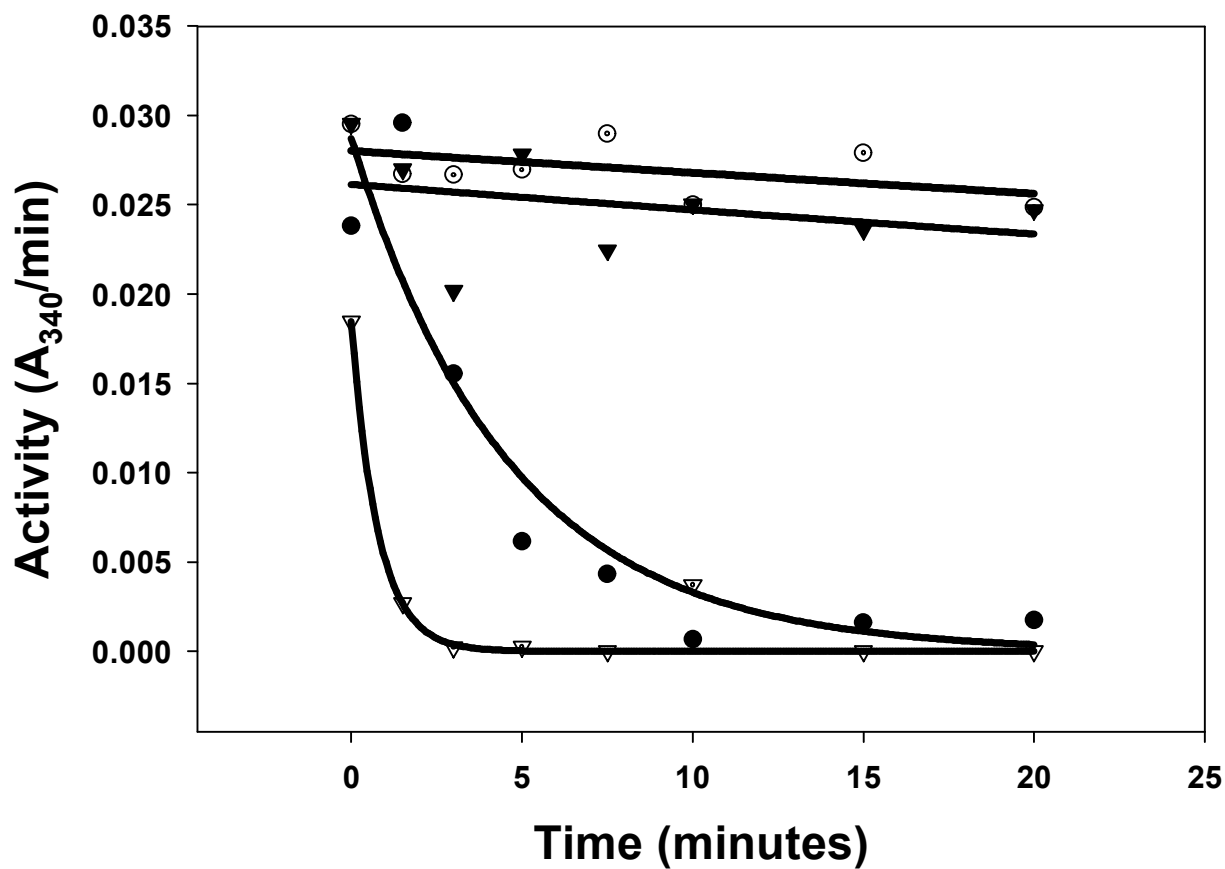


Figure 1. Heat inactivation curves of native glutamate dehydrogenase (black circle) and GDH enzyme associated with norvaline (white circle), ADP (black triangle), and NADPH (white triangle).

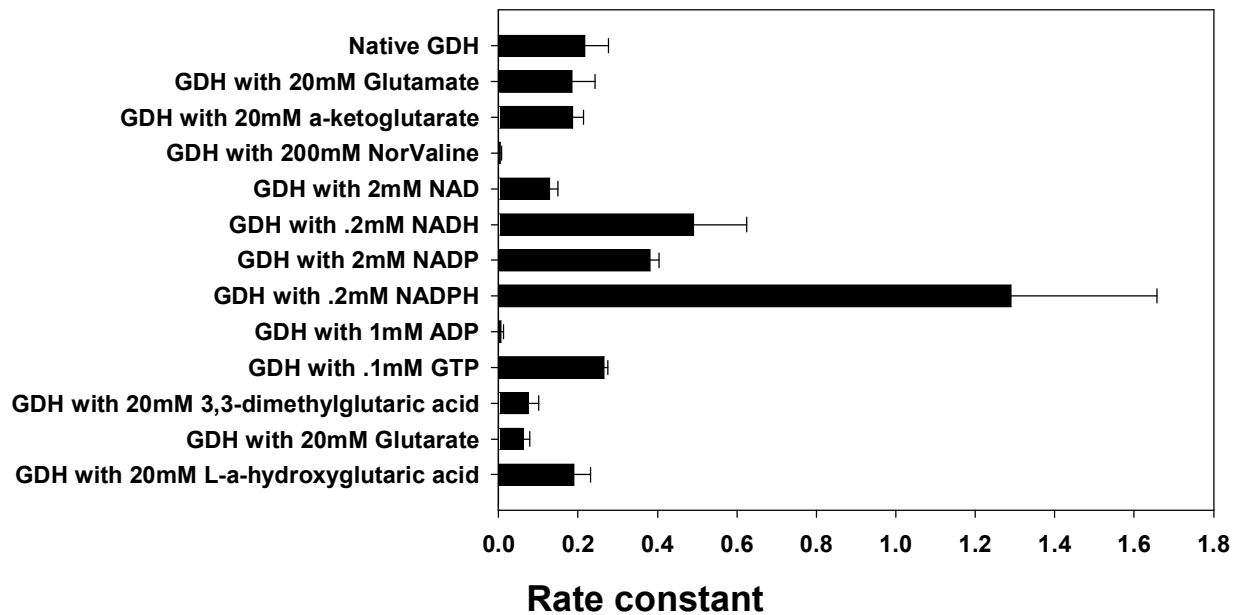


Figure 2. Comparison of rate constants for the heat inactivation of glutamate dehydrogenase associated with different ligands. Rate constants calculated from a 2 parameter exponential decay curve fit to the activity of GDH when exposed to a temperature of 50°C for a period of 20 minutes. Ligands selected included substrates (glutamate, α -ketoglutarate), substrate analogs (norvaline, 3,3-dimethylglutarate, glutarate, L- α -hydroxyglutarate), cofactors (NAD(H), NADP(H)), and known regulators (ADP, GTP). Exact values are reported in Table 1.

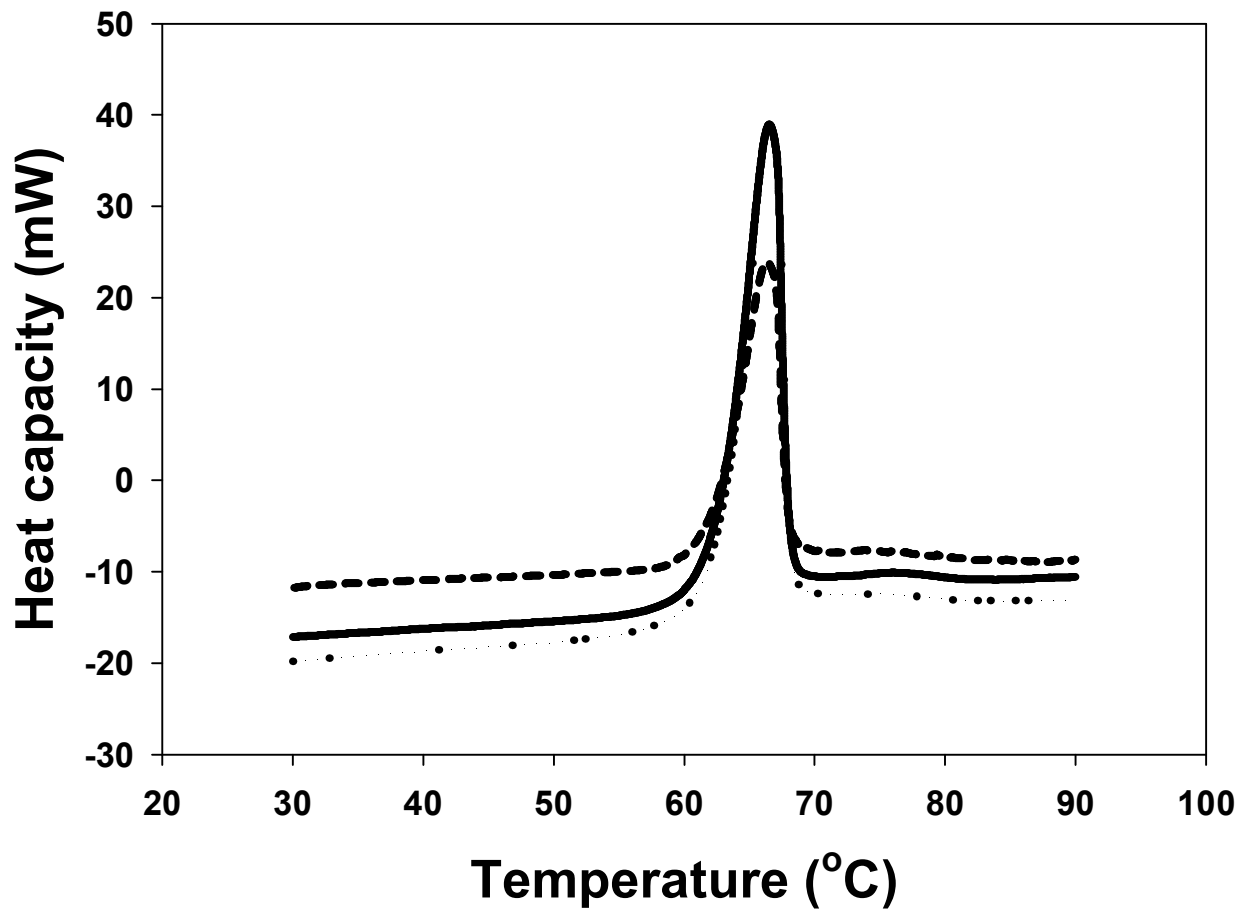


Figure 3. Multiple differential scanning calorimetry thermograms of 2 mg/ml glutamate dehydrogenase with 1mM ADP in 0.1M phosphate buffer, pH 7.0. Separate baselines were subtracted from each thermogram with scan rates of 1°C/min. Trial 1 (dashed) had a reported transition temperature of 66.3°C, trial 2 (dotted) had a transition temperature of 66.5°C, and trial 3 (solid) had a transition temperature of 66.6°C.

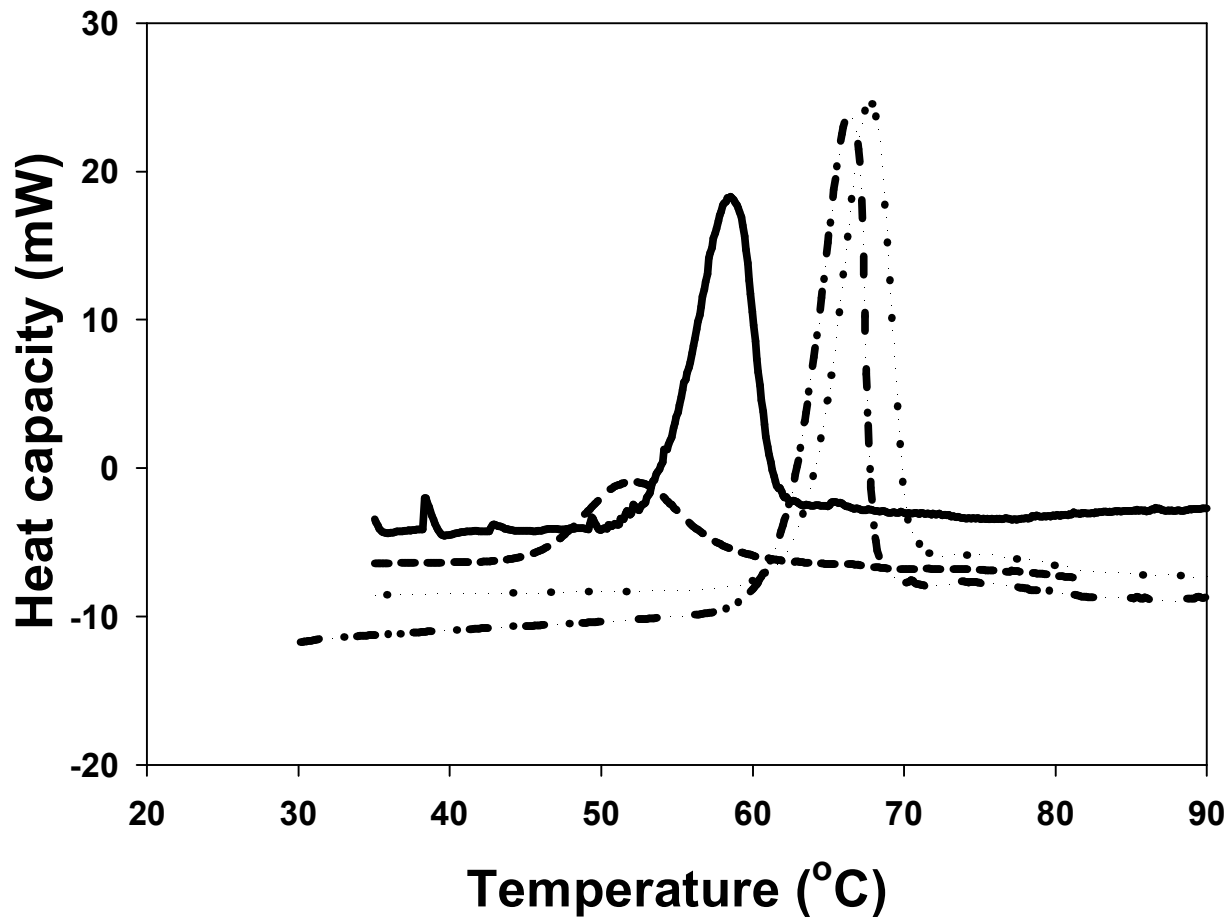


Figure 4. Differential scanning calorimetry thermograms of native glutamate dehydrogenase (solid) and GDH enzyme with ligands. The protein associated with 2mM NADPH (dashed) resulted in a much lower transition temperature, while 1mM ADP (dash-dot-dot), and 200mM norvaline (dotted) both increased the transition temperature of the protein. Protein concentrations for all samples were at 2mg/ml in 0.1M phosphate buffer, pH 7.0 with a scan rate of 1°C/min.

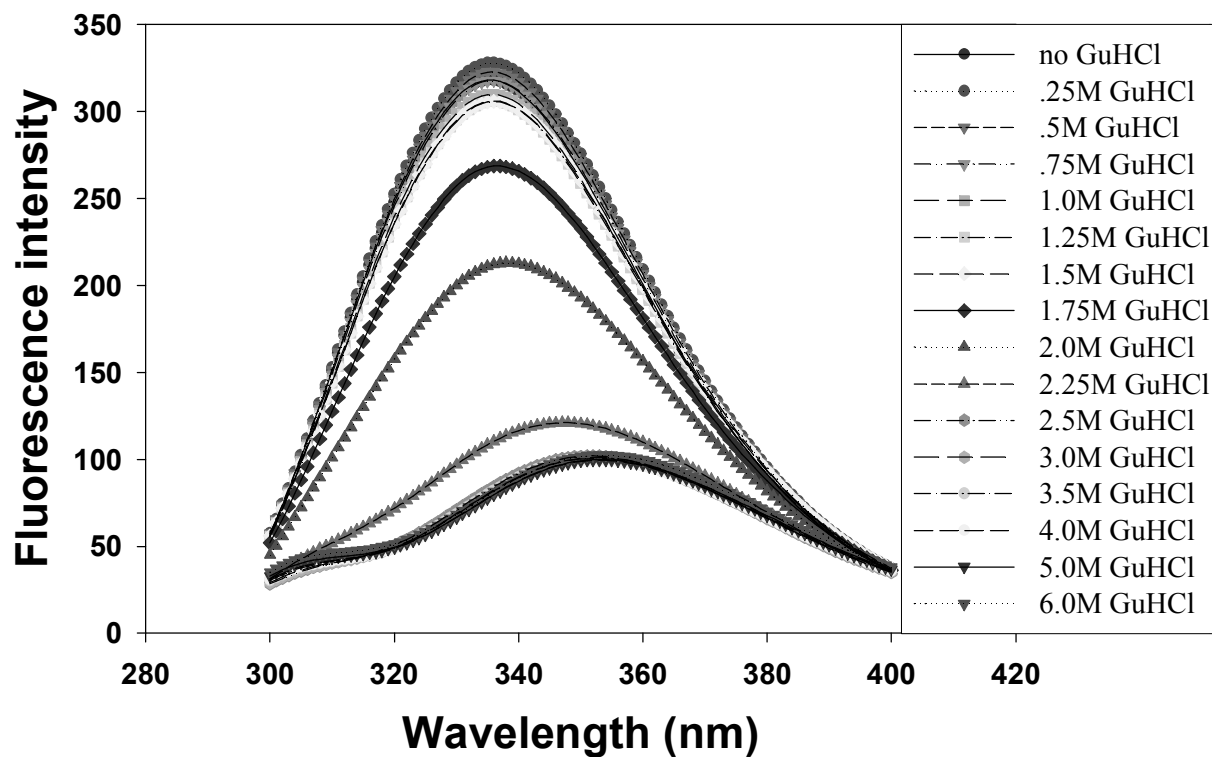


Figure 5. Fluorescence spectra showing guanidine hydrochloride unfolding of native glutamate dehydrogenase. The wavelength at the maximum fluorescence activity indicates whether tryptophan residues have moved, indicating the dissociation of GDH subunits. Protein concentrations for all samples were at 1mg/ml in 0.1M phosphate buffer, pH 7.0.

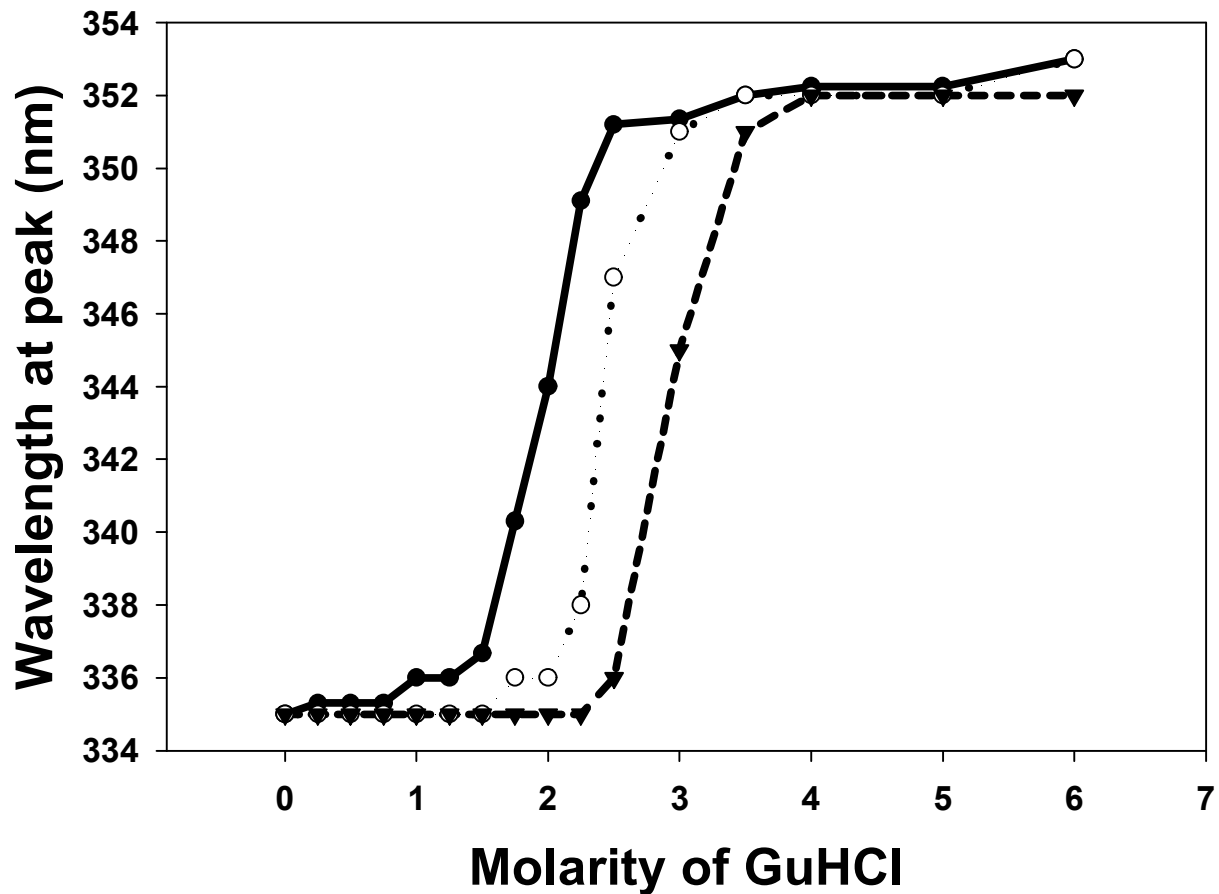


Figure 6. Guanidine hydrochloride unfolding curves of native glutamate dehydrogenase (black circle) and GDH enzyme associated with 20mM glutamate (white circle) and 200mM norvaline (black triangle). Two transitions can be seen in the native and GDH with glutamate curves, but only the second transition appears with norvaline. Previously, the first transition has been correlated with the dissociation of the GDH hexamer into two trimers while the second transition is the dissociation of the trimer into three monomers and immediate unfolding (which is irreversible).

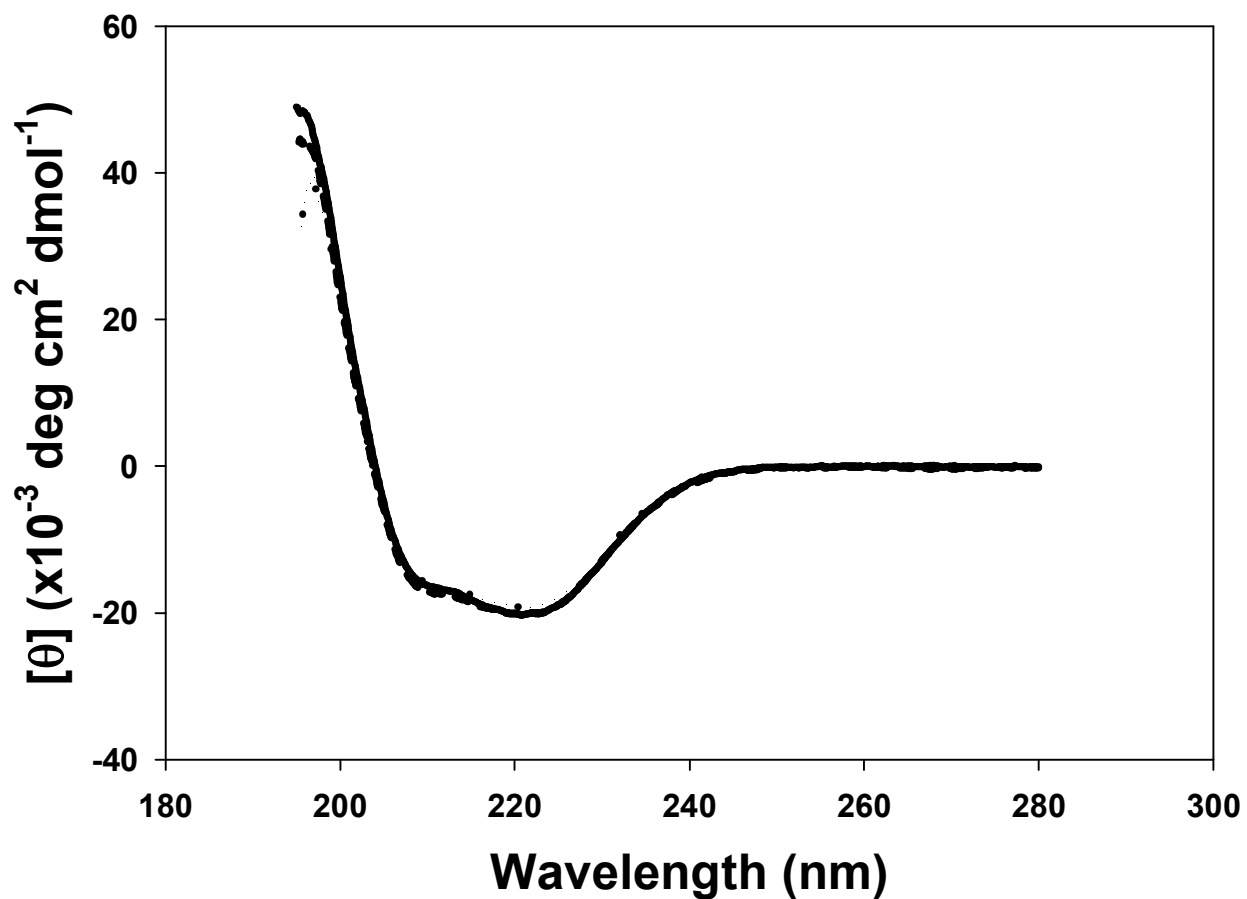


Figure 7. Circular dichroism spectra of native glutamate dehydrogenase (solid) and GDH enzyme associated with 20mM norvaline (dashed) and 20mM glutamate (dotted). None of the studied ligands appeared to affect the secondary structure, particularly the α -helical nature, of the protein.