Ultraviolet Spectrophotometric Characterization of a Glutamate Dehydrogenase-reduced Coenzyme-α-Ketoglutarate Complex*

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SUMMARY

Glutamate dehydrogenase binds α-ketoglutarate and TPNH in a ternary complex such that both ligands are more tightly bound than in binary complexes. TPNH, bound in this ternary complex, absorbs maximally at 332 nm with $E_{332} = 5.2$ mM$^{-1}$ cm$^{-1}$ in contrast to the absorbance of "free" TPNH ($E_{105} = E_{	ext{max}} = 6.2$ mM$^{-1}$ cm$^{-1}$) and TPNH bound in either a binary or in a glutamate dehydrogenase-TPNH-α-glutamate dead-end ternary complex ($E_{340} = E_{	ext{max}} = 4.8$ mM$^{-1}$ cm$^{-1}$). One molecule of TPNH per 47,000 to 54,000 molecular weight glutamate dehydrogenase (approximately one per peptide chain) was found to be bound in either the α-ketoglutarate or l-glutamate ternary complex. The spectral shifts associated with ternary complex formation were not observed when the coenzyme and substrate analog, 3-acetylpyridine DPNH or α-ketobutyrate, were used. Therefore, an intact amide on the reduced nicotinamide moiety and a gamma-carboxyl of the dicarboxylate substrate must be required for observable complex formation. The demonstration of the high stability of the glutamate dehydrogenase-TPNH-α-ketoglutarate complex and the correlation of the binding characteristics of the ligands involved in this complex with the effects of these ligands on catalytic events warrants the inclusion of this complex as either a reactive or a dead-end complex in all general mechanisms proposed for glutamate dehydrogenase catalysis.

The binding of TPNH to glutamate dehydrogenase in the absence and in the presence of L-glutamate was shown in this laboratory (1) to be characterized by a red-shifted difference-spectrum of the reduced nicotinamide absorbance. Since that time both red- and blue-shifted reduced nicotinamide spectra have been observed during the oxidative deamination of L-glutamate by glutamate dehydrogenase and TPN$^+$ with stopped flow spectrophotometry (2, 3). This blue-shifted species of TPNH was tentatively assigned by Fisher et al. (2) to either a transitory GDH-TPNH-NH$_3^+$-αKG complex or a GDH-TPNH-αKG complex on the basis of a deuterium isotope effect. Just prior to the completion of this investigation, di Francia and Iwatsubo (3) reported the GDH-TPNH-αKG complex to possess a blue-shifted spectrum using stopped flow displacement studies. This complex, however, has not been confirmed as obligatory for catalysis. Using evidence from steady state rates, Frieden (4) proposed a mechanism in which reduced coenzyme is first on and last off and Engel and Dukiel (5) propose a random mechanism for the reductive amination of α-ketoglutarate. Iwatsubo and Pataloni (6), using stopped flow evidence, suggest that the rate-limiting step of the oxidative reaction with L-glutamate is the release of reduced coenzyme subsequent to hydride transfer. This report presents the absolute spectrum of TPNH in the glutamate dehydrogenase-TPNH-α-ketoglutarate complex obtained with direct ultraviolet spectrophotometric techniques, shows the formation of this complex to involve an aromatic amine and acid residue on the enzyme, gives the stoichiometry of complex formation, describes some of the functional group requirements on the substrate and coenzyme necessary for complex formation, compares this complex to free TPNH and TPNH in other glutamate dehydrogenase complexes, and relates the characteristics of this complex to some of the properties of glutamate dehydrogenase catalysis.

MATERIALS AND METHODS

L-Glutamate dehydrogenase, L-glutamate: (TPN$^+$)DPN$^+$-oxidoreductase (deaminating) EC 1.4.1.3, was obtained as an ammonium sulfate suspension from Sigma and prepared as previously described (7). This resulted in an enzyme with a ratio of 280 to 260 nm absorbance of 1.95 to 1.98 and a specific activity of 3.5 ± 0.5 μmoles of DPN$^+$ reduced per min per mg of enzyme as assayed at 25°C in 0.2 M potassium phosphate buffer at pH 8.5 with 33.3 mM L-glutamate and 500 μM DPN$^+$—Enzyme concentrations were determined from 280 nm absorbance values with 0.97 (8) as the extinction coefficient of 1 mg per ml solution. α-Ketoglutarate, L-glutamate, α-ketobutyrate, and L-α-aminoacetobutyrate, all M.A. grade, were purchased from Mann. TPNH was also a product of Sigma. The 3-acetylpyridine analog of DPNH was obtained from Calbiochem.

All experiments were performed with 0.1 M potassium phosphate buffer, pH 7.6 in 1.000 cm cells thermostated to 20°C. A Cary model 14 double-beam spectrophotometer interfaced to a
Varian model 620/i computer was used to collect all spectral data. Each spectrum presented in this study represents an average of four to five spectra collected from consecutive runs. The absorbance values and peak positions given in the text were determined with the numerical values of each averaged spectrum.

All spectra were recorded as follows: an averaged base line was obtained with equal amounts of glutamate dehydrogenase in the sample and reference cells mixed with equal concentrations of the substrates as indicated in the text. Coenzyme, made up in an identical concentration of substrate, was added to the sample cell with Lang-Levy pipets and an equal volume of substrate, without coenzyme, was added to the reference cell. The spectra were recorded after mixing the contents of both cells with nonwettable polypropylene stirrers and waiting for 10 to 15 min. Since coenzyme is present only in the sample cells and enzyme is present in both the sample and reference cells, this arrangement results in the spectra of free and bound coenzyme and any difference spectra resulting from the perturbation of enzyme aromatic amino acid absorption usually observable in the 280- to 300-nm region and superimposed, of course, on any absorbance contributions of the coenzyme. This method also results in a slight dilution of the enzyme during the titration (each of the seven to ten additions was 0.01 ml to an initial volume of 3.00 ml). For calculations in the text this dilution is compensated by assuming that the binding signals were proportional to enzyme concentration. The error introduced in the calculations with this assumption is less than 3%.

**RESULTS AND DISCUSSION**

Fig. 1A depicts averaged spectra obtained by titration of a solution of glutamate dehydrogenase and L-glutamate with TPNH. Fig. 1B shows the results of a similar titration of glutamate dehydrogenase and α-ketoglutarate with TPNH.

The dashed lines in both figures indicate the changing position of the maximum absorbance during the titrations. Fig. 1, A and B, shows that for the first few titrations TPNH binds in the presence of glutamate exhibiting a spectrum with maximum absorbance at 348 nm and in the presence of α-ketoglutarate it binds exhibiting a spectrum with maximal absorbance at 332 nm. Both titrations show the expected shift of the maxima toward that of free TPNH at 339 nm upon accumulation of free TPNH in the system. In addition to the change in the reduced nicotinamide absorption spectrum, small features in the 280- to 300-nm region are difference spectra attributable to the perturbation of enzyme aromatic amino acid absorption. These same spectral features are also evident when TPNH binds to the enzyme in a binary complex (1, 9, 7) and have been previously reported as features of a difference spectrum resulting from the binding of glutamate to a glutamate dehydrogenase-TPNH complex (10). These spectral features are assigned to aromatic amino acid perturbation since neither the solvent perturbation difference spectra of reduced coenzyme, the adenine chromophore, nor that of reduced nicotinamide mononucleotide showed the 292 nm peak and 288 nm trough depicted in Fig. 1A and B (11). It is conceivable that the coenzyme absorbance is so altered as to show the 292 to 288 nm feature normally attributed to changes in aromatic amino acid absorbance (12, 13) but no such changes have been reported. The extinction coefficient of binding is the same for this feature whether measured for the binding of coenzyme in a GDH-TPNH binary complex or in the GDH-TPNH-G or GDH-TPNH-αKG ternary complexes.

Fig. 2 shows the change of free TPNH absorbance upon co-

![Fig. 1. Spectra of reduced nicotinamide absorbance (300 to 390 nm) and difference spectra of glutamate dehydrogenase aromatic amino acid perturbation (280 to 300 nm) resulting from the titration of enzyme and dicarboxylate substrate with TPNH. A, 32 mM L-glutamate and 2.2 mg per ml of glutamate dehydrogenase titrated with TPNH. B, 5.5 mM α-ketoglutarate and 1.85 mg per ml of glutamate dehydrogenase titrated with TPNH. Other experimental conditions are to be found in the text and in Fig. 2. The dashed line indicates absorption maximum as determined from the numerical values of absorption for each titration.](http://www.jbc.org/)

![Fig. 2. Dependence of the loss of TPNH absorbance on TPNH concentration. ΔA, was calculated by subtracting the absorbance shown in Fig. 1 from the absorbance of free TPNH added during the titrations. ΔA140 (C) resulted from the titration of 2.2 mg per ml of glutamate dehydrogenase and 32 mM L-glutamate with TPNH. ΔA280 (C) resulted from the titration of 1.85 mg per ml of glutamate dehydrogenase and 5.5 mM α-ketoglutarate with TPNH.](http://www.jbc.org/)
2.2 mg per ml of glutamate dehydrogenase or 1 molecule per binding in the GDH-TPNH complex to be 41 molecules per data at 41 ~.ll TPSII indicates the stoichiometry of TPNH oxidation, presumably due to the presence of ammonium ion. Following the initial loss there are two regions of data which are quite linear, again indicating the formation of a very stable complex. The extrapolation of the data in the first linear region to the ordinate gives the value of the loss of the $A_{331}$ due to TPNH oxidation which amounts to 8.5 $\mu M$ TPNH oxidized. Subtraction of this value from the value of the intersection of the two linear extrapolations indicates that 39.5 $\mu M$ TPNH was maximally bound to 1.85 mg per ml of glutamate dehydrogenase in a GDH-TPNH-αKG complex or one TPNH per 46,800 molecular weight of enzyme. This stoichiometry and the stoichiometry for TPNH binding in the GDH-TPNH-G complex agree reasonably well with the stoichiometry of the binding of reduced coenzyme to glutamate dehydrogenase in a binary complex, one per 52,000 molecular weight (14) and the molecular weight of each peptide chain, 56,100 (15). The dissociation constant of TPNH from glutamate dehydrogenase and 5.5 mM α-ketoglutarate was calculated from Equation 1 above after subtracting the loss of optical density and TPNH concentration due to TPNH oxidation and was found to be 0.5 ± 0.1 $\mu M$. The titration of glutamate dehydrogenase with TPNH in the absence of either dicarboxylate substrate results in a redshifted reduced nicotinamide absorbance quite similar to that shown above for the GDH-TPNH-G complex and the apparent dissociation constant of TPNH from this binary complex is 58.5 $\mu M$ (7) which is two magnitudes larger than the dissociation constant of TPNH from either GDH-TPNH-αKG or GDH-TPNH-αKG.

Fig. 3 shows the dependence of the 292 to 288 nm difference spectral feature on TPNH concentration for the formation of the GDH-TPNH-G and GDH-TPNH-αKG complexes. These figures show the same linear titration dependence as seen in Fig. 2 and indicate the same stoichiometries from the intersection of the extrapolation lines. This figure shows the 292 to 288 nm feature, attributed here to aromatic amino acid perturbation, to be the result of coenzyme binding in the GDH-TPNH-G and GDH-TPNH-αKG complexes.

The extinction coefficients for TPNH binding in the GDH-

 enzyme binding to glutamate dehydrogenase. The loss of 348-nm absorbance on TPNH concentration, when the coenzyme binds to the enzyme in the presence of 32 mM L-glutamate in this figure, shows two regions of linearity. The initial region indicates the formation of a very stable GDH-TPNH-G complex. The intersection of the two linear extrapolations of the $\Delta A_{292}$ data at 41 $\mu M$ TPNH indicates the stoichiometry of TPNH binding in the GDH-TPNH-G complex to be 41 molecules per 2.2 mg per ml of glutamate dehydrogenase or 1 molecule per 53,658 molecular weight of enzyme. Knowing this stoichiometry, the data in Fig. 2 were used in the following equation, derived from the equilibrium expression, to determine the dissociation constant of TPNII binding to glutamate dehydrogenase in the presence of 32 mM L-glutamate.

$$K_D = \frac{(E_t - \Delta A_\lambda / \Delta e_\lambda)(R_t - \Delta A_\lambda / \Delta e_\lambda)}{(\Delta A_\lambda / \Delta e_\lambda)}$$

where $\Delta e_\lambda$ = the differential extinction coefficient of binding, $E_t$ = the total enzyme concentration, $R_t$ = the total TPNH concentration, and $\Delta A_\lambda$ = the loss of optical density upon binding, and this dissociation constant was found to be 0.6 ± 0.1 $\mu M$. Also shown in Fig. 2 is the dependence of the loss of 332 nm absorbance on TPNH concentration for the binding of TPNH to glutamate dehydrogenase in the presence of 5.5 mM α-ketoglutarate. This dependence differs from that seen in the formation of the GDH-TPNH-G complex in that the loss of optical density during the initial part of the titration is the result of TPNH oxidation, presumably due to the presence of ammonium ion. Following the initial loss there are two regions of data which are quite linear, again indicating the formation of a very stable complex. The extrapolation of the data in the first linear region to the ordinate gives the value of the loss of $A_{331}$ due to TPNH oxidation which amounts to 8.5 $\mu M$ TPNH oxidized. Subtraction of this value from the value of the intersection of the two linear extrapolations indicates that 39.5 $\mu M$ TPNH was maximally bound to 1.85 mg per ml of glutamate dehydrogenase in a GDH-TPNH-αKG complex or one TPNH per 46,800 molecular weight of enzyme. This stoichiometry and the stoichiometry for TPNH binding in the GDH-TPNH-G complex agree reasonably well with the stoichiometry of the binding of reduced coenzyme to glutamate dehydrogenase in a binary complex, one per 52,000 molecular weight (14) and the molecular weight of each peptide chain, 56,100 (15). The dissociation constant of TPNH from glutamate dehydrogenase and 5.5 mM α-ketoglutarate was calculated from Equation 1 above after subtracting the loss of optical density and TPNH concentration due to TPNH oxidation and was found to be 0.5 ± 0.1 $\mu M$. The titration of glutamate dehydrogenase with TPNH in the absence of either dicarboxylate substrate results in a redshifted reduced nicotinamide absorbance quite similar to that shown above for the GDH-TPNH-G complex and the apparent dissociation constant of TPNH from this binary complex is 58.5 $\mu M$ (7) which is two magnitudes larger than the dissociation constant of TPNH from either GDH-TPNH-αKG or GDH-TPNH-αKG.

FIG. 3. Dependence of the enzyme aromatic amino acid difference spectrum on TPNH concentration. $A$, 292 - 288 nm differential absorption (□) resulting from the titration of 2.2 mg per ml of glutamate dehydrogenase and 32 mM L-glutamate with TPNH. $B$, 292 - 288 nm differential absorption (□) resulting from the titration of 1.85 mg per ml of glutamate dehydrogenase and 5.5 mM α-ketoglutarate with TPNH.
TPNH-G and GDH-TPNH-\(\alpha\)KG complexes were calculated from the differences in the spectra shown in Fig. 1 in the regions which exhibited linear binding in Figs. 2 and 3. Thus, for GDH-TPNH-G formation \(E_{\text{max}}^{\text{TPNH-G}} = 4.8 \, \text{mm}^{-1} \, \text{cm}^{-1}\) and for GDH-TPNH-\(\alpha\)KG formation \(E_{\text{max}}^{\text{TPNH-\(\alpha\)KG}} = 5.2 \, \text{mm}^{-1} \, \text{cm}^{-1}\). These contrast with the 6.2 mm extinction coefficient of “free” TPNH at 340 nm. Using stopped-flow spectrophotometry, di Franco (16) describes the spectrum of the GDH-TPNH-\(\alpha\)KG complex as having a \(\lambda_{\text{max}} = 335 \, \text{nm}\) and \(E_{140} = 5.3 \, \text{mm}^{-1}\) \, cm\(^{-1}\), whereas the \(\lambda_{\text{max}}\) reported herein was at 332 nm and \(E_{140}\) is 5.0 mm\(^{-1}\) cm\(^{-1}\).

The spectra of 1 mm TPNH in a GDH TPNH-\(\alpha\)KG complex, in a GDH-TPNH-G complex (both superimposed on enzyme perturbation in the 280- to 300-nm region), and as a free species in solution are shown in Fig. 4. It is apparent that the spectra of both complexes are hypochromic to that of free TPNH. A similar hypochromism is also exhibited when TPNH binds to glutamate dehydrogenase in a binary complex (9). Possible causes of hypochromism in this system include an orientation of the nicotinamide chromophore to another chromophore or an orientation of nicotinamide to a charged group (17). It has been shown previously that hyperchromism of the 340-nm band of TPNH absorption results when the native conformation of coenzyme in solution is changed with a concomitant disorientation of the coparallel, coplanar orientation of the nicotinamide and adenine rings (11). Thus, the hypochromism evidenced in both the GDH-TPNH-G and GDH-TPNH-\(\alpha\)KG complex formation is most likely due to an orientation of the nicotinamide ring to either the adenine portion of the coenzyme, to an enzyme aromatic amino acid chromophore or possibly to a charge on the enzyme or coenzyme. Because hypochromism is present in the spectra of both GDH-TPNH-G and GDH-TPNH-\(\alpha\)KG, it appears that the orientations of the reduced nicotinamide rings are relatively equivalent for both species.

In addition to the hypochromism exhibited upon coenzyme binding in the two ternary complexes, the binding of TPNH in the GDH-TPNH-\(\alpha\)KG and GDH-TPNH-G complexes produces blue- and red-shifted spectra, respectively. Causes of spectral shifts seen when reduced coenzyme associates with the various dehydrogenases have been discussed at length (9), but they are briefly: spectral shifts due to changes in the polarization of the general medium surrounding the chromophore, the effect seen in solvent perturbation experiments, and spectral shifts due to changes in the electronic configuration of a chromophore caused by specific interactions such as by hydrogen bond formation or breaking. To ascribe the specific causes of the red and blue shifts observed above is, of course, hazardous but in view of the constant hypochromicity and the large (8 mm) absorbance shifts seen in the spectra of the GDH-TPNH-G and GDH-TPNH-\(\alpha\)KG complexes, it now appears likely that these shifts are a result of specific changes in the electronic configuration of the nicotinamide ring rather than the result of general medium effects arising from changes in coenzyme configuration or the burying of the ring in a hydrophobic pocket.

A survey of the dehydrogenase-reduced coenzyme binding difference spectra (11) has shown that all dehydrogenases which stereospecifically transfer hydrogen to the A side of the nicotinamide ring exhibit blue shifts of the reduced nicotinamide absorbance and that most B-stereospecific dehydrogenases produce red shifts. Since both red- and blue-shifted reduced nicotinamide spectra have been demonstrated for two complexes of glutamate dehydrogenase, a B-stereospecific dehydrogenase, the relationship of dehydrogenase stereospecificity with the direction of the spectral shift now appears to be less direct.

2-Acetylpyridine DPN\(^+\) is an excellent coenzyme analog for the glutamate dehydrogenase reaction (18). The steady state rates using this analog are, in fact, higher than when either DPN\(^+\) or TPNH are used. It has been shown that the 363-nm maximum absorbance of this reduced coenzyme analog remains unshifted when bound to glutamate dehydrogenase (7). When 3-acetylpyridine DPN\(^+\) was substituted for TPNH in titrations similar to those described above, there was no observable red or blue shift of the 363 nm peak even though the concentrations of glutamate and \(\alpha\)-ketoglutarate were increased 10- and 50-fold over what was used to demonstrate the TPNH complexes. It has been previously shown that the red shift of the reduced nicotinamide absorption of TPNH was a consequence of the binding of the nicotinamide moiety to a subsite which forms a part of the coenzyme binding site for both TPNH and DPNH on the enzyme. This subsite was designated Subsite I (7).

In order for coenzyme to bind the nicotinamide moiety at Subsite I it must have an intact amide group; which is not the case with 3-acetylpyridine DPNH. Since neither red nor blue shifts were evident in the experiments using 3-acetylpyridine DPNH, the appearance of the shifted spectra associated with GDH-TPNH-\(\alpha\)KG as well as GDH-TPNH-G formation also requires the binding of the reduced nicotinamide moiety to Subsite I on the enzyme.

The binding of glutamate to the glutamate dehydrogenase-coenzyme complex has been characterized by Caughey, Smiley, and Hollerman (19) as requiring two carboxyl groups on the...
substrate. From these data the subsites which bind the alpha- and gamma-carboxyl groups have been designated as Subsites V-alpha and V-gamma and from other evidence the subsite which binds the amino group of glutamate was designated Subsite IV (7). A consequence of substrate binding to these subsites is the mutually enhanced binding of TPNH and dicarboxylate substrate since coenzyme and substrate are both afforded additional means of attachment to the enzyme surface.

It is well known that glutamate dehydrogenase can utilize a large number of monocarboxylic amino- and keto-substrates (20, 21). L-α-Aminobutyrate and α-ketobutyrate4 are both excellent glutamate dehydrogenase substrates. Therefore, these monocarboxylate substrates were examined to determine whether they formed ternary complexes with enzyme and TPNH. Base lines were recorded with 1.52 mg per ml of glutamate dehydrogenase and 40 μM TPNH present in both the sample and reference compartments of the spectrophotometer and additions of up to 25 mM α-ketobutyrate and 77 mM α-amino- butyrate were made to the enzyme-enzyme mixture in the sample compartment. Any shift of the reduced nicotinamide absorbance would be easily observable by the production of a difference spectrum upon addition of the monocarboxylate substrates but none was seen. The experimental conditions used to attempt to observe monocarboxylate complex formation were the same as those described for ternary complex formation by the dicarboxylate substrates which showed gross difference spectra using this same technique. This lack of enhanced TPNH binding in the presence of high concentrations of the monocarboxylate substrates, α-ketobutyrate and α-amino- butyrate, shows that the gamma carboxyl mieties of the dicarboxylate substrates, α-ketoglutarate and L-glutamate, are required for the mutually enhanced binding of TPNH and substrate observed in the formation of the blue-shifted GDH-TPNH-α-KG complex and the red-shifted GDH-TPNH-G complex. Thus, the gamma carboxyl of the dicarboxylate substrates and the amide of the reduced coenzyme are both necessary for the formation of observable GD TPNH α-KG and GDH-TPNH-G complexes.

A partial reaction scheme for catalysis by glutamate dehydrogenase, using part of a random mechanism scheme suggested by Engel and Dalziel (5) and including a dead-end GDH-TPNH-α-KG complex is shown in Fig. 5.

Frieden (4) and Iwatsubo and Pantaloni (6) have shown that the release of reduced coenzyme from the enzyme is the rate-limiting step of the reaction using TPN+ and α-glutamate. Iwatsubo and Pantaloni (6) also conclude that the rate-limiting step of the reaction, using monocarboxylic amino acid substrates, is not the release of coenzyme from glutamate dehydrogenase. This kinetic consequence is a result of the lack of a gamma-carboxyl group on the substrate and is consistent with the observed lack of enhanced TPNH binding when amounts of α-aminobutyrate or α-ketobutyrate, sufficient for catalytic activity, are added to a solution of glutamate dehydrogenase and TPNH. That is, the rate of release of coenzyme, using monocarboxylate substrates, is not lowered by formation of tight GDH-TPNH-α-KG or dead-end GDH-TPNH-G complexes. If it is assumed that the lack of enhanced TPNH binding is indicative of a lesser affinity of the monocarboxylate substrates for the enzyme-coenzyme complex, then the higher concentration requirements for catalytic activity using these analogs also reflect that lack of binding ability at Subsite V-gamma.

Fisher et al. (2) have reported both red- and blue-shifted reduced nicotinamide spectra as intermediates in the glutamate dehydrogenase reaction from stopped flow spectrophotometric studies. Furthermore, these authors have shown that the rate of formation of the blue-shifted complex was lowered nearly 2-fold when L-[2-3H]glutamate was used as a substrate, and that there was no isotope effect on the formation of the red-shifted complex which occurs subsequent to the formation of the blue-shifted complex. Because the GDH-TPNH and GDH-TPNH-G complexes are known to be red-shifted and because ammonium had no effect on the spectrum of GDH-TPNH in solution, the GDH-TPNH, GDH-TPNH-G, and GDH-TPNH-NH4+ complexes were eliminated as those possessing blue-shifted spectra.

The blue-shifted reduced nicotinamide spectrum was then assigned by these investigators to either the GDH-TPNH-NH4+-α-KG or GDH-TPNH-α-KG complex which remains unidentified in the reaction scheme shown above. di Franco and Iwatsubo (3) suggest the blue-shifted species observed as a transient complex to be either the GDH-TPNH-α-KG or the GDH-TPNH-NH4+-α-KG complex. The latter was considered by these investigators to be an enzyme-TPNH-α-iminoglutarate complex as suggested by Hochreiter and Schellenberg (22).

However, Olson and Anfinsen (23) showed, using equilibrium studies, that the concentration dependence of ammonium did not fit a mechanism which included free α-iminoglutarate. Thus, the GDH-TPNH-NH4+-α-KG complex has not yet been conclusively demonstrated nor shown to possess a blue-shifted reduced nicotinamide spectrum.

Insofar as the glutamate dehydrogenase-TPNH-α-keto- glutarate complex is characterized by a spectrum identical with that of a transient state intermediate, it is highly stable and has requirements for binding which relate to changes in catalytic rate; it must be included as an active or dead-end intermediate in any general mechanism proposed for glutamate dehydrogenase catalysis.

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