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THE BINDING OF α-KETOGLUTARATE IN A BINARY COMPLEX AND IN A TERNARY COMPLEX WITH NADP+ BY L-GLUTAMATE DEHYDROGENASE

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SUMMARY

L-Glutamate dehydrogenase (L-glutamate:NAD(P)+ oxidoreductase (deaminating EC 1.4.1.3)), cooperatively binds NADP+ and α-ketoglutarate in a highly stable dead-end complex exhibiting a near ultraviolet difference spectrum characteristic of red shifts of tryptophan and oxidized nicotinamide absorbance. The requirements of an intact amide on the nicotinamide moiety and two carboxyl groups on the substrate for a 200-fold heterotropic cooperativity in binding are demonstrated by the use of coenzyme and substrate analogs.

The agreement of the binary complex dissociation constants calculated from the concentration dependence of the formation of the abortive complex with those determined directly shows that both coenzyme and α-ketoglutarate are bound at the same site in the binary and ternary complexes. By analogy it is inferred that NADP+ and L-glutamate bind to the enzyme to form the active complex with a cooperativity similar to that demonstrated for the dead-end complex.

The ability of the enzyme to form the NADP+–α-ketoglutarate dead-end complex and other stable complexes is pertinent to the catalytic mechanisms proposed for glutamate dehydrogenase. This ability also provides a mechanism through which both the *in vivo* direction and rate of catalysis can be selectively and sensitively controlled by the cooperative binding of the reactants and products themselves.

INTRODUCTION

L-Glutamate dehydrogenase (L-glutamate:NAD(P)+ oxidoreductase (deaminating), EC 1.4.1.3.) catalyzes the oxidative deamination of L-glutamate by NADP+ to form α-ketoglutarate, NH_4^+ and NADPH.

The scheme below depicts the various complexes formed between the enzyme and its reactant ligands, including both those whose formation has been detected by direct evidence (designated by bold-face lettering) and those whose existence has been inferred from kinetic evidence alone.

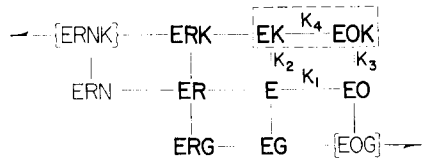


Fig. 1. Scheme showing complexes of glutamate dehydrogenase (E) with NAD(P)H (R), NAD(P)$^+$ (O), α-ketoglutarate (K), L-glutamate (G), and NH_4^+ (N). K_1, K_2, K_3 and K_4 are complex dissociation constants which are defined in the text.

The two "central" or "Michaelis" complexes indicated by the braces, for example, are more or less required by the stoichiometry of the reaction itself (since no evidence of a ping-pong mechanism for this reaction has ever been observed). On the other hand, there has been no evidence of the existence of the enzyme–NAD(P)H–NH_4^+ (or ERN) complex other than as an intermediate in the formation of the left-hand Michaelis complex, a postulation based solely on inferences from steady-state kinetic measurements[1,2].

Engel and Dalziel[3] have used steady-state kinetics to describe the righthand (Michaelis) complex as one which is formed during catalysis. Both fluorescence[4,5] and ultraviolet difference spectroscopy[6] have been used to characterize the glutamate dehydrogenase–NAD(P)H binary complex as well as the dead-end glutamate dehydrogenase–NAD(P)H–glutamate complex[4,5,7]. These complexes containing the reduced coenzyme together with the recently characterized enzyme–NAD(P)H–α-ketoglutarate complex[8,9] have been implicated in the steady-state rate limiting release of reduced coenzyme[10,11].

Recently the existence of binary complexes of L-glutamate[12] and NADP$^+$ (ref. 13) have been demonstrated by direct binding methods, but previous attempts to demonstrate an α-ketoglutarate binary complex have been inconclusive and thus far no direct characterization of the dead-end glutamate dehydrogenase–NAD(P)$^+$–α-ketoglutarate complex has been made. We present here direct differential spectroscopic evidence of the existence of both of these complexes (enclosed in the dashed line of the scheme) and evaluate the dissociation constants indicated in that scheme. From these data we show a 200-fold cooperativity in the binding of the two ligands in the ternary complex and that NADP$^+$ and α-ketoglutarate each bind to the same site in the binary complex as they do in the ternary complex.

METHODS AND MATERIALS

Bovine liver L-glutamate dehydrogenase, NADP$^+$, 3-acetylpyridine-NADP$^+$ were obtained from Sigma. The $(NH_4)_2SO_4$ suspension of the enzyme was extensively dialyzed against 0.1 M potassium phosphate buffer (pH 7.6), treated with approximately 5 mg/ml Norit A and filtered through an 0.45 μm/Millipore filter. This procedure resulted in an enzyme with a specific activity of 3.5 $\pm$ 0.5 μmoles NAD$^+$ reduced per min in 0.2 M phosphate buffer (pH 8.5) using 33 mM L-glutamate and 500 μM NAD$^+$. The ratio of 280 to 260 nm absorbance of the enzyme after this treatment was 1.95–1.98 and enzyme concentrations were determined using 0.97 as the extinction coefficient of a 1 mg/ml solution[14]. The normality of the enzyme was calculated using a molecular weight of 56 100 (ref. 15), the molecular weight of a single peptide chain

and the established stoichiometry of other ternary complexes[9]. Keto-acids and glutaric acid, all M.A. grade, were purchased from Mann Research Labs.

Difference spectra

All difference spectral experiments were performed using 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 620i computer was used to collect and average all spectral data. To generate difference spectra, four cells, arranged in tandem in the sample and reference compartments, were used as previously described[9]. Base lines were recorded with enzyme in both the sample and reference compartments for binary difference spectra and with enzyme plus α-ketoglutarate or other carboxylic acid in both the sample and reference compartments for ternary difference spectra. Binding difference spectra were recorded after additions of the varied constituent to the sample cell containing enzyme and to the reference cell containing only buffer. The spectral data were collected at one nm intervals with averages over a 0.4-nm range for each datum. In addition, from three to five difference spectra were averaged for each final difference spectrum.

RESULTS

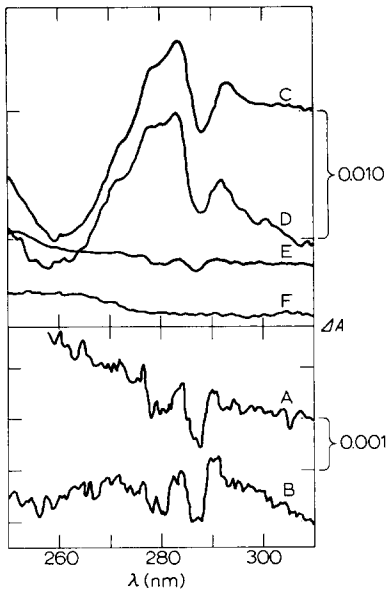
Glutamate dehydrogenase–α-ketoglutarate complex

The difference spectrum which results from the binding of α-ketoglutarate to glutamate dehydrogenase is shown in Fig. 2A. The primary features of this diminutive difference spectrum are the peaks at 291 and 283 nm with a trough at 287 nm. These features are characteristic of a difference spectrum resulting from a red shift of tryptophan absorbance[16]. Fig. 2 Curve B shows the difference spectrum resulting from the binding of glutarate to glutamate dehydrogenase exhibits qualitatively the same features as seen in Fig. 2 Curve A. Both difference spectra, Curves A and B, have been corrected for differential scatter by subtracting an absorbance value having a reciprocal fourth power dependence on wavelength, fitted to the 400 nm region of the difference spectra, from the difference spectra themselves.

The concentration dependence of the formation of the glutamate dehydrogenase–α-ketoglutarate difference spectrum is shown in the double reciprocal plot in Fig. 3. The line drawn through the data was calculated from a weighted least squares fit equation and the values calculated from the intercepts of this line are: $\Delta A_{max} = 0.0025 \pm 0.0005$ and a dissociation constant of 2.8 ± 0.5 mM.

Glutamate dehydrogenase–α-ketoglutarate–NADP$^+$ complex

A comparison of the difference spectrum resulting from the titration of glutamate dehydrogenase and α-ketoglutarate with NADP$^+$ (Fig. 2C) with the much smaller difference spectra resulting from the titrations of glutamate dehydrogenase with the same concentrations of α-ketoglutarate (Fig. 2 Curve E) or of NADP$^+$ (Fig. 2 Curve F) shows that a ternary complex has been formed. Although not shown, there was no significant difference spectrum when these concentrations of α-ketoglutarate and NADP$^+$ were mixed in the absence of enzyme. The 292 nm peak and 288 nm trough are again indicative of a red shift of tryptophan absorption but the extinction coefficient ($4.8 \cdot 10^{-4}$ mM$^{-1} \cdot$ cm^{-1}) of binding for this feature is much larger than for the α-



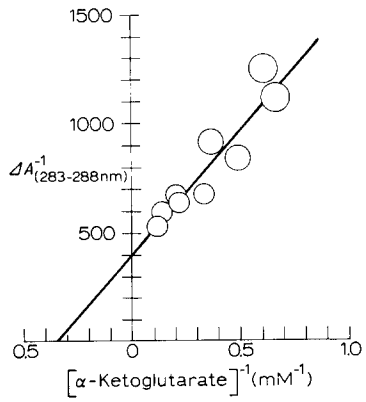


Fig. 2. Difference spectra of binary and ternary complexes of glutamate dehydrogenase. The concentration of glutamate dehydrogenase for the production of all difference spectra was 1 mg/ml. Curves A and E, titration of enzyme with 5 mM α-ketoglutarate; Curve B, titration of enzyme with 5 mM glutarate; Curve C, titration of enzyme and 5 mM α-ketoglutarate with 29 μM $NADP^+$; Curve D, titration of enzyme and 2 mM glutarate with 48 μM $NADP^+$; Curve F, titration of enzyme with 29 μM $NADP^+$.

Fig. 3. Double-reciprocal plot showing the spectrophotometric titration of glutamate dehydrogenase with α-ketoglutarate. Enzyme concentration is 1 mg/ml.

ketoglutarate binary ($0.8 \cdot 10^{-4}$ $mM^{-1} \cdot cm^{-1}$) complex. The ternary difference spectrum differs from the binary difference spectrum by the relatively large 283 nm peak and 260 nm trough at the lower wavelengths. Since the 283 to 288 nm, peak to trough, feature is distinctive and occurs at wavelengths where the total absorbance of the system is relatively low, the measurement of this feature was used to determine the concentration dependence of the formation of the ternary complex.

$\Delta A_{283-288\ nm\ (max)}$ was determined by titrating enzyme and α-ketoglutarate with $NADP^+$ and extrapolating to ΔA_{max} in a double reciprocal plot; the value was found to be 0.0142/mg per ml glutamate dehydrogenase. Using this value the stoichiometry was determined to be approximately one $NADP^+$ bound in the ternary complex per peptide chain, molecular weight 56 100 (ref. 15). This result is shown in the binding plot in Fig. 4.

The evaluation of the ternary complex binding data in terms of dissociation constants is made assuming four forms of the enzyme to be in equilibrium: E, free glutamate dehydrogenase; EK, the α-ketoglutarate complex shown above; EO, an enzyme–$NADP^+$ complex characterized by Dalziel and Egan[13] using equilibrium dialysis techniques; and EOK, the abortive ternary complex. Thus, $K_1 = [E][O]/[EO]$, $K_2 = [E][K]/[EK]$, $K_3 = [EO][K]/[EOK]$, and $K_4 = [EK][O]/[EOK]$. It will be further assumed that the 283 to 288 nm, peak to trough, feature of the ternary difference spectrum is directly proportional to only ternary complex formation, an

assumption supported by the approximately six-fold higher extinction of binding for the ternary complex as compared to that of the binary complex and by the use of low concentrations of α-ketoglutarate (relative to the 2.8 mM glutamate dehydrogenase–α-ketoglutarate binary dissociation constant shown earlier). Using these assumptions, the following equation was derived from the above equilibria:

$$\Delta A^{-1} = \Delta A^{-1}_{max} (1 + K_4/[O] + K_3/[K] + K_2K_4/[O][K]) \quad (1)$$

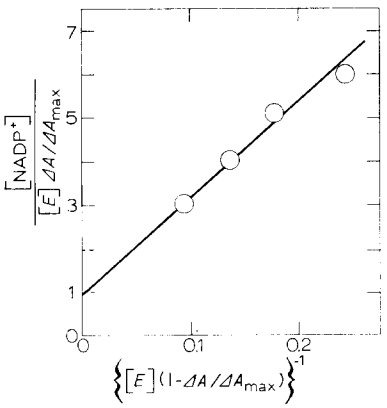


Fig. 4. Binding plot according to the method of Stockell[25] showing the binding of $NADP^+$ to 1 mg/ml glutamate dehydrogenase in the presence of 2.0 mM α-ketoglutarate. $\Delta A = (\Delta A_{283} - \Delta A_{288})$. Value of the extrapolated line to the ordinate is 0.93 sites per 56 100 molecular weight glutamate dehydrogenase.

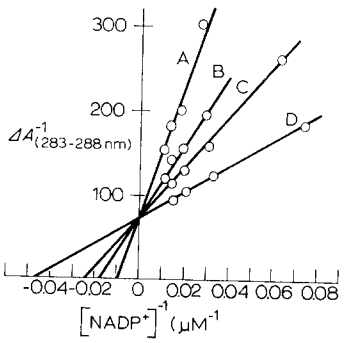


Fig. 5. Double-reciprocal plot showing the dependence of ternary complex formation on $NADP^+$ concentration at four concentrations of α-ketoglutarate. Glutamate dehydrogenase concentration is 1 mg/ml for all experimental points. α-Ketoglutarate concentrations are: Curve A, 245 μM; Curve B, 490 μM; Curve C, 740 μM; Curve D, 1990 μM.

The data in the double reciprocal plot in Fig. 5 show the dependence of the appearance of ternary complex difference spectra on $NADP^+$ concentration at four different α-ketoglutarate concentrations. From these data K_4, K_3 and K_2K_4 were calculated from a weighted, least squares fit to the reciprocal of Eqn 1 using the method of Box[17]. The values for these constants were determined to be: $K_4 = 9.6 \pm 2.0$ μM, $K_3 = 11 \pm 5.3$ μM, and $K_2K_4 = 23955$ μM (ref. 2). Since, by definition, $K_2K_4 = K_1K_3$, K_1 and K_2 were calculated from these data and were determined to be 2.2 ± 1.2 mM and 2.5 ± 0.4 mM, respectively. These values for the dissociation constants were then used to generate the theoretical lines drawn through the data in Fig. 5.

Ternary complex analogs of $NADP^+$ and α-ketoglutarate

NAD^+ is also a coenzyme for the glutamate dehydrogenase reaction and although not shown, this ligand also forms a ternary complex with α-ketoglutarate and enzyme. In addition to the features exhibited in the $NADP^+$ ternary difference spectrum, the NAD^+ ternary difference spectrum contained difference spectral features in the 260–280 nm region which result from the perturbation of adenine absorption when the coenzyme binds at a second site[18–20].

3-Acetylpyridine $NADP^+$, 3-acetylpyridine NAD^+ and pyridine-3-aldehyde NAD^+ are all excellent coenzyme analogs for the glutamate dehydrogenase reaction[18]. However, the reduced form of these analogs do not exhibit red shifts of reduced nicotinamide absorption which are typical of NADPH and NADH binding[6]. None of the oxidized analogs produce a difference spectrum indicative of ternary complex formation when titrated against a 1 mg/ml solution of glutamate dehydrogenase in the presence of up to 10 mM α-ketoglutarate.

Pyruvate and α-ketobutyrate are substrate analogs for the glutamate dehydrogenase reaction[21,22]. However, neither of these ligands produce ternary complex difference spectra in the presence of 1 mg/ml glutamate dehydrogenase and up to 100 μM $NADP^+$. Glutarate is a competitive inhibitor of the glutamate dehydrogenase reaction[23] and it has been reported to be bound cooperatively with NADPH[18] and $NADP^+$ [13] to the enzyme in ternary complexes exhibiting difference spectra. The difference spectrum in Fig. 2 Curve D shows that glutarate forms a ternary complex with $NADP^+$ qualitatively indistinguishable from the α-ketoglutarate ternary complex difference spectrum.

DISCUSSION

The binary complex dissociation constants for the glutamate dehydrogenase–α-ketoglutarate complex determined from the direct observation of the formation of the binary complex and calculated from the concentration dependence of the formation of the abortive ternary complex agree quite well (2.8 mM and 2.5 mM, respectively). This agreement indicates that α-ketoglutarate is bound at the same site in both the binary and ternary complexes. The binary dissociation constant for a glutamate dehydrogenase–$NADP^+$ complex, calculated from the concentration dependence of the formation of the abortive ternary complex was 2.2 ± 1.2 mM. Equilibrium dialysis studies by Dalziel and Egan[13] show that $NADP^+$ binds to glutamate dehydrogenase with a 2.5 mM dissociation constant at pH 7.0. These two values agree sufficiently to permit us now to describe $NADP^+$ as being bound to the same site on the enzyme in the binary complex as it is in the ternary complex.

Since steady-state kinetic studies have shown that L-glutamate is a competitive inhibitor of α-ketoglutarate[23] and since it has been recently shown in this laboratory that L-glutamate binds to the enzyme in the absence of coenzyme[12], it is reasonable to conclude, by analogy to the abortive complex results and in agreement with Dalziel and Egan[13], that the formation of the active glutamate dehydrogenase–$NADP^+$–L-glutamate complex is also cooperative. Using the same analogy we also conclude that the substrate and coenzyme are bound in binary complexes at the same sites as those to which they are bound in the active ternary complex. Any description of the formation of such a complex as either random or ordered, of course, must await the measurement of the rate constants for the various kinetic pathways.

A comparison of the dissociation constants of α-ketoglutarate and $NADP^+$ from binary and ternary complexes shows that both ligands are bound some 200-fold tighter in the ternary complex. The involvement of the enzyme in the formation of the ternary complex is indicated by the aromatic amino acid perturbation characterized by the red shift of tryptophan absorption. This same perturbation is also observed in the formation of either the glutamate dehydrogenase–NADPH complex[6] or

glutamate dehydrogenase–NADPH–L-glutamate complex[16]. The 292 to 288 nm feature in the latter two complexes has a millimolar extinction coefficient of binding of $4.2 \cdot 10^{-4}$ which agrees with the $4.8 \cdot 10^{-4}$ extinction coefficient we calculate for this feature for the formation of the enzyme–$NADP^+$–α-ketoglutarate complex. As shown here the binding of α-ketoglutarate in the absence of coenzyme also exhibits a tryptophan perturbation difference spectrum but the millimolar extinction of binding of the 292 to 288 nm feature for this binary complex is $0.8 \cdot 10^{-4}$. This much lower value indicates that the tryptophan perturbation observed in the formation of the ternary complexes does not arise solely from the enzyme–α-ketoglutarate interaction present in the binary complex. The apparent dissociation constant of $NADP^+$ from its binary complex with glutamate dehydrogenase is in the mmolar range, thus the high absorbances necessary to observe $NADP^+$ binding preclude our direct observation of the formation of the binary complex and our ability to ascertain whether $NADP^+$ binding perturbs tryptophan absorbance. However, since NADPH binding does show this feature we feel that the tryptophan perturbation arises as a result of coenzyme–enzyme interaction in the ternary complex. We have previously shown, using solvent perturbation techniques, that the number of tryptophan moieties available for direct contact by solutes greater than $6.5 \cdot 10^{-8}$ cm diameter is one per peptide chain, the same as the stoichiometry we have demonstrated for the ternary complexes. Thus, both the coenzyme and substrate, being larger than $6.5 \cdot 10^{-8}$ cm, must bind at or near a single tryptophan residue on the enzyme surface. The red shifts which produce the tryptophan difference spectra are consistent with the introduction of an environment with greater polarizability, possibly with the formation of a hydrogen bond by the aromatic amino acid, or from many other general causes[24]. Thus, the ability of two different ligands to perturb one chromophore is not perplexing and the tryptophan perturbation observed in the abortive complex formation may simply indicate the enhanced ability of $NADP^+$ to bind to the enzyme.

The large 260 nm trough and 283 nm peak are not typical of difference spectra arising from the perturbation of aromatic amino acid absorption. Since α-ketoglutarate absorbs very little in this region, this feature is most likely due to the perturbation of coenzyme absorption upon formation of the ternary complex. Since we have shown previously[6] that the adenine moiety of NADPH is not perturbed appreciably when the coenzyme binds to glutamate dehydrogenase and that the reduced nicotinamide moiety is red shifted, the above portion of the difference spectrum is consistent with and probably represents a red shift of the absorption of the oxidized nicotinamide moiety. The inability of the active coenzyme analog, 3-acetylpyridine-$NADP^+$, to exhibit a difference spectrum indicative of ternary complex formation reflects the inability of this coenzyme to form a stable, spectroscopically observable ternary complex.

The ability of glutarate to replace α-ketoglutarate in the formation of the abortive ternary complex shows that the keto-group of the substrate molecule is not necessary for the cooperativity nor for the spectral signals which are exhibited in the formation of the complex. This ability, together with the inability of monocarboxylate substrates to form the ternary complex, shows that the γ-carboxyl moiety is an absolute requirement for the heterotropic binding cooperativity exhibited in the ternary complex formation.

Since the constituents of the glutamate dehydrogenase–$NADP^+$–α-ketoglutarate

complex are nonreactive in the complex, it is by definition an inhibitory complex and under conditions of catalysis (where concentrations of $NADP^+$ and α-ketoglutarate are relatively large) it will be formed cooperatively and inhibit the reaction. This complex has been implicated kinetically through the inhibition of the L-glutamate reaction by α-ketoglutarate in steady-state studies. Because of the high stability of this complex it should be thus included in any total mechanism proposed for glutamate dehydrogenase catalysis.

The ability of glutamate dehydrogenase to form ternary complexes in which the coenzyme and substrate or product ligands are cooperatively bound is not limited to the glutamate dehydrogenase–$NADP^+$–α-ketoglutarate complex we demonstrate here since α-ketoglutarate and L-glutamate both bind cooperatively to the enzyme in the presence of NADPH[9]. The requirement of an intact amide group on the coenzyme for a spectrophotometric signal indicating binding and of a dicarboxylate ligand for the cooperative formation of the ternary complex are common requirements for all three of the above ternary complexes.

The concentration of glutamate dehydrogenase *in vivo* has been determined to be in the mg/ml range. At these levels and in the presence of coenzymes, substrates and products, the complexes which require just the binding of one additional ligand in order for a reaction to occur (glutamate dehydrogenase–NADH–α-ketoglutarate, enzyme–$NADP^+$ and enzyme–L-glutamate) will be in equilibrium with those nonreactive, abortive ternary complexes discussed above. This equilibrium offers a system with greater sensitivity to substrate and product concentration than a system in which very little or no cooperativity is present. Thus, the cooperative formation of both potentially active and abortive complexes offers a mechanism of control of catalysis in which the constituents of the reaction, themselves, can selectively control both the direction and rate of the reaction.

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