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Kinetic Studies To Determine the Mechanism of Regulation of Bovine Liver Glutamate Dehydrogenase by Nucleotide Effectors†

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ABSTRACT: A combination of kinetic and isotope effect studies in the presence and absence of the effectors ADP and GTP was used to elucidate the mechanism of regulation of bovine liver glutamate dehydrogenase. ADP at low concentrations of glutamate competes with TPN for free enzyme. GTP exhibits a similar effect at high concentrations (100 μM and above). When ADP binds at its allosteric site, it increases the off rates of both α-ketoglutarate and TPNH from their product complexes. This results in a decrease in V/K for both substrates, an increase in V, and an increase in the deuterium isotope effects for all three parameters so that they are all about 1.3. The rate of release of glutamate from E–TPNH–glutamate is also apparently enhanced since no substrate inhibition by glutamate is observed in the presence of ADP. The effect of GTP is in opposition to that of ADP in that GTP decreases the off rates for both TPN and glutamate from E–TPN–glutamate as well as the off rates for α-ketoglutarate and TPNH. This results in an increase in the V/K's for both substrates, a decrease in V, and a decrease in the deuterium isotope effects for all three parameters to a value of 1. Substrate inhibition by glutamate is also eliminated by GTP probably by preventing any significant accumulation of E–TPNH to which glutamate binds as an inhibitor.

Glutamate dehydrogenase catalyzes the reversible oxidative deamination of L-glutamate by TPN to TPNH, α-ketoglutarate, and ammonia. Frieden (1959) has shown that this enzyme is regulated allosterically by nucleotides. Activation is obtained with the nucleoside diphosphates ADP and GDP while inhibition is obtained with ATP and GTP.

The nucleoside diphosphates have been found to enhance the rate of formation of a dead-end E–TPNH–glutamate complex and inhibit the formation of the E–TPNH–α-ketoglutarate complex (Sanner, 1975) probably by increasing the off rate for α-ketoglutarate from this complex (Andree, 1978). Dalziel & Egan (1972) have shown that in the presence of 1 mM ADP, TPN does not bind at all in the absence of glutamate. In opposition, the nucleoside triphosphates inhibit the rate of formation of E–TPNH–glutamate and enhance the formation of the E–TPNH–α-ketoglutarate complex (Sanner, 1975) presumably by decreasing product off rates (di Franco, 1974). In addition, 0.8 mM GTP enhances the binding of TPN to free enzyme (Dalziel & Egan, 1972).

More recently, George & Bell (1980) have shown that ADP is a competitive inhibitor of TPN at low concentrations of glutamate. In addition, these authors have shown that ADP activates by destabilizing the E–TPNH–glutamate complex. Modification of a single imidazole residue by diethyl pyrocarbonate treatment yields an activation similar to that observed with ADP.

This paper presents studies which indicate that the mechanism of activation of an enzyme can best be determined by a complete and systematic study of the effect of modifiers on each of the kinetic parameters. Once these have been determined, results may be tested, if possible by the use of deuterium isotope effects.

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Materials and Methods

Chemicals. Bovine liver glutamate dehydrogenase was obtained from Sigma. L-Glutamate-*2-d* was prepared by the method of Rife & Cleland (1980). All other reagents were obtained from commercial sources and were of the best quality available. Glutamate concentrations were determined enzymatically according to Cook et al. (1980).

Initial Velocity Studies. Initial velocity studies were carried out with a Beckman DU monochromator and a Gilford OD converter by monitoring the change in absorbance at 340 nm due to production of TPNH. All studies were carried out at pH 7.1 in 70 mM phosphate buffer. Ionic strength was maintained at 0.4 M by addition of NaCl.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear except those in the absence of either GTP or ADP which exhibited substrate inhibition by glutamate. The data were fitted to appropriate equations with the FORTRAN programs of Cleland (1979). The initial velocity pattern obtained in the absence of GTP or ADP was fitted to eq 1, while all other

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB(1 + B/K_{II})} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (2)$$

$$y = \frac{a(1 + P/K_{I\,num})}{1 + P/K_{I\,denom}} \quad (3)$$

$$y = mx + b \quad (4)$$

$$v = \frac{VA}{K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (5)$$

initial velocity patterns were fitted to eq 2. Hyperbolic functions of kinetic parameters vs. ADP and GTP concentrations were fitted to eq 3. Linear functions of kinetic parameters vs. ADP concentration were fitted to eq 4. All data

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Table I: Kinetic Parameters for Glutamate Dehydrogenase in the Presence or Absence of ADP[a]

parameter[b]	ADP (mM)				
	0.0[c]	0.1	0.2	0.5	1.0
V	0.026 ± 0.002	0.068 ± 0.001	0.063 ± 0.012	0.071 ± 0.006	0.064 ± 0.006
V/K_{TPN}	0.59 ± 0.03	0.512 ± 0.011	0.31 ± 0.03	0.23 ± 0.02	0.206 ± 0.018
$V/K_{glutamate}$	0.0046 ± 0.001	0.00385 ± 0.00012	0.00345 ± 0.0002	0.0031 ± 0.0006	0.0029 ± 0.0006
$(K_{i\,TPN})(K_{glutamate})/V$	29.5 ± 1.2	41.0 ± 2.2	49 ± 5	78.7 ± 5.2	125.3 ± 9.7
$K_{i\,TPN}$	0.136 ± 0.03	0.158 ± 0.007	0.169 ± 0.014	0.244 ± 0.044	0.363 ± 0.070

[a] Assays were carried out at 25 °C, pH 7.1, in 70 mM phosphate at 0.4 M ionic strength. [b] Units are the following: V, μmol/min; V/K_{TPN} and $V/K_{glutamate}$, min^{-1}; $(K_{i\,TPN})(K_{glutamate})/V$, mM·min; $K_{i\,TPN}$, mM. [c] Glutamate exhibits uncompetitive substrate inhibition with K_I = 13.3 ± 1.8 mM.

obtained for deuterium isotope effects by direct comparison of initial velocities were fitted to eq 5, which allows for isotope effects on both V and V/K. In eq 1 and 2, all constants have their normal meaning, while K_{II} is the inhibition constant for glutamate combining as a dead-end inhibitor to some complex which is formed after both substrates are bound. In eq 3, P is either ADP or GTP, a is the value of y at P = O, and $K_{I\,num}$ and $K_{I\,denom}$ are inhibition constants for numerator and denominator. In eq 5, F_i represents the fraction of heavy-atom label in the substrate, while $E_{V/K}$ and E_V are isotope effects minus 1 for the respective parameters.

Theory

The use of initial velocity studies to determine the mechanism of regulation by a non-active-site effector is an adaption of the work of Viola & Cleland (1981) on the use of initial velocity studies to determine the kinetic mechanism of terreactant enzymes. This use of initial velocity studies is based on the fact that when the modifier is included in the reaction scheme of a bireactant enzyme it becomes pseudoterreactant. By analogy to the case where all three molecules are reactants, if initial velocities are obtained by varying A at several fixed levels of B and a single level of X (effector), an initial velocity pattern is obtained. If the kinetic mechanism is of the sequential type, the rate equation will be that given by eq 2. Slope and intercept replots vs. $1/B$ are obtained from this initial velocity pattern to obtain results graphically. If the initial velocity pattern is repeated at several fixed levels of effector (zero concentration should be included if possible), the slope and intercept replots are now composed of a family of lines (one for each effector concentration) such that new patterns are generated. From these two new patterns, secondary slope and intercept replots can be constructed from each of the two so that the effect of modifier concentration is observed on each of the kinetic parameters. Thus, a plot of the slopes (obtained from the slope replot vs. $1/B$) against effector concentration (X) gives the effect of X on $K_{ia}K_b/V$; a plot of the intercepts (obtained from the slope replot vs. $1/B$) against effector concentration gives the effect of X on K_a/V; a plot of slopes (obtained from the intercept replot vs. $1/B$) against effector concentration gives the effect of X on K_b/V; a plot of the intercepts (obtained from the intercept replot vs. $1/B$) against effector concentration gives the effect of X on $1/V$. Each of these parameters can be related to an enzyme form via the distribution equations. For example, in a random mechanism, $K_{ia}K_b/V$, K_a/V, and K_b/V represent E, EB, and EA while $1/V$ represents a combination of central and product complexes (for example, if two products are obtained and released in random fashion, $1/V$ represents EAB, EPQ, EQ, and EP). Thus, the dissociation constant for X from any of the above complexes with X can be determined. In the present study, values of the four parameters discussed above are generated from a fit of the data to eq 2, and a table of parameters vs. effector concentration is shown.

Results

The kinetic parameters obtained by varying glutamate concentration at several fixed levels of TPN and zero ADP and GTP are given in column 1 of Table I. Uncompetitive substrate inhibition is observed by glutamate vs. TPN, indicating that glutamate combines to an enzyme form produced after both substrates are bound to produce the inhibition [addition of glutamate and TPN is random (Rife & Cleland, 1980)].

The initial velocity pattern from which the data in column 1 of Table I were obtained was repeated at four different concentrations of ADP, and these data are also shown in Table I. No substrate inhibition by L-glutamate was observed even at the lowest ADP concentration.

From the data in Table I, it can be seen that V is increased, while all other kinetic parameters are decreased. $(K_{i\,TPN})(K_{glutamate})/V$ increases linearly, characteristic of active-site combination, while all other changes are partial. Thus, ADP competes with TPN for free enzyme (represented by $K_{ia}K_b/V$), in agreement with recent studies by George & Bell (1980). The dissociation constant for ADP binding to the active site of free enzyme [represented by $(K_{i\,TPN})(K_{glutamate})/V$] is 0.32 ± 0.01 mM. Binding of ADP at the allosteric site to either E–TPN ($V/K_{glutamate}$) or E–glutamate (V/K_{TPN}) gives dissociation constants of 151 ± 14 and 230 ± 170 μM, respectively, while binding to product complexes gives a dissociation constant of ≤10 μM.

The initial velocity pattern from which the data in column 1 of Table I were obtained was repeated at several GTP concentrations, and these results are shown in Table II. GTP dramatically increases $V/K_{glutamate}$. In addition, substrate inhibition was not observed until glutamate concentration was increased to 40 mM and higher. The dissociation constant for GTP binding to free enzyme is 20 ± 3 μM. Binding to E–glutamate gives a dissociation constant of 290 ± 220 μM while binding to product complexes yields a dissociation constant of 28 ± 2 μM. Negative cooperativity is observed for the binding of GTP to E–TPN with dissociation constants of about 10 and 60 μM.

Deuterium Isotope Effects. Isotope effects obtained by direct comparison of initial velocities are shown in Table III. In the absence of effectors, isotope effects on all parameters are small. In the presence of 1 mM ADP, the isotope effect on all three parameters, V, V/K_{TPN}, and $V/K_{glutamate}$, is about 30%. With 1 mM GTP, however, all isotope effects are not different than 1.0.

Discussion

Substrate inhibition by L-glutamate is quite pronounced at pH 7 and 0.4 M ionic strength, yielding a K_I of about 13 mM. This is consistent with previous work by Engel & Dalziel

Table II: Kinetic Parameters for Glutamate Dehydrogenase in the Presence of GTP[a]

parameter[b]	GTP (mM) 0.1	0.2	0.5	1.0
V	0.0118 ± 0.0004	0.0100 ± 0.008	0.009 ± 0.001	0.008 ± 0.0006
V/K_{TPN}	0.567 ± 0.073	0.26 ± 0.03	0.160 ± 0.009	0.083 ± 0.010
$V/K_{glutamate}$	0.035 ± 0.008	0.050 ± 0.006	0.063 ± 0.008	0.08 ± 0.003
$(K_{i\,TPN})(K_{glutamate})/V$	6.6 ± 0.5	5.6 ± 1.0	3.03 ± 0.17	2.3 ± 0.6
$K_{i\,TPN}$	0.231 ± 0.050	0.280 ± 0.030	0.191 ± 0.022	0.184 ± 0.048

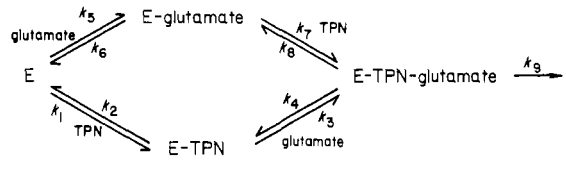
[a] Conditions are the same as those listed in the footnote to Table I. [b] Units for parameters listed are in Table I.

Table III: Primary Deuterium Isotope Effects in the Presence and Absence of Modifiers

ligand	V	V/K_{TPN}	$V/K_{glutamate}$
none	1.02 ± 0.02	1.05 ± 0.02	1.07 ± 0.01
1 mM ADP	1.34 ± 0.02	1.31 ± 0.03	1.30 ± 0.01
1 mM GTP	1.02 ± 0.02	1.01 ± 0.02	1.0 ± 0.02

(1969), who showed that substrate inhibition by glutamate is obtained with TPN concentrations higher than 10 μM. In addition, George & Bell (1980) have shown that substrate inhibition is obtained at high TPN concentrations. This inhibition has been attributed to formation of an abortive E–TPNH–glutamate complex (Engel & Dalziel, 1969; di Franco & Iwatsubo, 1971; di Franco, 1974). Since ADP eliminates the substrate inhibition by glutamate, it most probably increases the off rate for either glutamate, TPNH, or both from the abortive E–TPNH–glutamate complex. This was also concluded by George & Bell (1980).

Although ADP is always listed as an activator, the only parameter for which any activation is observed is the maximum velocity, which shows a 2.6-fold increase. The K_{ADP} for this change is $\leq$10 μM. All other parameters are progressively inhibited. Indeed, the $(K_{i\,TPN})(K_{glutamate})/V$ term exhibits linear inhibition by ADP, indicative of direct competition by ADP and TPN for free enzyme active sites. This is in agreement with previous observations by Dalziel & Egan (1972), who showed that DPN binds very weakly in the absence of glutarate and in the presence of 1 mM ADP. Changes in both V/K's are partial, consistent with ADP acting at the allosteric site. Some binding of ADP to E–glutamate cannot be ruled out, particularly since the total change in V/K_{TPN} (3.4) is larger than that observed for $V/K_{glutamate}$ (1.8). Since glutamate dehydrogenase has a random kinetic mechanism, the following simple model can be considered:



(6)

where k_9 represents catalysis and the off rate for the first product while k_{11} represents the off rate for the last products. The expressions for V and the V/K's for substrates are the following:

$$V = \frac{k_9 k_{11}}{k_9 + k_{11}} \tag{7}$$

$$V/K_{glutamate} = \frac{k_3 k_9}{k_4 + k_9} \tag{8}$$

$$V/K_{TPN} = \frac{k_7 k_9}{k_8 + k_9} \tag{9}$$

The overall reaction catalyzed by glutamate dehydrogenase is limited by the release of reduced nucleotide (Fisher et al., 1970). As can be seen from the above expressions, the simplest way to explain the reciprocal effect of ADP on V and the substrate V/K's is to propose an increase in the off rates for substrates and products (that is, an increase in k_4, k_8, and k_{11}). Since $k_{11} < k_9$, this would result in an increase in V while increasing k_4 and k_8 would decrease the V/K's. It is also possible, however, that ADP has an effect on both the off rates of reactant and products and the catalytic steps.

In support of the above interpretation of increased product off rates is the elimination of substrate inhibition by ADP as discussed above. In addition, di Franco (1974) has shown that ADP increases the rate of decomposition of E–TPNH–α-ketoglutarate. This work was extended by Sanner (1975), who showed that this increase in the dissociation rate was independent of glutamate concentration and partial. Andree (1978), using NMR, directly observed an increase in the dissociation of α-ketoglutarate from enzyme in the presence of ADP.

Finally, the deuterium isotope effects on all three parameters become equal to about 30% in the presence of ADP. The expressions for isotope effects on V and the substrate V/K's are as follows (Northrop, 1975; Cleland, 1978):

$$^{D}V = \frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}} \tag{10}$$

$$^{D}(V/K_{glutamate}) = \frac{^{D}k_9 + k_9/k_4}{1 + k_9/k_4} \tag{11}$$

$$^{D}(V/K_{TPN}) = \frac{^{D}k_9 + k_9/k_8}{1 + k_9/k_8} \tag{12}$$

where ^{D}V, $^{D}(V/K_{glutamate})$, and $^{D}(V/K_{TPN})$ represent deuterium isotope effects on the respective parameters, $^{D}k_9$ represents the maximum isotope effect on k_9 [not necessarily an intrinsic isotope effect (Cook & Cleland, 1981)], and the ratios k_9/k_{11}, k_9/k_4, and k_9/k_8 cause the observed isotope effect to be less than $^{D}k_9$. The reverse commitment does not have to be considered since ammonia is released very fast with respect to the reverse catalytic steps (Rife & Cleland, 1980). The maximum isotope effect observed in these studies is also the maximum isotope effect observed when catalysis becomes rate determining at high and low pH (Cook, 1981). Under these conditions, the deuterium isotope effect on V and $V/K_{glutamate}$ is about 1.25 while the ^{15}N isotope effect is 1.03[1] in the direction of glutamate oxidative deamination. These data, therefore, are consistent with increasing k_4, k_8, and k_{11} such that the observed isotope effects become equal to $^{D}k_9$. Thus, if $^{D}k_9$ = 1.3 and isotope effects in the absence of ADP are 1.05 and 1.07 on V/K_{TPN} and $V/K_{glutamate}$, respectively, k_9/k_4 and k_9/k_8 are calculated as 3.3 and 5. (Unfortunately, the value obtained for ^{D}V in the absence of ADP does not allow determination of k_9/k_{11}, but the value cannot be less than 15, which is calculated to yield a value of 1.02 for ^{D}V.) Thus, glutamate

[1] Thus, at high and low pH, hydride transfer only partially limits the reaction. The majority of the rate limitation of the reaction is a result of carbon–nitrogen bond cleavage.

is released slightly faster than TPN from E–TPN–glutamate. It is also possible to estimate the amount the off rates for glutamate and TPN were increased by ADP. The maximum changes in V/K_{TPN} and $V/K_{glutamate}$ as a result of the effect of ADP are

$$3.4 = \frac{k_7k_9/(k_8 + k_9)}{k_7k_9/(k_{8'} + k_9)} \quad (13)$$

$$1.8 = \frac{k_3k_9/(k_4 + k_9)}{k_3k_9/(k_{4'} + k_9)} \quad (14)$$

where $k_{4'}$ and $k_{8'}$ represent the off rates in the presence of ADP. Rearrangement of these equations gives $3.4 + 2.4(k_9/k_8) = k_{8'}/k_8$ and $1.8 + 0.8(k_9/k_4) = k_{4'}/k_4$. When 5 and 3.3 are substituted for k_9/k_8 and k_9/k_4, respectively, values of about 15.4 and 4.4 are obtained for $k_{8'}/k_8$ and $k_{4'}/k_4$. Thus, ADP increases the rates of dissociation of TPN and glutamate by 15- and 4.5-fold, respectively.

The binding constant for ADP to central and product complexes is about 10 μM and those to E–glutamate and E–TPN are comparable at about 150 μM, so that ADP binds tighter to central and product complexes than binary substrate complexes. Interestingly, Koberstein et al. (1973) have shown that ADP binds an order of magnitude tighter to free enzyme than to E–TPNH. This is consistent with the overall picture developed in these studies indicating that ADP favors free enzyme.

In direct opposition to the effect of ADP is the effect of GTP. This nucleotide triphosphate is considered an allosteric inhibitor, yet it can be seen that at low levels of glutamate the rate of the reaction is accelerated. It has also been reported that at very low concentrations of GTP (0.8 μM), the binding of TPN to enzyme at low glutamate concentrations is enhanced (Dalziel & Egan, 1972). Thus, it appears that at concentrations lower than those used in this study, GTP enhances $V/K_{glutamate}$ and probably also V/K_{TPN} and decreases the K_i for TPN binding to free enzyme, while at the higher concentrations used in this study GTP has the added effect of competing for enzyme with TPN, as does ADP. In addition, GTP, like ADP, eliminates substrate inhibition by glutamate at least until glutamate concentration is increased to greater than 40 mM, in agreement with Sanner (1975).

If the effects of GTP concentration on V, V/K_{TPN}, and $V/K_{glutamate}$ alone are considered, the allosteric effector increases both V/K's (at least at low concentration) and decreases V. These effects can be explained by proposing that GTP affects the same rate processes as does ADP but in the opposite direction. Thus, GTP decreases k_4, k_8, and k_{11}, which results in an increase in the V/K's and a decrease in V. $V/K_{glutamate}$ is activated about 17-fold, which indicates that the off rate for glutamate from E–TPN–glutamate is decreased approximately by this amount (since k_4 is already 4.5-fold less than k_9 in the absence of GTP). The decrease in V is about 3-fold so that the rates of release of the last two products are decreased by approximately this amount. As a result of decreasing the off rate for glutamate 17-fold, the forward commitment factor increases 17-fold to a value of 56. If the isotope effect is recalculated by using 56 as the forward commitment, a value not different than 1 is obtained, as was observed experimentally. In support of this interpretation of the mode of action of GTP, Andree (1978) has shown that GTP decreases the rate of release of α-ketoglutarate, while di Franco (1974) has shown that the release of not only α-ketoglutarate but also TPNH is decreased.

The binding constant for GTP to central and product complexes is quite tight as it appears to be to free enzyme and E–glutamate with binding constants around 20 μM. There is an indication of negative cooperativity in the binding of GTP in the presence of coenzyme (E–TPN complex), with one binding constant about 10 μM and the other 50 μM. This is in general agreement with the work of Pal & Colman (1979), who showed that there are two sites for GTP in the presence of reduced nucleotide (4.7 and 44 μM) and only one in the absence (42 μM).

The mode of action of these allosteric effectors with glutamate as substrate can also be used to explain their action with monocarboxylic acid substrates such as norvaline or alanine. When a monocarboxylic acid substrate like alanine is used with glutamate dehydrogenase, GTP increases while ADP decreases the $V/K_{alanine}$ value (Tomkins et al., 1961, 1965). Since ADP increases reactant off rates while GTP has the opposite effect, the $V/K_{alanine}$ value should be decreased when ADP is used and increased when GTP is used as an effector.

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