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Properties of the pyridoxaldimine form of glutamate semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase) and analysis of its role as an intermediate in the formation of aminolaevulinate

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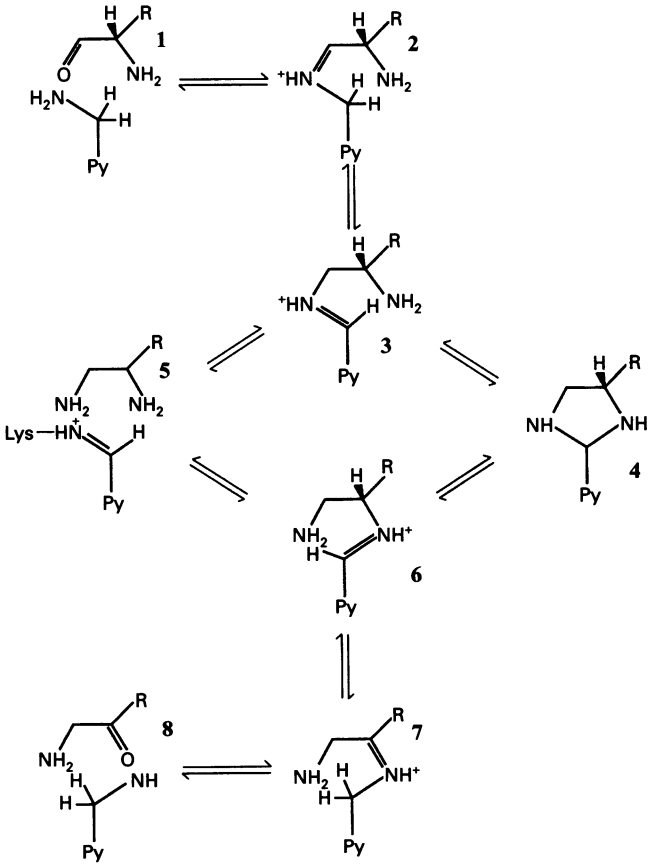
Glutamate semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase; EC 5.4.3.8) was converted into its pyridoxaldimine form by exhaustive replacement of endogenous pyridoxamine phosphate with pyridoxal phosphate. The isomerization of glutamate 1-semialdehyde to 5-aminolaevulinate by this form of the enzyme followed an accelerating time course which indicated that the enzyme initially had no activity but was converted into the active pyridoxamine phosphate form in an exponential process characterized by a rate constant (k) of 0.027 s^{-1}. The pyridoxaldimine form of the enzyme was converted rapidly into the pyridoxamine form by (*S*)-4-aminohex-5-enoate and much more slowly by 4-aminobutyrate. The steady-state velocity of the enzyme increased in a markedly non-linear fashion with increasing enzyme concentration, indicating that the extent of dissociation of an intermediate in the reaction to free diaminovalerate and the pyridoxaldimine form of the enzyme depends upon the concentration of the enzyme.

INTRODUCTION

The aminolaevulinate from which tetrapyrroles are synthesized in plants is derived from glutamate by a series of steps known as the 'C_5 pathway' (Kannangara et al., 1988). The last of these steps is catalysed by glutamate-1-semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1-aminomutase, EC 5.4.3.8; Grimm et al., 1988), an enzyme which is exceptional among transaminases because it transfers amino and oxo functions within the same molecule by interconverting O=CH–CH(–NH_2)–CH_2–CH_2–CO_2H and NH_2–CH_2–CO–CH_2–CH_2–CO_2H. Two recent papers (Smith et al., 1991; Pugh et al., 1992) report results which broadly agree that the major route for the catalysis takes place through the initial combination of the enzyme in its pyridoxamine form (E_M) with the aldehyde group of the substrate and that the reaction proceeds through interconversion of tautomers (**3** and **6** in Scheme 1) of the imine of diaminovalerate with the pyridoxaldimine phosphate form of the enzyme (E_L). However, the mechanisms proposed in the two papers differ in some points of detail, notably concerning the role and activity of E_L and in the requirement for release of the intermediate, diaminovalerate.

On the grounds that reduction of E_L with $NaBH_4$ produced no detectable loss of enzyme activity, Pugh et al. (1992) concluded that E_L did not contribute significantly to catalysis of the reaction. Conversely Smith et al. (1991), using enzyme converted into the E_L form by treatment with dioxovalerate, derived steady-state constants for this form of the enzyme which indicated that it has one third of the activity of E_M. Smith et al. (1991) also proposed that this activity arises only after conversion into E_M by a reaction in which E_L accepts the amino group of glutamate 1-semialdehyde.

The present paper presents results of experiments intended to determine the activity of the E_L form in the conversion of glutamate 1-semialdehyde into aminolaevulinate and its reactivity with other analogous amino acids. Experiments which assess the extent to which diaminovalerate dissociates from the enzyme are also reported.



Scheme 1 Possible mechanisms for the conversion of the glutamate 1-semialdehyde into aminolaevulinate

The lysine residue shown as an aldimine with pyridoxal-5′-phosphate (**5**) has been omitted from the other structures to simplify the diagram.

MATERIALS AND METHODS

Preparation of enzyme and substrate

Glutamate 1-semialdehyde aminotransferase was prepared from pea (*Pisum sativum*) leaves as described by Pugh et al. (1992). (*R,S*)-Glutamate 1-semialdehyde and (*S*)-glutamate 1-semialdehyde were prepared from (*R,S*)-4-aminohex-5-enoate and (*S*)-4-aminohex-5-enoate respectively by ozonolysis as described by Pugh et al. (1991). Both of the samples of aminohexenoate were obtained as gifts from the Marion Merrell Dow Research Centre, Strasbourg, France.

Enzyme assay, fluorimetry and absorption spectrophotometry

Glutamate-1-semialdehyde aminotransferase was assayed by maintaining a solution of substrate (150 mM) and enzyme at 37 °C for 30 s. The reaction was stopped by adding $HClO_4$, and the aminolaevulinate formed in the reaction was quantified by the colour developed with Ehrlich's reagent. The method was described in detail by Pugh et al. (1991).

Increases in fluorescence due to release of pyridoxamine phosphate were determined by using a Perkin–Elmer LS-5 luminescence spectrometer. Absorption spectra were determined with a Beckman model DU7 spectrophotometer.

Analysis of kinetic data

The results of kinetic experiments were best-fitted to the relevant equations (see the Results and discussion section) using the non-linear least-squares curve-fitting routine supplied with the data and analysis software of Sigmaplot 4 (Jandel Corporation, Corte Madera, CA, U.S.A.).

RESULTS AND DISCUSSION

Replacement of cofactor

The fact that the enzyme releases pyridoxamine phosphate when it is treated with strong phosphate solutions (Nair et al., 1991) suggested that, if this process were allowed to occur in the presence of a relatively high concentration of pyridoxal phosphate, a quantitative replacement might be achieved. Accordingly, the enzyme (1.8 μM) was treated with a solution of pyridoxal phosphate (10 μM) in 0.5 M sodium phosphate, pH 6.8, and the resulting progressive increase in fluorescence was monitored. Standard solutions of pyridoxamine phosphate were used for calibration. Figure 1 shows the course of release of pyridoxamine phosphate. After 50 h, when no further dissociation was apparent, the sample was concentrated to 100 μl and separated from low-molecular-mass material by gel filtration on Sephadex G-25. The sample was rediluted to 2 ml and subjected to a repeat of the treatment with pyridoxal phosphate in 0.5 M phosphate. No further increase in fluorescence occurred, indicating that no further pyridoxamine phosphate was available for release. The absorption spectrum of this preparation (Figure 2) has substantially lower absorbance at 330 nm than preparations of the E_L form obtained by treatment with dioxovalerate or other oxo acids (Smith et al., 1991; Pugh et al., 1992), suggesting that those preparations may still have contained some E_M. The fact that the amount of pyridoxamine phosphate released was approximately half of the enzyme concentration (expressed as monomer) indicates that the sample of enzyme was already about half in the E_L form. This observation is consistent with the presence of a substantial 420 nm-absorbing chromophore in the spectrum of the enzyme obtained at the end of the preparation (spectrum included in Figure 2 for comparison). The E_L form

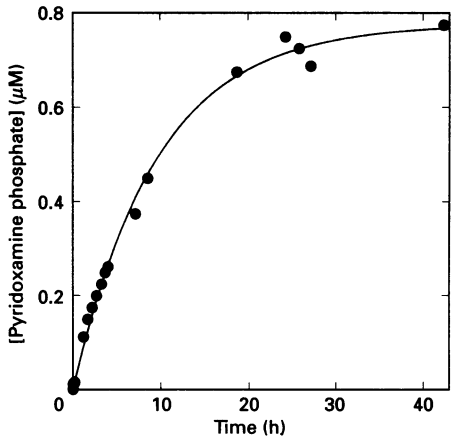


Figure 1 Release of pyridoxamine phosphate during its replacement by pyridoxal phosphate

The enzyme (1.8 μM in 0.1 M Tricine buffer, pH 7.9, containing 0.3 M glycerol) was concentrated to 60 μl and added to 2 ml of 0.5 M sodium phosphate, pH 6.8, containing 10 μM pyridoxal phosphate. Release of pyridoxamine phosphate was monitored by measuring increase in fluorescence (excitation 330 nm, emission 390 nm) and quantified with a standard solution of the same compound.

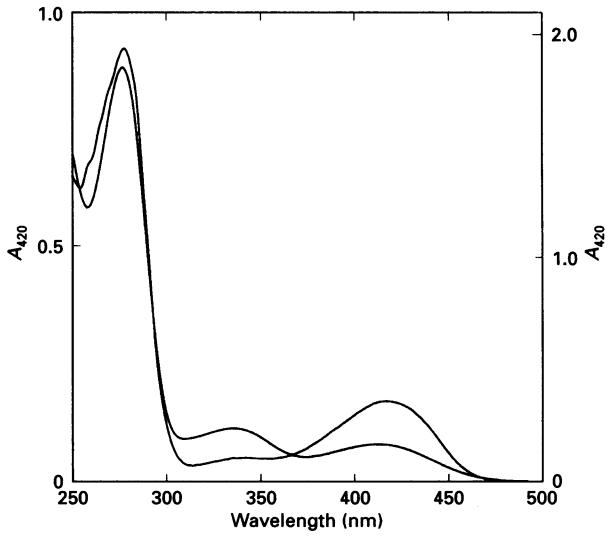


Figure 2 Absorption spectrum of glutamate-1-semialdehyde aminotransferase reconstituted with pyridoxal phosphate

The spectrum with higher absorbance at 420 nm than at 330 nm (absorbance scale 0–1) is that of the enzyme after replacement of pyridoxamine phosphate by pyridoxal phosphate. The other spectrum (absorbance scale 0–2) is that of the enzyme obtained at the end of the preparation.

was therefore present as a significant component of the preparation obtained by purification from pea leaves, indicating that this form of the enzyme exists naturally *in vivo*. It is also clear that, even after the exhaustive methods used to replace the pyridoxamine phosphate, a chromophore with an absorbance maximum at about 340 nm is still present. This could be the tautomer of the protonated aldimine that predominates in non-polar environments (Heinertt & Martell, 1963; Johnson & Metzler, 1970) or a covalently modified form of the coenzyme

similar to that which exists in the β-subform of aspartate aminotransferase (Martinez-Carrion et al., 1967; Metzler et al., 1991).

Course of the reaction with glutamate 1-semialdehyde

Measurement of the activity of glutamate-1-semialdehyde aminotransferase is difficult because the substrate is very unstable at pH values at which the enzyme is active (Pugh et al., 1991). For this reason we routinely employ an assay in which the course of the reaction is monitored for only 30 s. The assay is necessarily discontinuous, and we normally ensure that initial rates are linear by stopping the reaction at 0, 15, and 30 s before determining the concentration of aminolaevulinate produced. The proposal (Smith et al., 1991), that the E_L form of the enzyme is active only after conversion into the E_M form, requires that a transient accelerating phase be present in the progress curve for the reaction. However the assignment of steady-state constants to that form implies that any pre-steady-state transient is rapid. Figure 3 shows the results of an experiment in which the reaction was monitored by stopping it at frequent intervals. The data fit well to eqn. (1), which describes the formation of product by a system in which inactive E_L is converted into active E_M in a first-order process:

$$[P] = vt - v/k(1 - e^{-kt}) \quad (1)$$

Compared with the time of assay, the conversion is slow ($k = 0.027 \pm 0.006\ s^{-1}$), and it is clear that very little product is formed in the first 30 s, thus explaining why borohydride reduction did not give a significant fall in activity when the enzyme was assayed over 30 s (Pugh et al., 1992). The estimate for v given by the curve fit was $0.23 \pm 0.02\ \mu M \cdot s^{-1}$, a value which is close to that expected for an assay beginning with an untreated preparation of the enzyme at the same concentration. These results are consistent with our earlier conclusion (Pugh et al., 1992) that the initial rate of reaction catalysed by the E_L form of the enzyme is zero and that it is not therefore active as a catalyst of aminolaevulinate formation from glutamate 1-semialdehyde. The proposal of Smith et al. (1991) that E_L is converted into E_M in a reaction with the amino group of glutamate 1-semialdehyde is also confirmed by this experiment. However, the transition is relatively slow, and would be in progress throughout the 3 min duration of the single-point assay employed by Smith et al. (1991). We would argue that assigning steady-state kinetic constants to the E_L form implies, incorrectly, that this form itself has activity.

Reaction of E_L with 4-aminobutyrate and 4-aminohex-5-enoate

It is known that diaminovalerate converts the E_L form of the enzyme rapidly to the E_M form (Smith et al., 1991; Pugh et al., 1992). In an attempt to determine the structural requirements for this conversion, 4-aminobutyrate and 4-aminohex-5-enoate were assessed for their capacity to act as amino donors by observing the resulting changes in absorption spectrum. The reaction with 4-aminobutyrate was slow enough to observe directly by recording the associated decrease in A_{410}. The reactions observed were apparently first-order (Figure 4), and the concentration-dependence of the observed first-order constants fit reasonably well to a rectangular hyperbola (eqn. 2), indicating that the slow transamination ($k = 0.36 \pm 0.006\ min^{-1}$) is preceded by a rapidly reversible initial binding step ($K = 7.3 \pm 1.3\ \mu M$):

$$k_{obs.} = k[S]/(K + [S]) \quad (2)$$

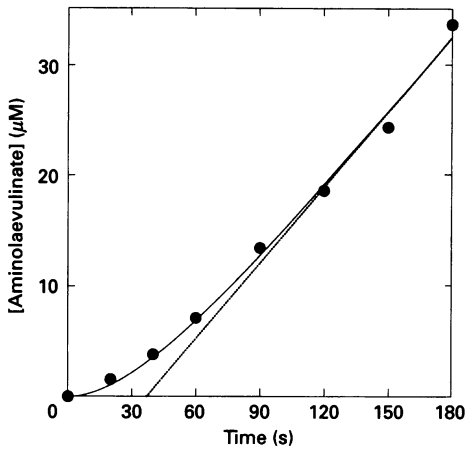


Figure 3 Time course of formation of aminolaevulinate from glutamate 1-semialdehyde catalysed by the E_L form of glutamate-1-semialdehyde aminotransferase

The reaction was initiated by mixing 120 μl of the E_L form of the enzyme (1.8 μM at pH 6.7 in 0.24 M glycerol/80 mM Tricine/130 mM Mops) with 15 μl of 0.81 mM (*S*)-glutamate 1-semialdehyde. Aminolaevulinate concentrations were determined as described in the text. The continuous line through the points was that which gave the best fit to eqn. (1) with $k = 0.027\ s^{-1}$ and $v = 0.23\ \mu M\ s^{-1}$. The straight line has a slope equal to v and intersects the time axis at 37 s ($1/k$).

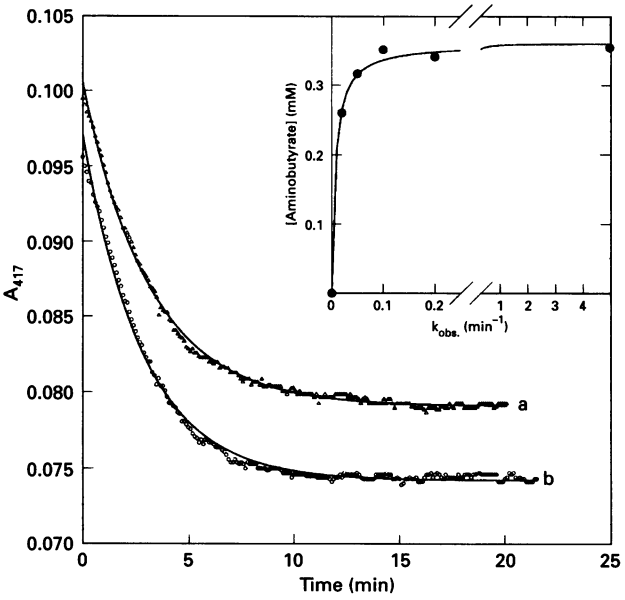


Figure 4 Reaction of the E_L form of glutamate-1-semialdehyde aminotransferase with 4-aminobutyrate

The enzyme (5 μM) was treated with 4-aminobutyrate at different concentrations, and the change in absorbance at 410 nm was monitored. The reactions observed at 0.05 μM (curve a) and 0.5 μM (curve b) aminobutyrate are shown. In the inset the dependence of the apparent first-order rate constants ($k_{obs.}$) on concentration is illustrated. The continuous line in the inset is that of best fit to eqn. (2).

Lower concentrations of 4-aminobutyrate were not used in this experiment in order that the reactions would be essentially pseudo-first-order.

When (*S*)-4-aminohex-5-enoate was used as the amino donor, spectral changes associated with the reaction (Figure 5a) were

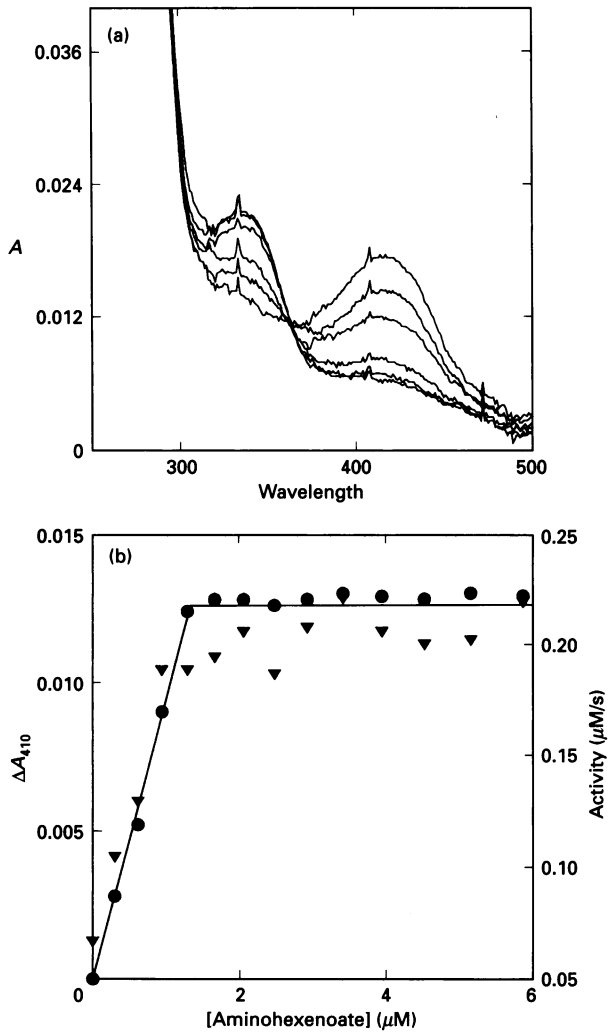
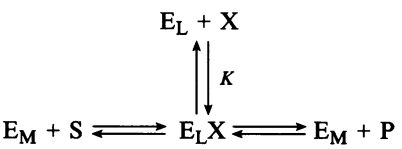


Figure 5 Reaction of the E_L form of glutamate-1-semialdehyde aminotransferase with 4-aminohex-5-enoate

The enzyme (1 ml, 3.4 μM) was treated with successive 1.8 μl portions of 167 μM (*S*)-4-aminohex-5-enoate so that stepwise increases in concentration of approx. 0.3 μM were obtained. After each addition the absorption spectrum was determined (**a**) and 50 μl of the mixture was removed for enzyme assay. The dependence of the extent of absorbance change at 410 nm and of enzyme activity on aminohexenoate concentration are shown (**b**).



Scheme 2

complete within the mixing time, even at very low concentrations of the compound. The activity of the enzyme in catalysing the glutamate 1-semialdehyde transamination reaction was measured after each addition of aminohexenoate and was found to increase in parallel with the absorbance changes (Figure 5b). Although the E_L form of the enzyme is converted into E_M with a concomitant increase in activity, the activities reached are much lower than when diaminovalerate is used to maintain the E_M form of the enzyme. One explanation of this observation would be that aminohexenoate remains reversibly bound to the enzyme and acts as an inhibitor.

The fact that aminohexenoate reacts much more rapidly than does 4-aminobutyrate is probably due to limitations in rotation about the C^{α}–N bond with the coenzyme caused by the presence of the vinyl group. Aminohexenoate is a close structural analogue of diaminovalerate, and it seems probable that occupancy of a site normally taken by CH_2–NH_2 of diaminovalerate by the vinyl group of the analogue maintains the C^{α}–H bond in a position orthogonal to the coenzyme imine π-electron plane. Abstraction of the proton on this carbon, the rate-limiting step of other transaminations, is facilitated by this orthogonal arrangement (Dunathan, 1966). Aminohexenoate resembles glutamate 1-semialdehyde even more than it does diaminovalerate. However, n.m.r. analysis (Hoober et al., 1988) shows that glutamate 1-semialdehyde exists very largely as the much bulkier hydrate, and it seems possible that the reaction of glutamate 1-semialdehyde with E_L is slow, because this structure cannot easily be accommodated.

Dependence of initial velocity on enzyme concentration

Inclusion of diaminovalerate increases the rate of formation of aminolaevulinate even when the reaction is initiated with the enzyme in the E_M form (Smith et al., 1991), indicating that free E_L is a component of the steady state during the reaction. Since this E_L must have arisen by transamination of glutamate 1-semialdehyde, it follows that diaminovalerate must also be present in equimolar amounts. Scheme 2 illustrates the essential features of a mechanism in which reversible dissociation of an intermediate, E_LX, occurs as a side reaction to the main pathway.

The dissociation of E_LX (governed by dissociation constant, *K*) conforms with eqn. (3). At saturating substrate concentration the expression relating $[E_LX]$ to total enzyme concentration, $[E_{tot.}]$, has a quadratic form (eqn. 4):

$$K = \frac{([E_{tot.}]-[E_LX])^2}{[E_LX]} \quad (3)$$

$$[E_LX]^2-(2[E_{tot.}]+K)[E_LX]+[E_{tot.}]^2 = 0 \quad (4)$$

At substrate concentrations below saturation the equation is still quadratic, but the coefficients of $[E_LX]^2$ and $[E_LX]$ are increased. For example, in the work described in the present paper, where the substrate concentration used was $2K_m$, the relevant equation is eqn. (5):

$$2.25[E_LX]^2-(3[E_{tot.}]+K)[E_LX]+[E_{tot.}]^2 = 0 \quad (5)$$

Thus, if dissociation occurs as a side reaction, as depicted in Scheme 2, the initial steady-state velocity of the reaction ($v = k[E_LX]$) will increase non-linearly with enzyme concentration. In an experiment intended to test this proposal for the transamination of glutamate 1-semialdehyde, we measured the rate of formation of aminolaevulinate from glutamate 1-semialdehyde at different enzyme concentrations in the range 0.5–4 μM, in both the presence and absence of added diaminovalerate. The results are shown in Figure 6. The upper level of enzyme concentration at which this experiment can be conducted by using conventional mixing methods is limited to about 4 μM, because increasing substrate depletion at higher enzyme concentrations gives non-linear reactions. Nevertheless, it is clear that, whereas in the presence of added diaminovalerate the measured

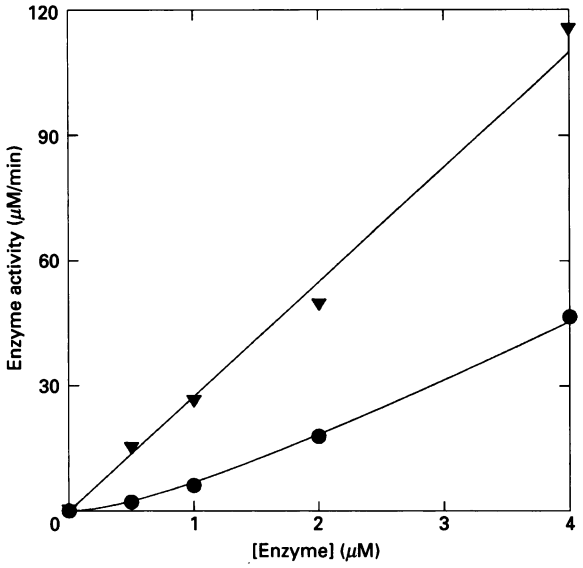


Figure 6 Dependence on enzyme concentration of the initial velocity of the glutamate-1-semialdehyde aminotransferase-catalysed formation of aminolaevulinate

The enzyme was assayed at the concentrations indicated by the standard method described in the Materials and methods section with (▼) and without (●) the inclusion of 0.22 μM diaminovalerate. The continuous line for the experiment conducted in the absence of added diaminovalerate was constructed by applying the standard solution for a quadratic equation to eqn. (5) using a value of 1.7 μM for the dissociation constant, K. The values of $[E_LX]$ obtained in this way were multiplied by 27 min^{-1}, this being the $k_{cat.}$ determined from the experiment in the presence of diaminovalerate (▼).

activity varies linearly with enzyme concentration, in the absence of added diaminovalerate measured activity follows an upward curve which approaches the line obtained in the presence of diaminovalerate. The data fit well to eqn. (5) using a K value of 1.7 μM. This result shows that, at enzyme concentrations up to 4 μM, dissociation of E_L and diaminovalerate is extensive. It can be predicted that this dissociation will be significant even at much higher concentrations. By considering the yield of enzyme in our preparation from pea leaf chloroplasts, it can be estimated that, if the enzyme is uniformly distributed throughout the chloroplast stroma, then its concentration would be approx. 2–4 μM and consequently dissociation would also be significant *in vivo*.

Two mechanisms have been proposed for the part played by diaminovalerate. Pugh et al. (1992) proposed that the reaction proceeds through interconversion of the two isomeric imines (**3** and **6** in Scheme 1) via the geminal diamine (**4**). However, Smith et al. (1991) proposed that the interconversion of **3** and **6** was through the intermediacy of diaminovalerate (**5**). Despite the appealing symmetry of the 'geminal diamine' mechanism, it is clear that, over the concentration range studied, the enzyme does dissociate extensively into diaminovalerate and E_L. Present evidence does not indicate whether the formation and release of diaminovalerate is an obligatory step in the formation of aminolaevulinate or simply a side reaction to the *geminal* diamine pathway.

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