Reactions of glutamate semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1 aminotransmutase) with vinyl and acetylenic substrate analogues analysed by rapid scanning spectrophotometry

Robin J. TYACKE,* Roberto CONTESTABLE,† Bernhard GRIMM,‡ John L. HARWOOD* and Robert A. JOHN*†

*School of Molecular and Medical Biosciences, University of Wales College of Cardiff, P.O. Box 911, Cardiff CF1 3US, Wales, U.K.
†Institute of Plant Genetics and Crop Plant Research, O-4325 Gatersleben, Germany

The reactions occurring when glutamate-1-semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1 aminotransmutase, EC 5.4.3.8) was treated with two potential mechanism-based inactivators, namely 4-aminohex-5-enoate and 4-aminohex-5-ynoate, have been investigated by monitoring rapid transient changes in the absorption spectrum of the enzyme’s prosthetic group, pyridoxal 5’-phosphate. In both cases a short-lived chromophore absorbing maximally at about 500 nm was formed in a few milliseconds. In the case of the vinyl analogue (4-aminohex-5-enoate) this chromophore, considered to be a quinonoid intermediate, converted rapidly into the pyridoxamine phosphate form of the co-enzyme in a single turnover which was accompanied by negligible inactivation. However, slow inactivation of the enzyme by this compound was observed when the enzyme was made to undergo multiple turnovers by including the efficient aldehyde substrate, succinic semialdehyde. The acetylenic compound, aminohexynoate, produced more complex spectral changes with the consecutive formation of compounds absorbing maximally at 496 nm, 450 nm, 564 nm and 330 nm. The enzyme was 90 % inactivated by aminohexynoate within 10 s and thereafter lost no further activity unless aldehyde substrate was added. Mechanisms and kinetic constants consistent with the observations are proposed for each compound. The observation that the acetylenic compound is a much more potent inactivator than its vinyl analogue is attributed to the occurrence of a conjugated allene as intermediate.

INTRODUCTION

Glutamate semialdehyde aminotransferase (glutamate-1-semialdehyde 2,1 aminotransmutase, EC 5.4.3.8) is an essential enzyme in the pathway that leads to the synthesis of chlorophyll and other tetrapyrroles in plants and some bacteria [1]. The mechanism whereby it catalyses its normal reaction, namely the isomerization of glutamate 1-semialdehyde (GSA) to 5-aminoalavulinate (ALA), has been described recently [2,3]. The reaction (Scheme 1) begins with the formation of an aldimine (E<sub>u</sub>-GSA) between the substrate aldehyde group and the amino group of the cofactor of the enzyme in its pyridoxamine 5’-phosphate form (E<sub>u</sub>). Transfer of a proton from C4’ of the cofactor to C1 of the substrate results in the formation of an external aldimine (E<sub>i</sub>-5-DAVA) of the co-enzyme with the 5-amino group of 4,5-diaminovalerate (DAVA). The continuing reaction entails isomerization to an imine (E<sub>i</sub>-4-DAVA) with the 4-amino group of DAVA by one of two possible routes. At least one of the two E<sub>i</sub>-DAVA intermediates dissociates into the free pyridoxalimine form (E<sub>i</sub>) and DAVA to an extent that is governed by the concentration of enzyme itself[4]. This process makes the enzyme vulnerable to reactions with analogues that react initially by forming an imine with the co-enzyme in its pyridoxalimine form.

The strategic position of glutamate semialdehyde aminotransferase in plant metabolism, and the absence of the enzyme from animals, which instead use 5-aminoalavulinate synthase to synthesize ALA from succinyl-CoA and glycine [5], makes this enzyme a potential target for selective herbicides. The substrate of the enzyme, GSA, is structurally similar to the neurotransmitter 4-aminobutyrate (GABA) and it is already known that mechanism-based inhibitors, known to be potent inactivators of mammalian 4-aminobutyrate aminotransferase, also inhibit glutamate semialdehyde aminotransferase [6,7]. Smith and Grimm [7] have proposed that gabaculine inactivates glutamate semialdehyde aminotransferase by a mechanism analogous to that found for the action of the same compound on 4-aminobutyrate aminotransferase [8] where, after transamination to the ketimine, the gabaculine adduct aromatizes to a stable 3-anthranilyl secondary amine of pyridoxamine phosphate. The vinyl and acetylenic analogues of GSA, 4-aminohex-5-enoate

![Diagram of Scheme 1](image1)

Scheme 1 Intermediates in the conversion of glutamate 1-semialdehyde into 5-aminoalavulinate

Abbreviations used: GSA, glutamate 1-semialdehyde; ALA, 5-aminoalavulinate; DAVA, 4,5-diaminovalerate; E<sub>i</sub>, glutamate semialdehyde aminotransferase with co-enzyme as aldime of pyridoxal 5’-phosphate; E<sub>u</sub>, glutamate semialdehyde aminotransferase in its pyridoxamine phosphate form.

† To whom correspondence should be addressed.
and 4-aminohex-5-ynoate (Figure 1) might be expected to react with glutamate semialdehyde aminotransferase by mechanisms analogous to those operating on 4-aminobutyrate aminotransferase and ornithine aminotransferase [9,10] where covalent modification of the enzyme protein occurs. However, it has been reported that the vinyl derivative converts the E_r form of the enzyme into E_m without significant inactivation [4].

The present paper reports the results of investigations into the reactions of glutamate semialdehyde aminotransferase with 4-aminohex-5-enoate and 4-aminohex-5-ynoate, and compares observations with those obtained using the dissociable intermediate DAVA. The study relies extensively on the observation of rapid transient changes in the absorption spectrum of the enzyme's cofactor, pyridoxal 5'-phosphate.

**EXPERIMENTAL**

**Reagents**

R and S enantiomers of 4-aminohex-5-enoate, and racemic 4-aminohex-5-ynoate were generously provided by Marion Merrell-Dow International Research Centre, Strasbourg, France. (S)-Glutamate 1-semialdehyde was synthesized from (S)-4-aminohex-5-enoate as described previously [11].

**Preparation and assay of glutamate semialdehyde aminotransferase**

Early experiments were conducted with the enzyme prepared from pea leaves as described earlier [2]. In later experiments, the enzyme used was that encoded by the gene from the photosynthetic bacterium *Synechococcus PCC 6301*, expressed in *Escherichia coli* and purified as described earlier [12] except that ammonium sulphate fractionation between 300 g l⁻¹ and 400 g l⁻¹ was included after the column chromatography. After the experiments were repeated with the enzyme obtained from both pea and *Synechococcus* and no significant differences were observed. The activity of the enzyme was assayed by the method of Pugh et al. [11] and its concentration was determined using ε_{350} 35000 M⁻¹·cm⁻¹ [3].

**Conversion of enzyme into the pyridoxalimine form**

At the end of the preparation, the enzyme is obtained as a mixture of E_r and E_m forms with E_m predominating. Conversion into E_r was achieved using succinic semialdehyde. To ensure virtually complete conversion, the equilibrium was displaced by gel filtration using the method of Dixon and Severin [13] as follows. The mixture of enzyme forms (2 ml, 200 μM) was treated with succinic semialdehyde to give a concentration of 2 mM. Succinic semialdehyde (1 ml, 1 mM) was added to a column of Sephadex G-25 (30 cm × 0.8 cm), and the enzyme loaded immediately after the succinic semialdehyde solution had completely entered the column. The high-molecular-mass fraction was collected in the void volume of the column, well separated from small molecules.

**Rapid scanning spectrophotometry and data analysis**

An SF-61 stopped-flow spectrophotometer fitted with an MG-6000 rapid scanning diode array detector (Hi-Tech, Salisbury, U.K.) was used to obtain absorption spectra within 2 ms of mixing. From each stopped-flow determination, 96 spectra were collected, each consisting of 366 data points spanning a spectral range from 290 nm to 580 nm. Data acquisition, visualization and single wavelength analysis were accomplished with the IS-2 kinetics software suite provided with the instrument or with the curve-fitting procedures of Sigmaplot (Jandel, Corte Madeira, CA, U.S.A.). By cross-sectioning the spectral data at specific wavelengths, time domain (kinetic) data were generated which were fitted to an appropriate rate equation. Goodness of fit was judged on the basis of an even distribution of residuals between experimental results and theoretically predicted data. Analysis of difference spectra was achieved by best fitting the difference between two log-normal curves using the equation described by Johnson and Metzler [14] and the curve-fitting software of Sigmaplot. Fixed values for width at half height (65 nm) and skewness (1.4) corresponding to the 418 nm chromophore of the enzyme itself were used in the fitting. Simulations of processes containing reversible steps were constructed using the data manipulation software 'Scientist' (Micromath, Salt Lake City, UT, U.S.A.)

**RESULTS AND DISCUSSION**

**Reaction with aminohexenoate**

It has already been reported [4] that racemic aminohexenoate converts E_r into E_m within the time taken for manual mixing and that the equilibrium is strongly in favour of E_m. To determine which of the enantiomers was reactive, (R)- and (S)-aminohexenoate (15 μM) were mixed separately with the E_r form of the enzyme (15 μM). Whereas the (S)-enantiomer produced complete conversion into E_m within the mixing time no reaction was observed with the (R)-enantiomer after several hours. Further experiments were therefore conducted exclusively with the (S)-enantiomer. Figure 2 shows the changes in absorption spectrum that occurred when E_r was mixed with aminohexenoate (4 mM) in the stopped-flow diode-array spectrophotometer. The main features are a large decrease in absorbance at 415 nm and an approximately equal increase in absorbance at 340 nm. Another process in which absorbance rose rapidly and then fell, was clearly observable at 495 nm (Figure 2). When the first 20 ms of reaction was excluded from the data analysis, the changes at all three wavelengths fitted very well to a single exponential with the same apparent first-order constant (k_app = 7.1 s⁻¹; Figure 2). At the end of this process both 420 nm and 490 nm chromophores had completely disappeared, showing that conversion into E_m was effectively irreversible. In a second experiment absorbance at a single wavelength (490 nm) was monitored over 2.5 s using an exponentially decreasing time base for data collection. The concentration of aminohexenoate was varied between 0.9 mM and 10 mM and the absorbance changes observed in the first 1.2 s are shown in Figure 3(a). Because the course of reactions at the higher concentrations were very similar only one example of the results obtained above 2 mM is shown. Although the reaction observed at each concentration fitted well to an equation describing the concentration of B in a system A→B→C in which both steps are irreversible, the best fit values for the rate
The enzyme (0.2 mM) was mixed in equal volume with 4-aminohex-5-enoate (4 mM). The first spectrum, that with highest absorbance at 420 nm, was taken at 3.6 ms and subsequent spectra are those observed at 40 ms intervals. The inserts show absorbance changes taking place at the single wavelengths indicated. The lower two panels of the insert show results collected over 1 s. Each is accompanied by the continuous line predicted for a first-order process characterized by \( k_{\text{mix}} = 7.1 \text{ s}^{-1} \). The upper panel shows results collected over 200 ms and a continuous line predicted for the [B] in a system A \( \rightarrow \) B \( \rightarrow \) C for which \( k_1 = 7.1 \text{ s}^{-1} \) and \( k_2 = 103 \text{ s}^{-1} \).

constants characterizing both steps increased significantly with increasing aminohexenoate concentration. Such behaviour is inconsistent with this simple mechanism and the results were therefore analysed according to a mechanism which included an isomerization in which both forward and backward steps are kinetically significant.

The rise and fall of EQ in such a mechanism can be described by the sum of two exponential terms (eqn 1) in which the relaxation times \( \tau_1 \) and \( \tau_2 \) are related to the rate constants of Scheme 2 by \( 1/\tau_1 = k_1[S]/(K_+ + [S]) \) and \( 1/\tau_2 = k_2[S]/(K_+ + [S]) + k_{-2} + k_{-1} \).

\[
\text{[EQ]} = A_1 \exp(t/\tau_1) + A_2 \exp(t/\tau_2)
\] (1)

The absorbance changes (A) were fitted to eqn (1) and the resulting values for \( 1/\tau_1 \) and \( 1/\tau_2 + 1/\tau_2 \) were best fitted to the above relationships by non-linear least-squares regression. The fit of \( 1/\tau_1 \) (Figure 3b, insert) gave a value of \( K_+ = 1.21 \pm 0.05 \text{ mM} \). This value was used in fitting \( 1/\tau_1 + 1/\tau_2 \) (Figure 3b, insert). Combining the two results gave solutions for the rate constants themselves: \( k_{-2} = 23 \text{ s}^{-1} \), \( k_{-1} = 45 \text{ s}^{-1} \), \( k_{-2} = 15 \text{ s}^{-1} \). The values for these rate constants and the dissociation constant \( K_+ \) were used to simulate the mechanism shown as Scheme 2. Figure 3(b) compares the simulation with the original results. The simulation required that an initial absorbance for E,, of 0.075 be assumed, but the true initial absorbance at 416 nm where E,, absorbs maximally was 0.25, implying that EQ has an absorption coefficient at 490 nm which is three times smaller than that of E,, at 416 nm. The 490 nm chromophore is almost certainly the quinonoid compound formed by proton abstraction and would be expected to have an absorption coefficient much higher than E,,[16]. It seems likely therefore that the ‘species’ EQ consists of

---

**Figure 2** Changes in absorption spectrum on mixing glutamate semialdehyde aminotransferase (E,) with 4-aminohex-5-enoate

The enzyme (0.2 mM) was mixed in equal volume with 4-aminohex-5-enoate (4 mM). The first spectrum, that with highest absorbance at 420 nm, was taken at 3.6 ms and subsequent spectra are those observed at 40 ms intervals. The inserts show absorbance changes taking place at the single wavelengths indicated. The lower two panels of the insert show results collected over 1 s. Each is accompanied by the continuous line predicted for a first-order process characterized by \( k_{\text{mix}} = 7.1 \text{ s}^{-1} \). The upper panel shows results collected over 200 ms and a continuous line predicted for the [B] in a system A \( \rightarrow \) B \( \rightarrow \) C for which \( k_1 = 7.1 \text{ s}^{-1} \) and \( k_2 = 103 \text{ s}^{-1} \).

---

**Figure 3** Dependence of absorbance changes at 490 nm on aminohexenoate concentration

The enzyme (50 \( \mu \text{M} \)) after mixing was reacted with aminohexenoate at different concentrations. (a) shows the results obtained at aminohexenoate concentrations of 0.45 mM, 0.58 mM, 0.86 mM, 2 mM and 6.7 mM. The trace reaching the highest absorbance is that obtained with the highest concentration. The inset of panel (b) shows plots used to determine the individual rate constants in Scheme 2 and the main panel (b) shows a simulation of the mechanism using the rate constants given in the text.
two chromophores in rapid equilibrium and that one of them is not spectrally distinguishable from $E_1$ itself.

When the enzyme in the $E_1$ form was treated with aminohexenoate alone, no significant loss of activity was detectable (less than 5%). However, when succinic semialdehyde (which converts $E_1$ into $E_2$) was added, enzyme activity was lost progressively. Assay of residual activity was complicated by the presence of high concentrations of aminohexenoate which acts as a competitive inhibitor. For this reason the enzyme was separated from low-molecular-mass material by gel filtration before assay. The specific enzyme activity was found to fall progressively, but very slowly, with a half-time of approximately 2 h.

Reactions with aminohexenoate

Changes in absorption spectrum occurring during the reaction of the $E_1$ form of the enzyme with (R,S)-4-aminohex-5-ynoate revealed multiple consecutive processes widely separated in time scale. The early phases of the reaction were examined by mixing the enzyme (200 μM) with aminohexenoate (4 mM) in the stopped-flow diode-array spectrophotometer. Samples of the spectra obtained from different periods in the reaction are shown in Figure 4. Initially the enzyme has no absorbance at 492 nm but the first spectrum (3.6 ms, Figure 4a) already shows a chromophore absorbing maximally at this wavelength. The concentration of this chromophore continued to rise for about 20 ms at the expense of the 420 nm-absorbing chromophore present at the start. Absorbance at 330 nm increased simultaneously but by much less. Figure 4(b) shows changes in absorption spectrum taking place in the period from 40 ms to 1 s. The main feature of this part of the reaction is the very large increase in absorbance at 564 nm and the accompanying smaller decrease at both 420 nm and 492 nm. No clear isosbestic point is present when all of the spectra are shown. However, the situation becomes clearer when the phases of the reaction are separated. The results shown in Figure 4(c) are taken from a separate experiment conducted over 200 ms. When the first 40 ms have been excluded, the sharp isosbestic point at 505 nm can be seen clearly and it is apparent that the increase at 564 nm is accompanied by decreases at both 420 nm and 492 nm. Figure 4(d) shows that the changes taking place in the period from 0.6 s to 1 s are characterized by another sharp isosbestic point at 455 nm. In this phase of the reaction the 564 nm chromophore appears to be arising from a chromophore absorbing at 420 nm.

Figure 5 shows the progress of the reaction at selected wavelengths. Although in the period from 40 ms to 200 ms the
increase at 564 nm was accompanied by a fall at 492 nm with a clear isosbestic point at 505 nm, the fact that the rate of formation of the 564 nm chromophore is low at around 20 ms when the 492 nm chromophore is at its maximum concentration, shows that there must be another intermediate between these two chromophores. Consideration of the spectral changes in the period from 0.6 s to 1 s (Figure 4d), when the complicating contribution of the 492 nm chromophore has become negligible, confirms that this is the case. We conclude that the 564 nm chromophore is not formed directly from the 492 nm chromophore but from an intermediate which, like the starting material, absorbs maximally at approximately 420 nm.

During this phase of the reaction the enzyme can be seen to have developed an intense purple colour but this too has only a transient existence. Figure 6 shows how the absorbance at 564 nm falls over a period of several minutes. During this phase there is a simultaneous, but much smaller, rise in absorbance at 330 nm. Measurements of enzyme activity show that activity of the enzyme had fallen to 10% of the original within 10 s of mixing. However, there is no further loss of activity thereafter. Addition of dioxovalerate (1 mM) to convert $E_{1a}$ into $E_{1b}$ resulted in further loss of activity to less than 5% of the original.

The process observed at 492 nm fitted well to an equation predicting the concentration of the intermediate B in two consecutive irreversible first-order reactions A→B→C (Figure 5). Best fit values for the rate constants were $148 \pm 6$ s$^{-1}$ and $7.5 \pm 0.3$ s$^{-1}$. Because the 492 nm chromophore, again presumed to be a quinonoid, does not accumulate to high concentrations we deduce that the lower of the rate constants characterizes the first step. The same rate constants also provided an adequate fit to the much smaller absorbance change seen at 330 nm. These constants are very similar to those characterizing the corresponding process seen with the vinyl analogue when fitted to this mechanism.

Analysis of the second half of the rise at 564 nm as a single exponential gave $k_{\text{app}} = 1.2 \pm 0.02$ s$^{-1}$ and predicted a final absorbance of 0.36. This process is very much faster than the subsequent fall in absorbance at this wavelength and is thus sufficiently well separated to make the estimate of rate constant reliable. The fall in absorbance at 420 nm does not fit well to a single exponential. Although a good fit was obtained to two exponential processes having the same sign the reciprocal relaxation times found were 16 s$^{-1}$ and 1.2 s$^{-1}$. Because of the kinetic behaviour observed for the 492 nm and 564 nm chromophores we fitted the results to a system consisting of three exponential processes having rate constants of 7.5 s$^{-1}$, 148 s$^{-1}$, and 1.2 s$^{-1}$. This gave an equally good fit (Figure 5) with the amplitudes of the processes being respectively 0.031, 0.043, and 0.09.

The increase in absorbance at 564 nm shows a clear lag as would be expected for a scheme composed of three consecutive reactions but, a good fit to the lag phase could not be obtained using 7.5 s$^{-1}$ as one of the rate constants. One reason for this difficulty is the small but definite contribution made at 564 nm by the 492 nm intermediate. The fit shown was achieved using constants of $17$ s$^{-1}$, $148$ s$^{-1}$, and $1.2$ s$^{-1}$ in an equation predicting the concentration of D in a sequence of reactions A→B→C→D. An equally good fit was obtained for a system consisting of only two consecutive components and the two lower rate constants.

The final fall in absorbance and accompanying increase at 330 nm (Figure 6) fit well to a single exponential ($k_{\text{app}} = 0.07$ min$^{-1}$). We therefore consider that a reaction scheme consisting of five consecutive intermediates together with a minor
side reaction that forms $E_m$ is the simplest that can satisfy these observations (Scheme 3). However, it is very likely that some of the steps are significantly irreversible.

**Reaction with DAVA**

When the enzyme in its $E_s$ form was reacted with the intermediate DAVA, no chromophore absorbing in the 500 nm region was detected. As expected from earlier reports [2,3] the 420 nm chromophore was converted into a 330 nm chromophore consistent with the conversion of $E_s$ into $E_m$. However, the course of the reaction was biphasic and was analysed as a double exponential (Figure 7). A good fit was obtained at all wavelengths.

The amplitude of the absorbance change occurring in the faster of the two processes was seen to change sign at 390 nm. Figure 7 shows the difference spectrum obtained when the amplitude of this change was plotted against wavelength. Fitting of log-normal curves [14] to this difference spectrum indicated that the best fit was obtained for a scheme in which a chromophore absorbing maximally at 418 nm is converted into one absorbing maximally at 386 nm. The most obvious assignment for the 386 nm chromophore is pyridoxal 5'-phosphate itself because this absorbs maximally at 388 nm [17]. However, participation of the co-enzyme as the free aldehyde seems mechanistically unlikely. Interestingly a chromophore with a similar absorbance maximum, 386 nm, has been observed in a Tyr-225 → Phe mutant of aspartate aminotransferase in which a hydrogen bond to O3' of the co-enzyme has been removed [18]. In that case it was clearly established that the cofactor is still bound as an internal aldimine [19], but with ground state ($S_0$) destabilized. It is thus possible that the transient 387 nm chromophore observed is one of the external aldimines of DAVA.

There is considerable similarity between the spectral changes observed when the two unsaturated compounds react with $E_s$. In each case there is evidence for the existence of transient chromophores absorbing in the region 420–440 nm and additionally at 490 nm. A major difference between the two reactions is that the acetylenic compound produces a 564 nm chromophore which is stable over many minutes and which is not seen at all with the vinyl compound. A second, probably related difference is that the acetylenic compound inactivates the enzyme within 10 s of mixing whereas the vinyl compound requires the addition of an aldehyde to bring about significant inactivation and even then the process is very slow.

We propose that the mechanisms shown in Scheme 4 provide broadly satisfactory explanations for our observations although it is recognized that other mechanisms, notably the 'enamine' mechanism proposed by Metzler and colleagues [20] are equally plausible for the steps that result in covalent modification of the enzyme. The mechanisms depicted in Schemes 4(a) and 4(b) differ in that IIa is protonated at C4' of the co-enzyme whereas IIb is protonated at C5 of the substrate. The fact that complete inactivation of the enzyme requires addition of an aldehyde substrate suggests that protonation of C4' of the co-enzyme does occur as a minor reaction when aminohexynoate is the substrate. Similarly the apparent existence of another aldimine in rapid equilibrium with the quinonoid suggests that protonation at C6 does occur when aminohexynoate is the substrate but that the intermediate formed is not sufficiently reactive to inactivate the

![Figure 7 Absorbance changes associated with the reaction with DAVA](image)

The enzyme in its $E_s$ form (0.2 mM) was mixed with DAVA (4 mM) and absorption spectra recorded at intervals. (a) Samples of results observed at 420 nm (●) and at 375 nm (■) together with the continuous line predicted by two exponential processes using $k_1 = 160$ s$^{-1}$ and $k_2 = 2$ s$^{-1}$. (b) Experimental points (●) represent the amplitude of the fast phase of the reaction at the wavelengths indicated. The solid line through the points is that predicted by best fitting the results to two log-normal curves with that having $\lambda_{\text{max}}$ at 386 nm being subtracted from that with $\lambda_{\text{max}}$ at 418 nm.
The most notable feature of a comparison of the reactions of the two unsaturated analogues with that of DAVA itself is the absence of any significant accumulation of quinonoid intermediate with DAVA. Despite this apparently major difference, the rate of conversion into Eₘₚ is faster with aminohexenoate than with the 'normal' amine substrate. The possibility exists that, as has been proposed for cytosolic aspartate aminotransferase in its reaction with aspartate [23], the reaction proceeds by a concerted 1,3-protoprotropic shift and that the quinonoid does not exist as a true intermediate on the pathway.

This work was supported by contract no. CHRX-CT93-0179 from the Human Capital and Mobility programme of the European Union and by a studentship from BBSRC (formerly SERC) to R.J.T.

REFERENCES


Received 12 December 1994/10 February 1995; accepted 28 February 1995