An investigation of transient intermediates in the reaction of 2-methylglutamate with glutamate decarboxylase from *Escherichia coli*

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Several intermediates in the reaction of 2-methylglutamate with glutamate decarboxylase from *Escherichia coli* were detected by stopped-flow spectrophotometry and by rapid-scanning spectrophotometry after conventional mixing. Structures were assigned to intermediates on the basis of kinetic and spectral evidence. In the early stages of the reaction an intermediate with the properties expected of a geminal diamine accumulated significantly. Changes consistent with the conversion of this species into the external aldimine were also observed. The course of product formation was determined and linked with spectral changes taking place in the bound coenzyme. The effect of the minor decarboxylation-dependent transamination that accompanies the major reaction was analysed.

INTRODUCTION

Amino acids methylated at the α -carbon atom have been useful in elucidating the catalytic mechanism of one group of pyridoxal 5'-phosphate-dependent enzymes, the aminotransferases, because they are able to undergo the initial steps required for formation of an external

aldimine with the coenzyme but cannot react further because of the absence of a proton on the α -carbon atom. The first three intermediates in the transamination reaction, namely Michaelis complex, geminal diamine and external aldimine (Scheme 1) also occur in the reactions catalysed by the other groups of pyridoxal 5'-phosphate-dependent enzymes. However, in the reac-

Scheme 1. Structures of intermediates in the decarboxylation of 2-methylglutamate

I, Michaelis complex with coenzyme bound as internal aldimine with lysine; $\lambda_{\text{max.}} = 420 \text{ nm}$. II, Geminal diamine in which nitrogen atoms from both substrate and lysine are bonded to 4'-C of coenzyme; $\lambda_{\text{max.}} = 350 \text{ nm}$. III, External aldimine with substrate; $\lambda_{\text{max.}} = 420 \text{ nm}$. IV, Quinonoid intermediate resulting from loss of CO_2 . V, External aldimine with product of simple decarboxylation, 4-aminovalerate. VI, Ketimine of transamination product, 4-oxovalerate, with enzyme; this dissociates to give enzyme in inactive pyridoxamine 5'-phosphate form.

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tions catalysed by the amino acid decarboxylases the reaction is not halted at the external aldimine stage because these enzymes abstract a carboxy group and not a proton. Nevertheless the presence of the 2-methyl group has a significant effect on catalysis by the decarboxylases in that it accentuates a minor reaction that leads to progressive loss of activity as the decarboxylation proceeds. This minor reaction has been well characterized in the case of pig kidney dopa (3,4-dihydroxyphenylalanine) decarboxylase (Sourkes, 1954; Borri-Voltattorni *et al.*, 1971; O'Leary & Baughn, 1975, 1977; Minelli *et al.*, 1979) and clearly shown to be due to an accompanying transamination that leads to simultaneous production of amine and keto products. The classical mechanism (Braunstein, 1973) proposed to explain catalysis by pyridoxal 5'-phosphate-dependent enzymes can readily be adapted to explain the formation of two products from the same substrate by proposing that the carbanion-quinonoid intermediate (Scheme 1, structure IV) formed after elimination of the carboxy group may be protonated either at the α -carbon atom from which the carboxy group was eliminated to give the amine, or at the 4'-carbon atom of the coenzyme to give the corresponding aldehyde or ketone.

Glutamate decarboxylase from *Escherichia coli* has been shown to undergo the decarboxylation-dependent transamination, and an early report (Huntley & Metzler, 1968) indicates that complex spectral changes accompany the reaction. The present paper is an account of investigations into the reaction aimed at characterizing the various steps.

EXPERIMENTAL

Materials

Glutamate decarboxylase was prepared from *E. coli* (A.T.C.C. 11246) by the method of Yang & Metzler (1979), with the following modifications. Streptomycin sulphate rather than protamine sulphate was used to precipitate nucleic acid, and the solution was heated at 37 °C for 60 min in the presence of 10 mM-glutaric acid. These modifications were made at the suggestion of Dr. Hiroshi Ueno. Ion-exchange chromatography was carried out on DEAE-Sepharose from Pharmacia. The final crystallization step was omitted. L-Glutamate, DL-2-methylglutamate, 4-aminobutyrate and pyridoxal 5'-phosphate were bought from Sigma Chemical Co. Dr. L. J. Fowler provided a sample of DL-4-aminovalerate. Other reagents were from BDH Chemicals.

Spectrophotometry and fluorimetry

Rapid-mixing experiments were performed on a Durrum-Gibson stopped-flow spectrophotometer interfaced with a BBC model B microcomputer. Interfacing was achieved by using a fast analogue-to-digital converter capable of sampling the analogue signal at 20 μ s intervals and of recording 5000 data points from each experiment. Slower experiments and absorbance-spectra determinations were carried out on a Beckman model DU7 spectrophotometer also interfaced with the BBC microcomputer. Fluorescence polarization measurements were made with a Perkin-Elmer model MPF-2A spectrofluorimeter equipped with polarizing filters.

Quenched-flow measurements

Analyses of the reaction products during the period from 0.5 s to 5 s were carried out with an apparatus

generously lent by Dr. David Yates of University of Bristol and described elsewhere (Eccleston *et al.*, 1980). Substrate solution (0.1 ml) was pneumatically driven into a stirred solution of enzyme, and after a prescribed interval the reaction was quenched by 25% (v/v) HClO₄ (0.1 ml). After centrifugation to remove protein, the solution was adjusted to pH 10 with 3.5 M-KOH (20 μ l) and again centrifuged, to remove KClO₄. Reactions lasting longer than 10 s were quenched manually and processed as above.

Measurement of aminovalerate

Aminovalerate formed by the decarboxylation of 2-methylglutamate was quantified as the *o*-phthaldialdehyde derivative by using h.p.l.c. with a 25 cm $\times$ 4.6 mm C₁₈ column developed by an isocratic system with 60% (v/v) methanol in 0.1 M-sodium acetate buffer, pH 6.2. The flow rate during separation was 1 ml/min, and pre-column formation of derivatives was carried out for 1 min in a solution of 0.1 M-NaHCO₃ adjusted to pH 10 with NaOH and containing *o*-phthaldialdehyde (0.1 mg/ml) and 2-mercaptoethanol (0.1%, v/v).

Kinetic analyses

First-order rate constants for reactions in which there appeared to be no interference from other reactions were determined by using the stored data and a program based on the method of Guggenheim (1926). We have found this method to be useful in that no subjective assessment of end point need be made. More complex reactions were best fitted to the relevant kinetic scheme by using non-linear least-squares methods provided by programs from the Numerical Algorithms Group (NAG), Oxford, U.K. These programs (NAG-EO4FCF, NAG-EO4FDF and NAG-EO4JAF) were run on a Honeywell DPS-8/70M computer with a Multics operating system, or as an external job on an ICL 2980 computer. Experimentally obtained data, stored on discs by using the interfaced BBC microcomputer, were transferred directly to the mainframe computer via the RS 232 port by using an EPIC operating system developed by University College Cardiff Computing Centre. When an analytical solution describing the relevant kinetic scheme was available, then this was used to represent the theoretical model for curve-fitting. When no analytical solution could be found, as for example in Scheme 2, the model was represented by numerical integration by using the NAG library sub-routine DO2BBF.

RESULTS AND DISCUSSION

Absorbance changes at 420 nm

At pH 4.6 glutamate decarboxylase has an absorbance band centred at 420 nm having a molar absorption coefficient of 10000 M⁻¹·cm⁻¹ (Fonda, 1971). When the reaction with 2-methylglutamate was monitored at this wavelength by both stopped-flow and manual mixing methods several consecutive phases were observed. An initial fall in absorbance, complete within the mixing time of the stopped-flow apparatus (2.5 ms), was followed by a slower fall (Fig. 1) lasting roughly 10 s. After remaining constant for several seconds, the direction of the absorbance change was reversed, and absorbance began to rise at an increasing rate. Before the full initial absorbance at 420 nm had returned, the direction of the change reversed once more, rather

Fig. 1. Early phase of the reaction of glutamate decarboxylase with 2-methylglutamate

Absorbance changes at 420 nm due to the early phases of reaction between enzyme ($5 \mu\text{M}$) and 2-methylglutamate (20 mM) are shown. The reaction was carried out in the stopped-flow apparatus at 22°C and pH 4.6 in pyridine/HCl buffer ($[\text{Cl}^-] = 0.2 \text{ M}$).

Fig. 2. Later phases of the reaction of glutamate decarboxylase with 2-methylglutamate

Glutamate decarboxylase ($20 \mu\text{M}$) was treated with 2-methylglutamate (0.25 M) and the course of the reaction was monitored ($\blacktriangle$) at 420 nm in a cell of 0.5 cm pathlength and ($\bullet$) by stopping the reaction at the intervals indicated and measuring the 4-aminovalerate formed. Experimental conditions were as described for Fig. 1.

abruptly, and absorbance fell to a constant level (Fig. 2). The phases of the reaction were sufficiently discrete to permit their separate investigation.

Phase 1. Analysis of the substrate concentration dependence of the initial observable fall in absorbance revealed that both the extent of this absorbance change (ΔA) and the apparent first-order constant characterizing

Fig. 3. Dependence of the early phases of the reaction of glutamate decarboxylase with 2-methylglutamate on 2-methylglutamate concentration

The enzyme ($5 \mu\text{M}$) was treated with 2-methylglutamate at the concentrations indicated and the observed absorbance change ($\blacksquare$) and apparent first-order rate constant for the change ($\bullet$) were measured. Points represent the mean of three measurements and bars indicate 1 s.d. The continuous lines are the least-squares best fit to eqns. (1) and (2) respectively. Relevant constants are given in the text.

it ($k_{\text{obs.}}$) increased with substrate concentration (Fig. 3). The different dependence of these two measurements on substrate concentration suggests a two-step reversible process, as depicted by the first two steps of Scheme 2. The first-order rate constants ($k_{\text{obs.}}$) observed at each substrate concentration were best fitted to eqn. (1) by using the least-squares method described in the Experimental section:

$$k_{\text{obs.}} = k_{-1} + \frac{k_{+1}[\text{S}]}{K_s + [\text{S}]} \quad (1)$$

The values returned by the analysis were $K_s = 652 \pm 350 \text{ mM}$, $k_{+1} = 3.9 \pm 1.4 \text{ s}^{-1}$ and $k_{-1} = 0.38 \pm 0.05 \text{ s}^{-1}$. ΔA was fitted to the hyperbola given by eqn. (2) by using the method of Wilkinson (1961):

$$\Delta A = \frac{\Delta A_{\infty}[\text{S}]}{K' + [\text{S}]} \quad (2)$$

ΔA_{∞} represents the amplitude change expected at infinite glutamate concentration. If the reactions seen are due to two consecutive reversible reactions as in Scheme 2, then K' of eqn. (2) has the value $K_s/(1 + k_{+1}/k_{-1})$. The value of K' determined from the separate constants was 58 mM, whereas that derived from the amplitude data was 10 mM. The value for ΔA_{∞} was 0.076.

Fig. 4(a) compares the absorption spectrum of free enzyme with that of the mixture 5 s after mixing. The major change is an increase in a species absorbing maximally near 350 nm at the expense of the 420 nm-absorbing free enzyme. Quenched-flow experiments carried out over this period demonstrated that no aminovalerate product was formed, although the sensitivity of the method was sufficient to detect a molar amount equivalent to that of the enzyme present. It is clear therefore that during this phase no decarboxylation

Fig. 4. Spectral changes occurring during the reaction of glutamate decarboxylase with 2-methylglutamate

(a) Comparison of spectra of the free enzyme with that of the mixture of intermediates existing after 5 s. The enzyme in the absence of substrate has a single absorbance maximum at 420 nm. Collection of data for the spectrum with absorbance maxima at 350 nm and 420 nm was begun 5 s after mixing. (b) Spectra taken during phase 2. Collection of data for the spectrum with lowest absorbance at 420 nm and highest at 350 nm was begun 5 s after mixing and completed at 20 s. The remaining spectra were taken at 27 s intervals. (c) Spectra taken during phase 3. The first spectrum with highest absorbance at 420 nm and lowest at 330 nm was taken 5 min after mixing. Remaining spectra were taken at 6, 7, 10 and 12 min. All spectra were determined with 15 μ M-enzyme and 0.25 M-2-methylglutamate. Reaction conditions were as for Fig. 1 except that for (c) the temperature was 15 $^{\circ}$ C.

of the substrate has taken place. The most likely interpretation of the proposed absorbance changes is that ES represents an initial Michaelis complex and that E'S is the geminal diamine intermediate that must precede the external aldimine. An absorbance maximum

at about 350 nm is fully consistent with the structure proposed (Johnson & Metzler, 1970).

Phase 2. The sharp rise in A_{420} that occurs after about 20 s was first reported by Huntley & Metzler (1968). The full spectral changes occurring during this phase are shown in Fig. 4(b). Clearly the species formed at the expense of the 350 nm-absorbing species has an absorbance maximum at 420 nm, suggesting strongly that it is an aldimine. The parallel increase in 4-aminovalerate concentration (Fig. 2) suggests that it may be the external aldimine formed with the substrate and that the rate of decarboxylation to 4-aminovalerate is proportional to its concentration. Alternatively it could be an aldimine formed with the accumulating product. We have eliminated this second alternative by an experiment in which fresh enzyme was added to a solution of enzyme that had already been treated for 4 min with 2-methylglutamate. The changes at 420 nm followed exactly the same course. We suggest therefore that the intermediate formed in this process is simply the external aldimine with the substrate and that the presence of the 2-methyl group greatly slows its formation. Analysis of the time course of absorbance changes such as those of Fig. 2 showed that the data do not fit a simple mechanism in which several steps precede the formation of an intermediate absorbing at 420 nm. Essentially the amplitude of the rise cannot be reconciled with the subsequent relatively rapid fall. A good fit to the results can, however, be obtained if the rate constant governing conversion of the 420 nm intermediate is made to increase in proportion to the concentration of the intermediate itself (Scheme 2). Glutamate decarboxylase is a hexamer (Strausbauch & Fischer, 1970) and thus has the potential for interaction between subunits. A mechanism that would give rise to the type of kinetic behaviour observed is one in which the rate constant for the formation of the 420 nm-absorbing intermediate increases with the occupancy of sites by this species. Scheme 2 is intended to represent this mechanism by assuming that the observed value for the rate constant governing this step increases linearly with the fraction of the enzyme existing as external aldimine. The best fit of data to Scheme 2 was derived by numerical integration. E, ES and E'S were assumed to be at equilibrium. The constants derived were $k_2 = 0.0017 \text{ s}^{-1}$, $k_3 = 0.013 \text{ s}^{-1}$ and $k_4 = 0.03 \text{ s}^{-1}$. Molar absorption coefficients at 420 nm determined for the intermediates were for EX $9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$, for EY $1550 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and for the initial complexes assumed to be in rapid equilibrium (E, ES and E'S) a mean of $3400 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Phase 3. Much evidence indicates that the final fall in A_{420} is due to the transamination reaction, which results in conversion of the coenzyme into the pyridoxamine form and thus renders the enzyme incapable of combining further with amino acid substrate. The results of Fig. 2 show that decarboxylation slows and finally stops during this phase. Additionally the spectral change associated with this stage (Fig. 4c) shows the formation

Scheme 2

Fig. 5. Changes in polarized fluorescence during reaction of glutamate decarboxylase with 2-methylglutamate

(a) Glutamate decarboxylase ($10 \mu\text{M}$) was treated with 2-methylglutamate (0.2 M). Polarized fluorescence emitted at 390 nm was monitored perpendicular (lower line) and parallel (upper line) to the plane of the exciting light (330 nm). Experimental conditions were as for Fig. 1 except that the temperature was 4°C . Arbitrary units of fluorescence are expressed relative to that obtained with free pyridoxamine 5'-phosphate with polarizer and analyser parallel. (b) Formation of bound ($\bullet$) and free ($\blacktriangle$) pyridoxamine 5'-phosphate deduced from polarized fluorescence as described in the text.

of a 330 nm -absorbing species, as expected for pyridoxamine 5'-phosphate.

Dissociation of coenzyme

After several minutes' reaction at 20°C with 2-methylglutamate the enzyme solution became noticeably turbid. At lower temperatures (4°C) this turbidity, which is presumably due to denaturation of the apoenzyme formed as pyridoxamine 5'-phosphate dissociates, did not occur. Fluorescence polarization was used to distinguish between bound and free pyridoxamine 5'-phosphate. The results shown in Fig. 5 show fluorescence-intensity measurements obtained either with polarizer and analyser parallel ($I_{\parallel}$) or with polarizer and analyser perpendicular ($I_{\perp}$). Fig. 5(b) shows the concentrations of bound and free pyridoxamine 5'-phosphate calculated from these results. The calculation is based on the assumption that only pyridoxamine 5'-phosphate-

enzyme and free pyridoxamine 5'-phosphate contribute to the fluorescence intensity and that they are formed consecutively. The values of $I_{\parallel}$ and $I_{\perp}$ at any time are therefore due to the concentrations of bound and free pyridoxamine 5'-phosphate and the respective fluorescence-intensity coefficients for each species with analyser parallel or perpendicular. By using a standard solution of free pyridoxamine 5'-phosphate the ratio of parallel and perpendicular fluorescence intensity coefficients was found to be:

$$\phi_{\parallel}^f/\phi_{\perp}^f = 1.08$$

Assuming that at the beginning of the reaction free pyridoxamine 5'-phosphate is negligible a corresponding ratio for enzyme-bound pyridoxamine 5'-phosphate was obtained:

$$\phi_{\parallel}^b/\phi_{\perp}^b = 2.64$$

This value approaches that expected for a totally rigid system (Cantor & Schimmel, 1980), and is consistent with the model of a small fluorescing species bound to a high- M_r , and therefore slowly rotating, protein. After 1 h the fluorescence changes follow a simple first-order process and are assumed to be due entirely to dissociation. The changes in parallel and perpendicularly polarized fluorescence should therefore conform to the relationship:

$$(\phi_{\parallel}^f - \phi_{\parallel}^b)/(\phi_{\perp}^f - \phi_{\perp}^b) = 5.44$$

These three relationships were used to evaluate the respective fluorescence-intensity coefficients, which, on taking $\phi_{\parallel}^f$ arbitrarily as 1, are $\phi_{\perp}^f = 0.93$, $\phi_{\parallel}^b = 0.74$ and $\phi_{\perp}^b = 2.0$ respectively. The concentrations of bound and free pyridoxamine 5'-phosphate were derived from the data of Fig. 5(a) by using equations derived from these coefficients and are presented in Fig. 5(b).

DISCUSSION

Although the formation of 4-aminovalerate occurs only transiently, results such as those of Fig. 2 show that a minimum value for k_{cat} for the decarboxylation of 2-methylglutamate is 4 s^{-1} . Bearing in mind that at least one step leading to the initial formation of the external aldimine is very much slower than this, it is clear that such a step is not included in the many turnovers that must occur during the transient production of 4-aminovalerate. This observation suggests that once the enzyme exists substantially as an external aldimine with 2-methylglutamate the slow initial step no longer occurs. This would appear to be an example of hysteresis as described by Frieden (1970).

An estimate of the ratio of simple decarboxylation to decarboxylation-dependent transamination may be obtained by comparing the course of formation of enzyme-bound pyridoxamine 5'-phosphate with the estimate of k_{cat} above. The value obtained is 150, which compares with a value of 100 estimated by Huntley & Metzler (1968) on the basis of the ratio of the amine and keto products formed. The ratio of these two reactions when glutamate is the substrate is 10000 (Sukhareva & Braunstein, 1971), whereas for the reaction catalysed by free pyridoxal these two reactions occur approximately equally (Kalyankar & Snell, 1962). It appears therefore that a major function of the apoprotein in glutamate decarboxylase is to limit the extent of the unwanted side reaction and that steric considerations imposed by the

2-methyl group reduce the enzyme's efficacy in this respect.

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REFERENCES

- Borri-Voltattorni, C., Minelli, A. & Turano, C. (1971) *FEBS Lett.* **17**, 231–235
- Braunstein, A. E. (1973) *Enzymes* 3rd Ed. **9**, 479–480
- Cantor, C. R. & Schimmel, P. R. (1980) *Biophysical Chemistry: Part 1, The Conformation of Macromolecules*, pp. 454–457, Freeman Press, New York
- Eccleston, J. F., Messerschmidt, R. G. & Yates, D. W. (1980) *Anal. Biochem.* **106**, 73–77
- Fonda, M. L. (1971) *J. Biol. Chem.* **246**, 2230–2240
- Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788–5799
- Guggenheim, E. A. (1926) *Philos. Mag. Ser. VII* **2**, 538–543
- Huntley, T. E. & Metzler, D. E. (1968) in *Symposium on Pyridoxal Enzymes* (Yamada, K., Katunuma, N. & Wada, H., eds.), pp. 81–90, Maruzen, Tokyo
- Johnson, R. J. & Metzler, D. E. (1970) *Methods Enzymol.* **18A**, 433–471
- Kalyankar, G. D. & Snell, E. E. (1962) *Biochemistry* **1**, 594–600
- Minelli, A., Charteris, A. T., Borri-Voltattorni, C. & John, R. A. (1979) *Biochem. J.* **183**, 361–368
- O'Leary, M. H. & Baughn, R. L. (1975) *Nature (London)* **253**, 52–53
- O'Leary, M. H. & Baughn, R. L. (1977) *J. Biol. Chem.* **252**, 7168–7173
- Sourkes, T. L. (1954) *Arch. Biochem. Biophys.* **51**, 444–456
- Strausbauch, P. H. & Fischer, E. H. (1970) *Biochemistry* **9**, 226–233
- Sukhareva, B. S. & Braunstein, A. E. (1971) *Mol. Biol. (Moscow)* **5**, 241–252
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Yang, B. I.-Y. & Metzler, D. E. (1979) *Methods Enzymol.* **62**, 528–551

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