Pyridoxal Phosphate-Sensitized Photoinactivation of Glutamate Decarboxylase from *Clostridium perfringens*

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(Received 28 January 1974)

1. L-Glutamate decarboxylase (EC 4.1.1.15) from *Clostridium perfringens* was inactivated by exposure to visible light at pH 6.2. 2. Inactivation does not occur at pH 4.6 or in the absence of bound pyridoxal phosphate. 3. On prolonged photo-oxidation six histidine residues per molecule of enzyme were destroyed. 4. The loss of six cysteine residues per molecule occurred both in irradiated samples and in controls oxygenated in the dark. 5. This dark-oxidation of cysteine residues is apparently required before the photo-oxidation process. 6. The absorbance, fluorescence and circular-dichroism properties of the enzyme as well as its elution volume during Sephadex gel-filtration were unaffected by prolonged irradiation. 7. However, an apparently homogeneous product of photo-oxidation could be separated from the control enzyme by ion-exchange chromatography. 8. The K_m for L-glutamate was unchanged in an irradiated sample retaining 22% of control activity. 9. These data and the catalytic role of imidazole residues at the active sites of amino acid decarboxylases are discussed.

Pyridoxal phosphate bound to specific lysine residues was shown to sensitize the photo-oxidation of histidine residues present at the active site of 6-phosphogluconate dehydrogenase (Rippa & Pontremoli, 1969) and aldolase (Davis *et al.*, 1970). Thus the protein-bound pyridoxal phosphate was proposed as a specific photosensitizer of the amino acids at the binding site not only of enzymes originally bearing this chromophore, but also of those enzymes that are inhibited by binding with this compound. However, irradiation of pig heart aspartate aminotransferase at wavelengths corresponding to the absorbance maxima of the bound pyridoxal phosphate did not appreciably affect the catalytic activity and the spectral properties of this enzyme, whereas irradiation of the apoprotein in the presence of lumichrome caused irreversible loss of catalytic activity, owing to photo-oxidation of histidine residues (Burrige & Churchich, 1970).

In view of these conflicting results, it was decided to test photosensitization by pyridoxal phosphate with another purified and characterized pyridoxal phosphate-enzyme, L-glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) from *Clostridium perfringens* (Cozzani, 1970; Cozzani *et al.*, 1970; Cozzani & Bagnoli, 1971). Further, the demonstration that the active sites of bacterial amino acid decarboxylases contain histidine residues in close proximity to the chromophore would deserve

special interest because of its mechanistic and evolutionary implications (Strausbauch & Fischer, 1970; Boeker & Snell, 1972).

Since the binding site of the coenzyme appears to be of crucial importance for both structural and functional properties of the whole molecule of glutamate decarboxylase from *Cl. perfringens* (Cozzani *et al.*, 1970; Cozzani & Bagnoli, 1973), we decided to investigate, in addition to the light-induced modifications of catalytic activity and amino acid composition, some comparative spectroscopic and kinetic properties of the native and irradiated enzyme.

Experimental

Photo-oxidation of glutamate decarboxylase

This was performed by exposing enzyme solutions in 0.05M-sodium acetate buffer, pH 6.2, containing about 2mg of protein/ml, to the light of four 300W tungsten bulbs, placed on either side of a water bath with Plexiglass walls (Benassi *et al.*, 1967). The temperature was maintained at about 20°C. A slow stream of O₂ was flushed through the irradiated solution. Samples were taken at fixed intervals for enzyme-activity and spectral studies, as well as for amino acid analyses. As a control, protein solutions were kept in the dark either in the presence or in the absence of a stream of O₂.

Spectral measurements

Absorption spectra were recorded with a Hitachi-Perkin-Elmer model 139 or a Cary 15 spectrophotometer. Fluorescence spectra were monitored by a Hitachi-Perkin-Elmer MPF2A spectrophotofluorimeter, equipped with a 150W xenon arc lamp; the absorbance of the analysed solutions was kept at about 0.1 at the excitation wavelength to minimize inner-filter and concentration quenching effects. The spectra were corrected for the wavelength-dependence of the excitation and detector systems. Circular-dichroism spectra were obtained by means of a Cary 60 recording spectropolarimeter equipped with a 6001 circular-dichroism accessory. Cylindrical quartz cells were used with 10cm, 1.0cm and 0.05cm optical path. The usual instrumental precautions were taken to avoid artifacts. The data were expressed in terms of $[\Theta]$, the mean residue molecular ellipticity, in units of $\text{degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

Amino acid analyses

These were performed on a Carlo Erba 3A27 amino acid analyser. Acid hydrolyses were done in 6M-HCl at 110°C in sealed evacuated Pyrex tubes for 22h. Phenylalanine, valine and leucine were used as references in calculating the number of amino acid residues susceptible to photo-oxidation. The content of methionine and of methionine sulfoxide was evaluated after hydrolysis in 3.75M-NaOH (Jori *et al.*, 1968). Cysteine thiol groups were determined by selectively converting them into cysteic acid by Crystal Violet-sensitized photo-oxidation (Jori *et al.*, 1969). Tryptophan content was determined spectrophotometrically by the method of Bencze & Schmid (1957).

Glutamate decarboxylase activity

This was measured as a routine by the manometric procedure described by Cozzani *et al.* (1970). The K_m for L-glutamate of a sample of glutamate decarboxylase submitted to prolonged photo-oxidation and of its control were determined by a sensitive spectrophotometric method previously reported (Cozzani, 1970).

Materials

Apoenzyme-free glutamate decarboxylase was purified from *Cl. perfringens* as previously described (Cozzani *et al.*, 1970; Cozzani & Bagnoli, 1971). Crystal Violet was a reagent-grade product of Fluka A.G., Basel, Switzerland. Other reagents and buffer salts were reagent-grade products.

Results

Photoinactivation of glutamate decarboxylase and destruction of histidine residues

Glutamate decarboxylase from *Cl. perfringens*

undergoes progressive inactivation during irradiation with visible light, at pH 6.2. However, the loss of catalytic activity does not follow first-order kinetics, as is usual for the photosensitized oxidation of amino acid residues (Spikes & Straight, 1967). As shown in Fig. 1, the glutamate decarboxylase activity is not appreciably affected by 30min irradiation with respect to the unirradiated control. During the next 90min the glutamate decarboxylase activity of the irradiated sample falls to about 30% of that of the control, and, after 6h photo-oxidation, over 20% of the enzyme activity is still recovered. The glutamate decarboxylase activity of the control preparation, oxygenated in the dark, does not significantly change throughout the whole experiment, but appreciable inactivation occurs early after dilution at pH 6.2 of the stock enzyme solution stored at pH 4.6, according to previous observations (Cozzani *et al.*, 1970). The lag shown in Fig. 1 is abolished when a control preparation, oxygenated in the dark, is submitted to irradiation.

The inactivation observed in the above experimental conditions is dependent on the presence of bound pyridoxal phosphate, as shown by experiments of irradiation of the apoenzyme. Apo-(glutamate decarboxylase) was obtained from the pyridoxamine phosphate form of the enzyme, as described by Huntley & Metzler (1968) on dialysis at pH 6.2. A sample of apoenzyme irradiated for 6h and a control preparation oxygenated in the dark recovered 67 and 65%, respectively, of the original activity of the holoenzyme, on reconstitution with pyridoxal phosphate.

Fig. 1. Time-course of photoinactivation of glutamate decarboxylase from *Clostridium perfringens*

The enzyme samples were irradiated with visible light at pH 6.2. The residual activity was calculated as a percentage of the activity of the controls oxygenated in the dark.

Table 1. *Amino acid composition of irradiated and unirradiated glutamate decarboxylase from Clostridium perfringens*

The enzyme sample was irradiated for 6 h at pH 6.2, as described in the text. The control preparation was oxygenated in the dark. The amino acids were determined chromatographically on a Carlo Erba 3A27 amino acid analyser, after 22 h hydrolysis in 6M-HCl at 110°C. Methionine, cysteic acid and tryptophan were determined as reported in the Experimental section. Aspartic acid and glutamic acid include both amide and free acid.

Amino acid	Irradiated sample			Control		
	(g of amino acid residues/100 g)		Average no. of residues/49 000 g	(g of amino acid residues/100 g)		Average no. of residues/49 000 g
	Run 1	Run 2		Run 1	Run 2	
Lysine	8.26	8.19	31.9	8.21	8.33	32.1
Histidine	1.48	1.50	5.8	1.76	1.78	6.9
Arginine	4.18	4.24	16.3	4.05	4.14	15.9
Aspartic acid	11.77	12.08	46.2	12.04	11.71	46.1
Threonine	6.95	6.75	26.7	6.78	7.09	26.9
Serine	6.48	6.69	25.7	6.71	6.44	25.6
Glutamic acid	12.36	12.62	48.4	12.17	12.41	47.7
Proline	3.72	3.46	13.9	3.43	3.70	13.8
Glycine	8.23	7.86	31.2	8.02	7.91	30.9
Alanine	5.62	5.87	22.3	5.51	5.73	21.8
Valine	7.16	7.21	29.6	7.19	7.24	30.1
Methionine	1.73	1.83	6.8	1.69	1.94	7.0
Isoleucine	5.48	5.19	20.7	5.49	5.22	20.8
Leucine	9.12	8.77	34.7	8.95	9.08	34.9
Tyrosine	3.45	3.61	13.7	3.72	3.57	14.1
Phenylalanine	4.06	4.20	16.0	4.21	3.94	15.8
Cysteic acid	5.19	5.42	20.6	5.49	5.25	20.8
Tryptophan	1.82		7.1	1.77		6.9

In our experimental conditions several amino acid residues, namely histidine, methionine, cysteine and tryptophan, could undergo photodestruction sensitized by pyridoxal phosphate (Jori *et al.*, 1969), provided that they were situated sufficiently close to the sensitizer. Amino acid analyses and spectro-photofluorimetric studies on glutamate decarboxylase samples submitted to photo-oxidation for different times indicated that only histidine residues were destroyed by exposure to light, whereas loss of cysteine occurred both in the irradiated samples and in controls kept in the dark. This result is shown in Table 1, which lists the amino acid composition of a glutamate decarboxylase sample irradiated for 6 h and that of the control oxygenated in the dark. The loss of one histidine residue per 49 000 g of glutamate decarboxylase after prolonged photo-oxidation is consistent with previous data on the quaternary structure of this protein, indicating that the native enzyme contains six polypeptide chains, each of 48 000–50 000 daltons, with one molecule of pyridoxal phosphate bound per monomer (Cozzani & Bagnoli, 1971, 1973). Failure to inactivate the enzyme after 6 h irradiation at pH 4.6 confirmed the selective photo-destruction of histidine, which is sensitive to photo-oxidation only in its unprotonated form. On the basis of these results we assume that one histidine residue is present

at the active site of each monomer, in close proximity to the pyridoxal phosphate.

The loss of cysteine was investigated by Crystal Violet-sensitized photo-oxidation of the native, dark-oxygenated and irradiated enzyme: as previously shown (Jori *et al.*, 1969), this procedure yields a selective conversion of cysteine into cysteic acid, whereas cystine is unaffected. After treatment of stock enzyme solutions either with performic acid (Hirs, 1967) or by irradiation in the presence of Crystal Violet, we obtained the same number of cysteic acid residues by amino acid analysis thus ruling out the presence of any disulphide bridge in the native enzyme molecule. On the other hand, no cysteic acid was detected on amino acid analysis of the dark-oxygenated or irradiated samples before Crystal Violet-sensitized photo-oxidation. However, after Crystal Violet-sensitized photo-oxidation, a loss of cysteic acid residues was observed in the irradiated and oxygenated glutamate decarboxylase compared with the untreated enzyme. As shown in Table 2, this loss occurs within the first 30 min both in the irradiated samples and in their oxygenated controls. In contrast, the kinetics of histidine photodestruction fairly well parallel that of inactivation of the irradiated samples. The involvement of cysteine oxidation in the photodestruction of histidine was confirmed by the following experiment.

A sample of glutamate decarboxylase was treated in 0.05 M-sodium acetate buffer, pH 6.2, with *p*-chloromercuribenzoate at a molar ratio of 1:6, conditions providing complete but reversible inhibition of catalytic activity (Cozzani & Bagnoli, 1971). This sample and its control not treated with *p*-chloromercuribenzoate were irradiated for 6h, then assayed for activity before and after dialysis against 10 mM-cysteine in 0.05 M-sodium acetate buffer, pH 4.6 at 4°C. On dialysis the *p*-mercuribenzoate-treated enzyme recovered over 90% of its original activity, whereas the control, which lost about 75% of its activity during the photo-oxidation process, was practically unaffected by the treatment with cysteine.

Structural and kinetic properties of the native and irradiated enzyme

Spectroscopic studies on glycogen phosphorylase (Shaltiel & Cortijo, 1970) provided evidence that the enzyme-bound pyridoxal phosphate is a sensitive built-in probe for the active site and its micro-environment. Therefore the absorbance and fluorescence properties of the bound pyridoxal phosphate were checked in irradiated samples of glutamate decarboxylase and in their controls. At pH 6.2 pyridoxal phosphate is bound to glutamate decarboxylase mainly in a form that absorbs at 333 nm, whose structure is not yet clarified, and only in minor proportion with a hydrogen-bonded aldimine linkage showing maximum absorbance at 410 nm (Cozzani *et al.*, 1970). As shown in Table 3, there was no significant change in the light-absorption and emission parameters of glutamate decarboxylase as a consequence of irradiation.

Circular dichroism was used to detect any

Table 2. *Comparative kinetics of photo-oxidation of histidine and oxidation of cysteine during irradiation of glutamate decarboxylase from Clostridium perfringens*

Conditions for irradiation of the samples and oxygenation of the controls are reported in the text. The proteins were then subjected to Crystal Violet-sensitized photo-oxidation before amino acid analysis. Apart from the variations of histidine and cysteic acid reported in the Table, no appreciable change was found in the other amino acids analysed. Each value is the average of two determinations.

Treatment of the enzyme	Amino acid content (residues per enzyme monomer)	
	Cysteic acid	Histidine
Native enzyme stored at pH 4.6	22.2	6.9
Unirradiated, oxygenated for 30 min	20.8	7.1
Irradiated for 30 min	20.8	6.8
Irradiated for 2 h	20.7	5.8
Irradiated for 6 h	20.6	5.8

Table 3. *Fluorescence data for irradiated and unirradiated glutamate carboxylase from Clostridium perfringens*

The spectra were recorded by a Perkin-Elmer MPF 2A spectrophotofluorimeter, by using solutions of absorbance about 0.1 at the exciting wavelength. The data were corrected for the wavelength-dependent response of the excitation and detector systems. Fluorescence quantum yields were calculated by using L-tryptophan as a standard ($Q = 0.13$ on excitation at 290 nm).

Treatment of the enzyme...	Unirradiated	Irradiated for 6 h
Fluorescence parameters		
Absorption $\lambda_{\max}$ (nm)	333	333
Emission ($\lambda_{\text{excitation}} = 333$ nm)		
$\lambda_{\max}$ (nm)	380	380
Quantum yield	0.30	0.29
Polarization	0.45	0.45
Emission ($\lambda_{\text{excitation}} = 290$ nm)		
$\lambda_{\max}$ (nm)	323	323
Quantum yield	0.22	0.22
Polarization	0.11	0.10

conformational alteration of the protein moiety of glutamate decarboxylase as a consequence of the photo-oxidation process. The pattern of dichroic bands from 200 to 310 nm for glutamate decarboxylase at pH 4.6 and 6.2 is reported in Fig. 2. The two spectra are practically coincident except in the highly structured aromatic region around 295 nm, where the small positive band disappears with increasing pH. The dichroic spectrum of the irradiated sample was superimposable with that of its unirradiated control at pH 6.2.

The elution profiles of irradiated samples and their controls passed through Sephadex G-100 and G-200 gel columns were also superimposable. However, chromatographic analysis on cellulose ion-exchanger allowed us to distinguish between the irradiated samples and their controls, either analysed separately, as in Fig. 3, or as a mixture. By this procedure it was ascertained that the residual activity of enzyme samples submitted to prolonged irradiation is not due to the presence of corresponding amounts of unmodified enzyme.

The apparent homogeneity of the photo-oxidation product encouraged us to study the catalytic properties of the selectively modified enzyme molecule. The main kinetic parameters of an irradiated sample of glutamate decarboxylase and its control are reported in Fig. 4. The K_m for L-glutamate was determined by a sensitive spectrophotometric method allowing accurate measurements of reaction rates at low substrate concentrations (Cozzani, 1970). As shown in the double-reciprocal plot, the glutamate decarboxylase sample irradiated for 6h, retaining 22% of the control activity, has an unchanged K_m for L-glutamate.

Fig. 2. Circular-dichroism spectra of glutamate decarboxylase at pH 4.6 (○—○), at pH 6.2 (○—○), and of enzyme irradiated for 6 h at pH 6.2 (●—●)

The spectra were monitored at room temperature, at protein concentrations of 0.5 mg/ml in the far-u.v. range and of 1 mg/ml in the near 1-u.v. range. The data are expressed in terms of $[\theta]$, the mean residual molecular ellipticity, in units of degrees $\cdot$ cm² $\cdot$ dmol⁻¹.

Fig. 3. Ion-exchange chromatography of irradiated and unirradiated glutamate decarboxylase

Samples (5 mg) of purified glutamate decarboxylase from *Cl. perfringens* were either irradiated or oxygenated in the dark for 6 h at pH 6.2, as described in the text. After equilibration against 0.05 M-sodium acetate buffer, pH 5.0, the irradiated sample (●, ▲) and its control (○, △) were separately submitted to ion-exchange chromatography, on a Whatman DE 11 DEAE-cellulose column (1.6 cm $\times$ 30 cm), previously equilibrated with 0.05 M-sodium acetate buffer, pH 5.0. The column was eluted with a linear gradient between 0.05 M- and 0.5 M-sodium acetate buffer, pH 5.0, at 4°C, with a flow rate of 22 ml/h. Fractions (2.25 ml) were collected and analysed for extinction at 280 nm (○, ●) and for glutamate decarboxylase activity. The specific activities (△, ▲) of both protein peaks were then determined.

Discussion

The comparative analysis of glutamate decarboxylase inactivation and histidine photodestruction, both occurring at the same rate when the holoenzyme

Fig. 4. Lineweaver-Burk plots of irradiated and unirradiated glutamate decarboxylase

The double-reciprocal plots were obtained with an enzyme sample irradiated for 6 h (△), and with the corresponding control oxygenated in the dark (○). By a spectrophotometric method previously described (Cozzani, 1970), the initial reaction velocities (v) (μ mol of γ -amino-butyrate formed/min) were measured with 0.1–1.0 mM-sodium L-glutamate concentrations.

is irradiated at pH 6.2 and in the absence of other detectable modification of the structural properties of the enzyme molecule, strongly suggests that the selective photo-oxidation of this amino acid residue, sensitized by the coenzyme bound at the active site, is the event responsible for the fall of catalytic activity of glutamate decarboxylase from *Cl. perfringens* when irradiated with visible light. The loss of one cysteine residue per monomer, occurring faster than the destruction of histidine and observed also in the controls kept in the dark,

cannot be attributed to photo-oxidation, but is probably due to spontaneous oxidation of thiol groups at pH values over 5.5, according to previous observations (Cozzani *et al.*, 1970; Cozzani & Bagnoli, 1971). The oxidation of this thiol group could account both for the lower activity of the enzyme diluted at pH 6.2 compared with that of the native enzyme stored at pH 4.6, and for the initial lag observed in the rates of glutamate decarboxylase inactivation and histidine photodestruction. The product of this oxidation of the thiol group remains unidentified beyond the fact that it is not cysteic acid. Actually it was demonstrated that the oxidative processes by protein-bound sensitizers usually occur within a narrow spatial range and are critically dependent on the mutual orientations of the sensitizer and the photo-oxidizable side chains. Therefore it is conceivable that in the native glutamate decarboxylase the histidine residue is either too far from the pyridoxal phosphate or unfavourably oriented. The critical distance and spatial relationships required for photo-oxidation sensitized by the intrinsic chromophore could also explain the failure of the bound pyridoxal phosphate to sensitize the photo-oxidation of histidine at the active site of aspartate aminotransferase from pig heart (Burrige & Churchich, 1970).

Anyway, the photo-oxidation of histidine sensitized by the pyridoxal phosphate bound to glutamate decarboxylase from *Cl. perfringens* appears to be a highly selective and localized event, as indicated by the invariance of all the spectroscopic parameters measured before and after irradiation. The difference reported above between dichroic spectra of the protein at different pH values, although unrelated with the photo-oxidation process, confirms the sensitivity of the spectroscopic investigations used. In conclusion the photodegraded histidine residue appears to play a small part, if any, in maintaining enzyme structure but is important for catalytic function.

The involvement of the imidazole residue in reactions catalysed by pyridoxal phosphate enzymes was originally proposed by Bruice & Topping (1963), and the catalytic role of histidine residues of pig heart aspartate aminotransferase was demonstrated by dye-sensitized photo-oxidation (Martinez-Carrion *et al.*, 1967). However, no evidence of involvement of histidine in amino acid decarboxylation reactions was so far reported. Distinct mechanisms can be conceived to explain the catalytic role of this amino acid residue at the active site of glutamate decarboxylase from *Cl. perfringens*: (1) it can provide a cationic site to bind one ionized carboxyl group of the substrate, or (2) it can contribute to the formation or stabilization of reaction intermediates, possibly by appropriate interaction with the cofactor. The invariance of the K_m for L-glutamate

after photo-oxidation is more compatible with the latter assumption. (3) The additional possibility that the imidazole ring participates in a proton-transfer reaction, cannot be excluded for this glutamate decarboxylase, which has optimum pH at 4.7 (Cozzani *et al.*, 1970). Whatever the true mechanism of the catalysis by histidine, it is noteworthy that in the comparative sequences of the active sites of glutamate, arginine and lysine decarboxylases from *Escherichia coli*, a histidine residue invariably precedes the lysine bearing the cofactor (Boeker & Snell, 1972). It would be interesting to verify whether sequence homologies exist among corresponding amino acid decarboxylases from different bacterial species.

This work was supported by grant from the Italian Consiglio Nazionale delle Ricerche.

References

- Benassi, C. A., Scoffone, E., Galiazzo, G. & Jori, G. (1967) *Photochem. Photobiol.* **6**, 857–868
- Bencze, W. L. & Schmid, K. (1957) *Anal. Chem.* **29**, 1193–1196
- Boeker, E. A. & Snell, E. E. (1972) *Enzymes*, 3rd edn., **6**, 217–253
- Bruice, T. C. & Topping, R. M. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P., Braunstein, A. & Rossi-Fanelli, A., eds.), pp. 29–56, Pergamon Press, New York
- Burrige, N. & Churchich, J. E. (1970) *J. Biol. Chem.* **245**, 6258–6263
- Cozzani, I. (1970) *Anal. Biochem.* **33**, 125–131
- Cozzani, I. & Bagnoli, G. (1971) *Ital. J. Biochem.* **20**, 179–190
- Cozzani, I. & Bagnoli, G. (1973) *Ital. J. Biochem.* **22**, 36–45
- Cozzani, I., Misuri, A. & Santoni, C. (1970) *Biochem. J.* **118**, 135–141
- Davis, L. C., Brox, L. W., Gracy, R. W., Ribereau-Gayon, G. & Horecker, B. L. (1970) *Arch. Biochem. Biophys.* **140**, 215–222
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62
- Huntley, T. E. & Metzler, D. E. (1968) *Symp. Pyridoxal Enzymes. Proc. 3rd* (Yamada, K., Katunuma, N. & Wada, H., eds.), pp. 81–84, Maruzen, Tokyo
- Jori, G., Galiazzo, G., Marzotto, A. & Scoffone, E. (1968) *J. Biol. Chem.* **243**, 4272–4278
- Jori, G., Galiazzo, G. & Scoffone, E. (1969) *Int. J. Protein Res.* **1**, 289–298
- Martinez-Carrion, M., Turano, C., Riva, F. & Fasella, P. (1967) *J. Biol. Chem.* **242**, 1426–1430
- Rippa, M. & Pontremoli, S. (1969) *Arch. Biochem. Biophys.* **133**, 112–118
- Shaltiel, S. & Cortijo, M. (1970) *Biochem. Biophys. Res. Commun.* **41**, 594–600
- Spikes, J. D. & Straight, R. (1967) *Annu. Rev. Phys. Chem.* **18**, 409–436
- Strausbauch, P. H. & Fischer, E. H. (1970) *Biochemistry* **9**, 233–238