Structural interpretation of the binding of 9-azidoacridine to D-amino acid oxidase

Bruce H. NICHOLSON* and Surendra P. BATRA†

Department of Physiology and Biochemistry, University of Reading, P.O. Box 228, Whiteknights, Reading RG6 2AJ, U.K.

D-Amino acid oxidase (EC 1.4.3.3) forms an inhibited complex with the nucleotide- and aromatic-binding-site affinity reagent 9-azido[³H]acridine. Tryptic digestion of the photolysed complex yielded two radioactive peptides, 222–265 (T23) and 298–328 (T29), which core and secondary structure analysis revealed to be exposed, but which also comprised the propargylglycine-binding residues. This suggests that at least parts of the peptides containing these residues are in the active centre and that they are spatially close to the flavin-binding site.

INTRODUCTION

The flavoprotein D-amino acid oxidase (EC 1.4.3.3) from pig kidney has been the subject of a large amount of mechanistic research. Despite the fact that its biological role is not yet understood, it has been studied extensively as a model for flavoprotein oxidases, especially with respect to its catalytic mechanism and the properties of the non-covalently bound FAD cofactor (Bright & Porter, 1975; Porter *et al.*, 1977; Miura & Miyake, 1987). The enzyme catalyses the oxidation of the D-isomer of a number of amino acids to the imino acid, which is then hydrolysed non-enzymically to the corresponding α -oxo acid, whose enol form reacts with borate to give a complex that can be measured spectrophotometrically at 300 nm.

The use of group-specific reagents has provided considerable information concerning the structure of the active site of D-amino acid oxidase. Three specific modifications have identified residues that are required for activity. Thus it has been shown that an essential histidine residue at position 217 can be modified by dansyl chloride (Nishino *et al.*, 1980; Swenson *et al.*, 1983), that Sanger's reagent, 1-fluoro-2,4-dinitrobenzene, will almost completely inactivate the protein with the modification of tyrosine-55 and lysine-204 (Swenson *et al.*, 1982) and that cyclohexanedione modifies a residue (possibly arginine) with concomitant loss of enzyme activity (Ferti *et al.*, 1981). In the present paper we report results obtained with the nucleotide analogue 9-azidoacridine (Batra & Nicholson, 1982a), together with structural predictions, and discuss the implication for the active centre of D-amino acid oxidase. Some preliminary results have been discussed elsewhere (Batra & Nicholson, 1983).

EXPERIMENTAL

Materials

[ring-G-³H]Aniline (specific radioactivity 150 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Pig kidney D-amino acid oxidase (EC 1.4.3.3) and diphenylcarbamoyl chloride-treated trypsin (DPCC-trypsin) (EC 3.4.21.4) were obtained

from Sigma (London) Chemical Co. Sephadex G-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. All other reagents were A.R. grade or the highest purity readily obtainable.

9-Azidoacridine syntheses

9-Azidoacridine was synthesized by the procedure of Reynolds (1968) from 9-chloroacridine and NaN₃, modified in the case of 9-azido[³H]acridine as described previously (Batra & Nicholson, 1982a). The specific radioactivity was 4.12×10^{12} d.p.m./mol (Packard Tri-Carb 3255 instrument).

Crude 9-azidoacridine was recrystallized by dissolving it in acetone and precipitating it as light-yellow crystals with cold water. The yellow crystals, m.p. 86 °C (uncorr.), gave an i.r. spectrum [ν_{max} (KBr) 2140 cm⁻¹ (N₃)] on a Perkin-Elmer 157 spectrophotometer. Mass-spectral data typically showed a relatively small (< 1%) molecular-ion peak at m/z 220 and 213, compared with a large peak at m/z 192. The m/z 192 peak is consistent with the loss of N₂ from the azidoacridine (m/z 220), giving the nitrene. A small amount of unmodified 9-chloroacridine would give m/z 213/5.

9-Azidoacridine is sparingly soluble in water but readily dissolves in acetone, ethanol and methanol. The absorption spectrum of 9-azidoacridine in ethanol, pH 7.4, gave a characteristic peak at 357 nm (ϵ_{275} 7900 M⁻¹·cm⁻¹). The change in absorption at this wavelength was less than 1% after 30 min in the spectrophotometer, so that photolysis during the course of enzyme assay was negligible. However, throughout the synthesis and subsequent operations care was taken to exclude daylight. On exposure to daylight and on photolysis, it decomposes to a reddish-brown product with gaseous evolution. 9-Azidoacridine is stable indefinitely in the dark at –20 °C.

Assay of D-amino acid oxidase (Wellner & Lichtenberg, 1971)

To a 0.7 ml mixture containing pyrophosphate buffer (5.0 μ M), catalase (60 units), FAD (0.4–2.0 μ M), bovine serum albumin (1 mg) and D-amino acid oxidase (0.1–0.5 unit) was added 0.1 ml of 0.1 M-D-phenylalanine, and

* To whom correspondence should be addressed.

† Present address: Department of Chemistry, University of Delaware, Newark, DE 19716, U.S.A.

the reaction vial was shaken in a water bath at 37 °C for 15 min. The reaction was stopped by adding 0.2 ml of 25% (w/v) trichloroacetic acid. The reaction mixture was centrifuged, and a 0.5 ml sample of the supernatant was added to 2.5 ml of the boric acid (1 M)/sodium arsenate (1 M) solution (previously adjusted to pH 6.5 with HCl) and mixed well. The solution was kept for at least 30 min at room temperature before the absorbance of the phenylpyruvate-borate complex was measured at 300 nm against a blank, in which enzyme was omitted. Inhibition of the oxidase was studied with unlabelled 9-azidoacridine. One unit of activity is taken as the amount of enzyme required to give an absorbance of 0.030 at 300 nm under the above conditions.

Incorporation of 9-azidoacridine into D-amino acid oxidase

A small volume of a concentrated ethanolic solution of 9-azidoacridine was added to the enzyme. The final concentration of ethanol was always less than 1% and without effect on enzyme activity. No precipitation or turbidity was observed either with brief visual examination or in the spectrophotometer. On the basis of the inhibition observed at different concentrations of 9-azidoacridine, a final concentration of 0.1 mM was employed for the photolysis. Accordingly 2 mg of D-amino acid oxidase (0.8 mg/ml in 0.1 M-pyrophosphate buffer, pH 8.3) was incubated in the dark with 0.1 mM-9-azido[³H]acridine (specific radioactivity 4.12×10^6 d.p.m./ μ mol) for 60 min (see the Results and discussion section) followed by photolysis. This long preincubation time was chosen to ensure that binding of 9-azidoacridine by the enzyme was complete. Solutions to be irradiated were placed in a glass tube and held in a beaker of water at 10 °C, and the temperature of the solution was monitored. A 150 W tungsten-filament lamp with heat filter was used to provide a collimated light-source at a distance of about 40 mm from the lens, typically for 30 min (Batra & Nicholson, 1982a). From observation of gas evolution on more concentrated solutions of 9-azidoacridine, and the absorption at 357 nm, generation of the nitrene was judged to be complete in under 30 min. The modified enzyme was separated from excess reagent by gel filtration with Sephadex G-25 equilibrated with 50 mM-NH₄HCO₃ and followed by exhaustive dialysis against the same buffer. The amount of 9-azidoacridine incorporated into this dialysed enzyme was determined by measuring the radioactivity of samples. The protein determinations were carried out by the method of Bradford (1976).

Tryptic digestion of carboxymethylated 9-azido[³H]acridine-modified enzyme

9-Azido[³H]acridine-modified D-amino acid oxidase containing 1.09 mol of reagent/mol of enzyme was denatured in 6 M-urea. A 10-fold molar excess of dithiothreitol was added over thiol groups in the enzyme. After 1 h iodoacetic acid was added in a 2-fold molar excess over dithiothreitol, followed 10 min later by the addition of excess 2-mercaptoethanol to quench the reaction. The carboxymethylated modified enzyme was dialysed against 50 mM-NH₄HCO₃, pH 8.0, to remove excess reagents and was then digested with DPCC-trypsin (2%, w/w) at 37 °C for 12 h. The digest was completely soluble at that time. Finally it was freeze-dried. In separate experiments with chymotrypsin and

glyceraldehyde-3-phosphate dehydrogenase, analysis of the products of exhaustive digestion by DPCC-trypsin followed by a two-dimensional combination of paper electrophoresis and chromatography gave no evidence of non-trypsin-specific cleavage. This establishes that the trypsin was sufficiently pure for analytical digestion of D-amino acid oxidase. Details of peptide isolation and characterization are given in the legends to the Figures.

The purification of peptide by preparative peptide mapping is both rapid and sensitive. Thus the tryptic digest (obtained above from modified and carboxymethylated D-amino acid oxidase) was subjected to a two-stage process involving a two-dimensional combination of electrophoresis and chromatography (Ingram, 1959). This extremely simple two-step procedure could readily be performed within a 24 h period, and separated most of the components of the tryptic digest of the enzyme.

RESULTS AND DISCUSSION

In previous papers we have shown that 9-azidoacridine binds to aromatic- and nucleotide-binding sites on a number of proteins (Batra & Nicholson, 1982a,b; Nicholson & Batra, 1982). Furthermore, this binding is restricted and specific in scope; enzymes that do not bind aromatic groups or nucleotides do not bind 9-azidoacridine, e.g. amylase and alkaline phosphatase (Batra & Nicholson, 1982a). The mode of binding and the earlier observed correlation with inhibition of enzymes by acridine and related heterocycles have been reviewed (Nicholson, 1973). In particular the combination of the area of the planar ring system, its partial hydrophobicity and the slight positive charge on one edge would appear to account for its affinity for specific sites.

At the start of the present investigation, the folding of the protein chain around both the adenine moiety and the isoalloxazine ring system were considered as potential binding sites for 9-azidoacridine (Batra & Nicholson, 1983; Batra, 1984).

Incorporation of 9-azido[³H]acridine by D-amino acid oxidase

Incubation of D-amino acid oxidase (0.8 mg/ml) with 100 μ M-9-azidoacridine at pH 8.3 results in incorporation of 1.09 mol of reagent/mol of D-amino acid oxidase. In previous experiments with chymotrypsin it had been observed that preincubation with 9-azidoacridine was necessary to achieve inhibition (Batra & Nicholson, 1982a; Batra, 1984). Examination of the inhibition of the chymotrypsin-catalysed hydrolysis of *N*-acetyltyrosine ethyl ester showed that inhibition by 9-azidoacridine was not observed in the absence of preincubation, but was maximal after preincubation for 30 min. The mechanism for preincubation-dependent inhibition is not known, but the results are suggestive of a rapid diffusion-controlled loose binding followed by tighter binding when the ligand is successfully oriented to enter the hydrophobic 'pocket'. Similar findings have been reported with other inhibitors (see, e.g., Russell *et al.*, 1976). Therefore in all further experiments it was decided to preincubate the enzymes with 9-azidoacridine for 30 min.

9-Azidoacridine was found to inhibit D-amino acid oxidase strongly when the concentration of FAD was low, but had little effect in the presence of high

Fig. 1. Peptide map of the tryptic digest of 9-azido[^3H]acridine-labelled pig kidney D-amino acid oxidase

Separation of tryptic peptides (1.5 mg) derived from the labelled enzyme was by electrophoresis followed by chromatography (Ingram, 1959). The sample was dissolved in running buffer, applied to Whatman 3MM paper and electrophoresed for 3 h at 40 V/cm in pyridine/acetic acid/water (25:1:225, by vol.) buffer, pH 6.5 (Evenberg *et al.*, 1977). Chromatography in the second dimension was in the pyridine/isopentanol/water (7:7:6, by vol.) solvent system as described by Baglioni (1961). Peptides were identified by using fluorescamine [2 mg in 100 ml 1% (v/v) pyridine in acetone], which could detect as little as 10 pmol (Vandekerckhove & Van Montagu, 1974). The spots were cut out, stitched to a strip of paper and the peptides eluted by descending chromatography with 50% (v/v) acetic acid at 4 °C to a final volume of 19 ml. Where spots appeared close together the distal areas and the overlapping intervening region were eluted separately. Fluorescamine testing of the eluted chromatography papers for residual peptides indicated recoveries of over 90%. Recoveries of radioactivity (94% overall) were: A, 69000 d.p.m. (16.7 nm, 40%); B, 65000 d.p.m. (15.8 nm, 38%); and the intervening region next to B, 27600 d.p.m. (6.7 nm, 16%).

concentrations of FAD. Furthermore, the inhibition could be reversed by increasing concentrations of FAD. Typically, at 66 μM 9-azidoacridine inhibition was 88% (FAD concentration 0.4 μM) and this declined to 30% on increasing FAD concentration to 2.0 μM . The enzyme activity at 0.4 μM FAD and 10 mM-D-phenylalanine was taken as 100% activity, measured as the phenylpyruvate formed. Photolysis of 9-azido[^3H]acridine when bound to the enzyme resulted in irreversible inhibition, the extent of which could be decreased by the presence of 2.0 μM FAD.

Localization of ^3H -labelled oligopeptides

Separation of the products of tryptic digestion by two-dimensional electrophoresis and chromatography yielded 27 peptides (Fig. 1), of which two were found to be radioactive. Some material remained at the origin, possibly as a result of incomplete digestion. The complete

347-amino-acid-residue sequence (Fig. 2) of D-amino oxidase from pig kidney cortex has been determined (Swenson *et al.*, 1982) by the isolation and characterization of 32 peptides from tryptic digests. Comparison of the amino acid compositions of these two radioactive peptides (Table 1) with the published sequence (Fig. 2) showed their derivations to be residues 226–265 and 298–328, corresponding to tryptic fragments T23 and T29 respectively. Altogether 27 oligopeptides were detected out of a possible 32 (some are di- or tri-peptides). The amino acid composition from the sequence showed that most contained only a few amino acid residues so that identity of these with the radioactive fragments could be ruled out on qualitative differences, e.g. the complete absence of several amino acid residues. Comparison of molar ratios established the identity of the labelled peptides, and this was confirmed by paired *t* tests, but with three significant deviations. Thus in Table 1 the peaks eluted at or close to the position nominally expected for methionine and lysine have been quantified relative to those standards, but their values are way outside the expected data range. Since these values are only observed after labelling, the acridine adduct or a breakdown product thereof is clearly involved.

Massey *et al.* (1986) have published their results on the binding of 6-azido-FAD to D-amino acid oxidase. 6-Azido-FAD appears to bind almost quantitatively to a catalytically important residue, but unfortunately the covalent linkage is broken on denaturation of the protein. Apart from the possibility of internal cyclization (of the azide group), a possible disadvantage with probes based on the natural ligand is that the evolutionary optimization of the stereochemistry of the flavin-binding site allows little room for the bulky azide group, or for movement of the heterocyclic base, so that the preferred or limited reaction is with a single side chain or, in the case of the derivatives that failed to label, water.

The T23 and T29 peptides have been analysed for secondary structure (Chou & Fasman, 1978; Garnier *et al.*, 1978; Ronchi *et al.*, 1982) and for side chains in the hydrophobic core of the protein (Nicholson, 1982). This suggests the existence of a looping out of the polypeptide backbone. Interestingly, the secondary-structure predictions confirm the idea of a looped-out structure bounded at either end by two α -helices. There is thus plenty of scope for contributing to a three-dimensional binding site. Some of the non-core isoleucine could be expected to participate in this, providing a considerable hydrophobic environment, in which one or more of the five aromatic side chains, together with spatially adjacent side chains at sequentially remote locations, may contribute to a site accommodating the 0.38 nm² (38 Å²) of the planar acridine ring.

In contrast with the thorough investigation of physicochemical and kinetic properties of this flavo-enzyme (Walsh *et al.*, 1973; Porter *et al.*, 1973; Yagi *et al.*, 1974; Hofsteenge *et al.*, 1980), the structural data are very limited. However, D-amino acid oxidase has primary-sequence similarities to the FAD-containing *p*-hydroxybenzoate hydroxylase, whose three-dimensional structure has been determined (Hofsteenge *et al.*, 1980). The latter enzyme comprises three domains, each of which contributes to the binding of the isoaalloxazine ring, whereas the binding of AMP is restricted to a $\beta\alpha\beta$ fold near the *N*-terminal. Thus the absence of labelled peptides from the *N*-terminal of D-amino acid oxidase

Table 1. Tryptic peptides of D-amino acid oxidase

The Table gives the expected fragments from a tryptic digest of the sequence shown in Fig. 2. Arg-Pro peptides are resistant. Fragments 8, 12 and 17 are lysine. The amino acid composition of the radioactive peptides has been normalized to aspartate (A) and alanine (B) and is shown next to the matching peptide. Samples containing 2–3.5 nmol of peptide were hydrolysed in 0.5 ml of 6 M-HCl containing 25 μ l of 2-mercaptoethanol under N₂ at 110 °C. The hydrolysates were dried under vacuum and redissolved in 1 mM-HCl for analysis on a JEOL (Tokyo, Japan) model JLC-6AH auto-analyser. The 24 h and 72 h values were expressed as mol/100 mol and averaged. Threonine, serine and methionine values were extrapolated to zero time. Tyrosine and other residues subject to decomposition were not determined (N.D.). No allowance has been made for modified residues. The analyses are for the purpose of peptide identification only and should not be construed as correction to the sequence data. Paired *t* tests of the difference between the indicated sequence value and experimental values gave for degrees of freedom, mean S.D., S.E.M. and *t*: (peptide A, excluding 'Met') 14, 0.067, 0.403, 0.108, 0.62; (peptide B, excluding 'Met') 12, 0.087, 0.289, 0.084, 1.04; (for peptide A, excluding 'Met' and 'Lys') 13, 0.036, 0.120, 0.033, –1.09. (The values for the omitted residues lie outside the expected data range by 9, 72 and 11 times the standard deviation respectively. For an assumed normal distribution, 95% of the data should fall within ± 2 times the standard deviation.)

	1	2	3	4	5	6	7	9	10	11	13	14	15	16	18	19	20	21	22	23	A	24	25	26	27	28	29	B	30	31	32
Lys	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	(1.41)	1	1	1	1	1	1	0.97	1	1	1
His	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.09	1	1	1	1	1	1	3	3.11	1	1
Arg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.97	1	1	1	1	1	1	2	1.96	1	1
Asp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	7.00	1	1	1	1	1	1	2	1.74	1	1
Thr	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2.95	1	1	1	1	1	1	2	2.09	1	1
Ser	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.05	1	1	1	1	1	1	2	2.16	1	1
Glu	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4.95	1	1	1	1	1	1	2	2.00	1	1
Gly	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5.62	1	1	1	1	1	1	6	6.27	1	1
Ala	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.04	1	1	1	1	1	1	2	1.78	1	1
Val	4	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2.08	1	1	1	1	1	1	2	(18.45)	1	1
Met	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	(3.84)	1	1	1	1	1	1	2	2.86	1	1
Ile	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5.89	1	1	1	1	1	1	2	2.17	1	1
Leu	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.94	2	1	1	1	1	1	2	0.93	1	1
Tyr	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	N.D.	1	1	1	1	1	1	2	N.D.	1	1
Phe	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.02	1	1	1	1	1	1	1	N.D.	1	1
Cys	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	N.D.	1	1	1	1	1	1	1	N.D.	1	1
Pro	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1.93	1	1	1	1	1	1	1	N.D.	1	1
Trp	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	N.D.	1	1	1	1	1	1	1	N.D.	1	1

Fig. 2. Amino acid sequence of pig kidney D-amino acid oxidase showing the predicted structures

The two lines below the sequence (Miyake & Yamano, 1970) are the secondary structures by the method of Chou & Fasman (1978) (lower) and Garnier *et al.* (1978) (upper): ----, helical; ---, extended; ····, turns (upper) and ~, turns (lower). Three letters in upper-case type indicate residues whose side chains constitute the hydrophobic core of the protein (Hofsteenge *et al.* 1980). The location of tryptic peptides T23 and T29 is shown, together with the residues (P) previously reported to be modified by the selective inhibitor propargylglycine.

suggests that 9-azidoacridine is not binding at the AMP-binding site, but rather is acting as an analogue of the isoalloxazine ring. Because of the finite size of FAD, this in turn implies that peptides T23 and T29 contain residues that are near the AMP-binding terminal region and the catalytic site. Several specific reagents have been tested with the aim of labelling essential amino acid residues in the active centre of the enzyme (Miyake & Yamano, 1970; Hellerman & Coffey, 1967; Rudie *et al.*, 1980; Horiike *et al.*, 1975). His-307 and Tyr-228 have previously been shown to be modified by the acetylenic inhibitor propargylglycine (Horiike *et al.*, 1975), although it has been suggested that they are not in the active centre. The present finding strongly suggests that at least parts of the peptides containing these residues are actually in the active centre and that they are spatially close to the flavin-binding site.

REFERENCES

- Baglioni, C. (1961) *Biochim. Biophys. Acta* **48**, 392–396
- Batra, S. P. (1984) Ph.D. Thesis, University of Reading
- Batra, S. P. & Nicholson, B. H. (1982a) *Biochem. J.* **207**, 101–108
- Batra, S. P. & Nicholson, B. H. (1982b) *Biochem. Soc. Trans.* **10**, 536–537
- Batra, S. P. & Nicholson, B. H. (1983) *Abstr. FEBS Meet.* 15th, Brussels, 209
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bright, H. J. & Porter, D. J. T. (1975) *Enzymes* 3rd ed. **12**, 421–505
- Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol.* **47**, 45–148
- Evenberg, A., Maijer, H., Gastra, W., Verheij, H. M. & de Hass, G. H. (1977) *J. Biol. Chem.* **252**, 1189–1196

- Ferti, C., Curti, B., Simonetta, M. P., Ronchi, S., Galliano, M. & Minchiotti, L. (1981) *Eur. J. Biochem.* **119**, 553–557
- Garnier, J., Osguthorpe, D. J. & Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120
- Hellerman, L. & Coffey, D. S. (1967) *J. Biol. Chem.* **242**, 582–589
- Hofsteenge, J., Vereijken, J. M., Weijer, W. J., Beintema, J. J., Wierenga, R. K. & Drenth, J. (1980) *Eur. J. Biochem.* **113**, 141–150
- Horiike, K., Nishima, Y., Miyake, Y. & Yamo, T. (1975) *J. Biochem. (Tokyo)* **78**, 57–63
- Ingram, V. M. (1959) *Biochim. Biophys. Acta* **28**, 539–545
- Massey, V., Ghisla, S. & Yagi, K. (1986) *Biochemistry* **25**, 8095–8102
- Miura, R. & Miyake, Y. (1987) *J. Biochem. (Tokyo)* **101**, 581–589
- Miyake, J. & Yamano, T. (1970) *Biochim. Biophys. Acta* **198**, 438–448
- Nicholson, B. H. (1973) in *Acridines* (Acheson, R. M., ed.), pp. 759–787, John Wiley and Sons, New York
- Nicholson, B. H. (1982) *Biochem. Soc. Trans.* **10**, 387–388
- Nicholson, B. H. & Batra, S. P. (1982) *Biochem. Soc. Trans.* **10**, 540
- Nishino, T., Massey, V. & Williams, C. H., Jr. (1980) *J. Biol. Chem.* **255**, 3610–3616
- Porter, D. J. T., Voet, J. G. & Bright, H. J. (1973) *J. Biol. Chem.* **248**, 4400–4416
- Porter, D. J. T., Voet, J. G. & Bright, H. J. (1977) *J. Biol. Chem.* **252**, 4464–4473
- Reynolds, G. A. (1968) Fr. Patent 511 485; cited in *Chem. Abstr.* (1969) **70**, 120041f
- Ronchi, R., Minchiotti, L., Galliano, M., Curti, B., Swenson, R. P., Williams, C. H. & Massey, V. (1982) *J. Biol. Chem.* **257**, 8824–8834
- Rudie, N. G., Porter, D. J. T. & Bright, H. J. (1980) *J. Biol. Chem.* **255**, 498–508
- Russell, J., Jeng, S. J. & Guillory, R. J. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1225–1234
- Swenson, R. P., Williams, C. H., Jr. & Massey, V. (1982) *J. Biol. Chem.* **257**, 1937–1944
- Swenson, R. P., Williams, C. H., Jr. & Massey, V. (1983) *J. Biol. Chem.* **258**, 497–502
- Vandekerckhove, J. S. & Van Montagu, M. C. (1974) *Eur. J. Biochem.* **44**, 279–288
- Walsh, C. T., Krodal, E., Massey, V. & Abeles, R. H. (1973) *J. Biol. Chem.* **248**, 1946–1955
- Wellner, D. & Lichtenberg, L. S. (1971) *Methods Enzymol.* **17B**, 593–605
- Yagi, K., Nishikimi, M., Takai, A. & Ohishi, N. (1974) *J. Biochem. (Tokyo)* **74**, 451–454

Received 20 November 1987/25 May 1988; accepted 7 July 1988