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IMPROVED PURIFICATION, AMINO ACID ANALYSIS AND MOLECULAR WEIGHT OF HOMOGENEOUS D-AMINO ACID OXIDASE FROM PIG KIDNEY

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SUMMARY

A highly purified preparation of D-amino acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) from hog kidney has been characterized catalytically, spectrally, and by amino acid content. Polyacrylamide-gel electrophoresis revealed a single band of protein. The subunit molecular weight has been determined to be 39 600 by amino acid analysis based on the flavin content of enzyme spectrally analyzed in the presence of excess benzoate. The flavin content was 25.3 nmoles/mg and the ratio of the ultraviolet to the visible absorbance was 9.5. The specific activity was 173 units/mg at 25 °C. Each of these parameters represents an improvement over the previously published purification procedures. Thiol determinations by several methods revealed five cysteine residues/FAD and no disulphides were indicated. N-terminal methionine and C-terminal leucine have been confirmed and the amide content is reported.

INTRODUCTION

Early approaches in our laboratory to structural studies on D-amino acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) from hog kidney revealed that the purification of the enzyme by the procedure of Brumby and Massey[1] failed to give a homogeneous preparation as indicated by polyacrylamide-gel electrophoresis. Henn and Ackers[2] have also reported difficulty in obtaining a homogeneous preparation with this method. On the other hand several amino acid analyses of D-amino acid oxidase have been published and the results have varied widely[3–5]. Since an enzyme suitable for structural studies must have a constant amino acid composition in addition to the usual criteria of homogeneity, we tried to improve the purification procedure of Brumby and Massey[1] by introducing a further

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

chromatographic step. The enzyme so obtained is homogeneous and has a constant amino acid composition. Some chemical characteristics of the purified protein have been studied.

MATERIALS AND METHODS

Standard assay

Enzyme activity has been tested at 25 °C or at 38 °C by the oxygen electrode. The incubation mixture contained 40 mM pyrophosphate buffer (pH 8.3), 45 mM DL-alanine, 4 μm FAD, 2.4 mM EDTA, 4 mg of bovine plasma albumin, 100 μg of beef liver catalase and water to a final volume of 2.45 ml. The reaction was started by the addition of 50 μl of diluted enzyme (5–10 μg of protein). One unit of activity corresponds to the consumption of 1 μl of O_2 per min[1].

Protein determination

The protein concentration was determined by a modification of the biuret method[1]. Bovine serum albumin was used as a standard giving an absorbance of 0.095 $\pm$ 0.005/mg.

Enzyme purification

1200 g of cleaned cortex of fresh pig kidneys were usually processed at one time. The use of kidneys stored in the deep freeze at −20 °C for 2–3 weeks usually gives a lower yield of enzyme. All the steps have been carried out at 0–5 °C unless otherwise stated. The procedure of Brumby and Massey[1] through Step E has been used. A minor modification was introduced at Step D. The supernatant, obtained after pH treatment and centrifugation, was brought to 25% $(NH_4)_2SO_4$ saturation prior to the subsequent heat treatment at 52 °C.

Chromatographic step (new stage F)

The eluate obtained from the calcium phosphate column is checked spectrophotometrically: only the fractions showing a $A_{274\ nm}/A_{462\ nm}$ ratio $\leq$ 10 are collected and brought to 80% $(NH_4)_2SO_4$ saturation. After standing for 20 min the precipitate is collected by centrifugation at 24 000 $\times$ g for 20 min. The enzyme is dissolved in a small volume of 25 mM sodium phosphate buffer (pH 6.3) containing 200 μM sodium benzoate and dialysed against 4 times 500-ml changes of 10 mM Tris–HCl buffer (pH 8) containing 125 mM KCl and 200 μM sodium benzoate. The dialysed solution (freed of small amounts of denatured protein by centrifugation) is applied on a 1.9 cm $\times$ 25 cm DEAE-Sephadex A-50 column previously equilibrated with the same Tris buffer used for the dialysis. The enzyme moves down the column as a yellow band leaving a white impurity at the top. All the yellow fractions are collected, sodium benzoate is added (1 g/l) and dissolved by vigorous stirring. The solution is brought to pH 6.5 with 1 M acetic acid and $(NH_4)_2SO_4$ slowly added to 50% satn. After standing for 20 min the solution is centrifuged at 24 000 $\times$ g for 20 min and the precipitate dissolved in a small volume of 100 mM pyrophosphate buffer (pH 8.3) containing 200 μM sodium benzoate. A dialysis against 4 times 250-ml changes of the same buffer followed by a centrifugation at 24 000 $\times$ g for 20 min yields a pure enzyme which may be stored for several weeks at −20 °C.

Apoprotein preparation

The apoprotein has been prepared starting from the pure benzoate–enzyme complex according to Massey and Curti[6] and stored at −20 °C in 100 mM pyrophosphate buffer (pH 8.5) + 300 μM EDTA.

Gel electrophoresis and amino acid analysis

Polyacrylamide gel electrophoresis has been carried out according to Davis[7]. Polyacrylamide gel electrophoresis in sodium dodecylsulphate has been performed according to the procedure of Weber and Osborn[8].

Hydrolysis of the protein samples was performed with 6 M HCl in sealed evacuated tubes for 18, 39 and 61 h at 106 °C (ref. 9). Amino acid analysis was carried out using a Beckman 120 B amino acid analyzer equipped with long path length cells, according to Spackman[10]. Tryptophan has been determined by a modification of the method of Spies and Chambers[11]. Cysteine has been determined: (a) by spectrophotometric titration with either 4-4'-dipyridyldisulphide at 324 nm ($\varepsilon = 19.4 \cdot 10^3$) (ref. 12) or DTNB at 412 nm ($\varepsilon = 13.6 \cdot 10^3$) (ref. 13); (b) by performic acid oxidation[14]; and (c) by the dimethylsulphoxide method of Spencer and Wold[15].

End group analysis and amide determination

The N-terminal amino acid was identified as the dansyl derivative[16] by chromatography on polyamide layer sheets[17]. The C-terminal amino acid has been characterized by hydrazynolysis[18] and by carboxypeptidase A digestion[19] using a ratio of carboxypeptidase A/protein of 1/50. The amide content of the enzyme has been evaluated according to Hoare and Koshland[20] using 1-ethyl-3-dimethylaminopropylcarbodiimide as the -COOH activating reagent. All the chemicals used in this work were of analytical grade.

RESULTS

Enzyme purification

The purification of the enzyme according to our method is summarized in Table I. The recovery of enzyme is 25% and a yield of 150–200 mg can be obtained from a typical preparation (2400 g of kidney). The final specific activity at 38 °C of the enzyme is also reported in Table I: the average values at the oxygen electrode give 400–430 μl $O_2 \cdot min^{-1} \cdot mg^{-1}$ in presence of catalase and 800–850 μl $O_2 \cdot min^{-1} \cdot mg^{-1}$ without catalase in the assay mixture. The DEAE-Sephadex chromatographic step increases the specific activity by about 30% more than the previous published data[1] while not lowering the final yield. The apoprotein prepared directly from the holoenzyme–benzoate complex[6] with a yield of 90–95% reaches essentially the specific activity of the native enzyme (95%) when fully saturated with FAD.

Polyacrylamide-gel electrophoresis

The acrylamide gel electrophoresis of the purified holoenzyme and apoprotein are shown in Fig. 1. Both forms give a single band. The differing electrophoresis migration further illustrates the observation of Antonini *et al.*[21] that D-amino acid oxidase apoprotein undergoes polymerization more readily than the holoenzyme does. These differences disappear when the two forms of D-amino acid oxidase are

TABLE I

PURIFICATION OF D-AMINO ACID OXIDASE

2400 g of hog kidneys were used. The initial steps of the purification procedure (A–E) were performed as previously described[1]. The recoveries and purification factors are calculated in relation to the homogenate. The standard activities were measured at 25 °C. For details see under Material and Methods.

Purification steps	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Spec. act. (units/mg)*	*Purification factor*	*Recovery (%)*
A. Homogenate of 2400 g of pig kidney cortex	10 345	561 804	117 417	0.209		100
B. First heat $(NH_4)_2SO_4$ fraction	1 100	19 413	85 417	4.40	21	73
C. Second heat $(NH_4)_2SO_4$ fraction	70.5	2 035	78 652	38.65	185	67
D. Third heat $(NH_4)_2SO_4$ fraction	23	952	55 977	58.80	281	47.6
E. Calcium phosphate-chromatographed enzyme	15.05	335	39 697	118.50	567	33.8
F. DEAE-Sephadex A-50 chromatographed enzyme	11.05	180	31 140	173*	827	26.5

* The corresponding value at 38 °C is 429.

Fig. 1. Polyacrylamide-gel electrophoresis of purified D-amino acid oxidase. A, apoprotein, 50 μg; B, holoenzyme–benzoate complex, 50 μg; C, apoprotein + holoenzyme–benzoate complex, 100 μg. The gels were stained with Comassie blue. For details see under Material and Methods.

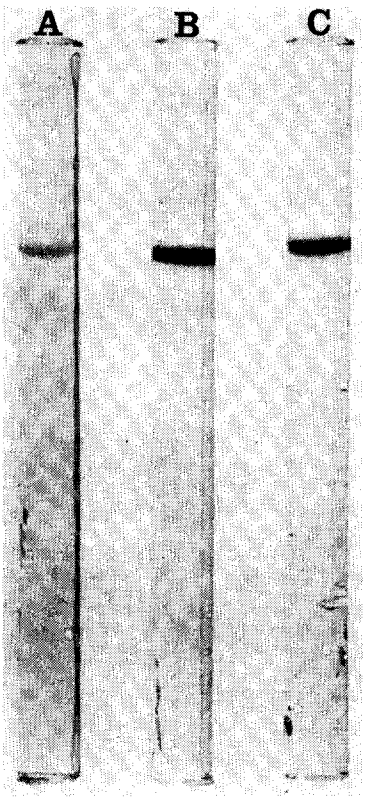

Fig. 2. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of purified D-amino acid oxidase. A, apoprotein, 50 μg; B, holoenzyme–benzoate complex, 100 μg; C, apoprotein + holoenzyme–benzoate complex, 100 μg. For details see under Material and Methods.

electrophoresed in the presence of sodium dodecylsulphate on a polyacrylamide gel (Fig. 2).

Amino acid composition and sulfhydryl groups content

The amino acid content of D-amino acid oxidase is reported in Table II. The protein taken for hydrolysis was the holoenzyme–benzoate complex which had been carefully characterized spectrophotometrically, assuming an $\varepsilon = 11\,300\ M^{-1} \cdot cm^{-1}$ for the enzyme bound at 462 nm. The tryptophan determination shows the presence of 8 moles/mole FAD. This high content agrees largely with the fluorescent studies of Massey and Curti[6].

The sulphydryl content of D-amino acid oxidase is summarized in Table III. Comparison of the data shows that no disulphides are present and the enzyme contains 5 sulphydryl groups per mole FAD.

Amide content and terminal amino acids

The amide determination has been carried out on 10 mg of apoprotein: 31 free carboxyl groups have been found which by difference gives a total of 38 residues of asparagine + glutamine/mole FAD. The results are the average of two experiments.

TABLE II

AMINO ACID COMPOSITION OF D-AMINO ACID OXIDASE

Amino acid	*Residues per mole of FAD*			
	18-h hydrolysis	*39-h hydrolysis*	*61-h hydrolysis*	*Average or extrapolated*
Lys	11.8	11.9	12.0	11.9
His	7.6	7.4	7.5	7.5
Arg	22.2	20.7	20.8	21.2
Trp*	—	—	—	8.0
Asp	32.1	31.8	32.8	32.2
Thr	21.8	21.7	21.1	22.6
Ser	13.0	12.5	12.2	13.6
Glu	36.6	37.0	37.2	36.9
Pro	23.0	22.9	24.2	23.4
Gly	32.9	33.0	33.7	33.2
Ala	18.1	17.9	17.8	17.9
Cys**	—	—	—	5.0
Val	20.9	24.0	25.6	24.8
Met	4.8	5.3	4.8	5.0
Ile	12.8	16.4	17.2	16.8
Leu	34.9	36.1	36.3	36.2
Tyr	13.4	14.0	14.2	13.9
Phe	14.8	14.9	15.1	14.9
		Total residues/mole FAD		345

* Determined according to a modification of the method of Spies and Chambers[11].
** Determined as cysteic acid after oxidation with performic acid[14] or dimethylsulphoxide[15].

The $-NH_2$ terminal amino acid, determined by the dansyl procedure[16], either on the native or on the performic acid-oxidized protein gives, respectively, methionine and methionine sulphone. The –COOH determination with carboxypeptidase A (ref. 19) gives 0.7 mole of leucine per mole of enzyme monomer after 24 h of digestion. Leucine was also found as the –COOH terminal using the hydrazine method but in this case only 0.2 mole of amino acid has been detected per mole of enzyme monomer.

TABLE III

DETERMINATION OF SULPHYDRYL GROUPS IN D-AMINO AICD OXIDASE

Treatment of enzyme	*Sulphydryl groups (moles/mole FAD)*
Oxidation with performic acid or dimethylsulphoxide	5.00 (cysteic acid)
Reduction with dithiothreitol + iodoacetic acid in 8 M urea	4.98 (carboxymethylcysteine)
Titration with DTNB in 6 M guanidine	5.07 (free sulphydryl groups)
Titration with 4,4′-dipyridyl-disulphide in sodium dodecyl sulphate	5.18 (free sulphydryl groups)

Optical properties

The correlation of the protein content obtained by the biuret procedure and the amino acid analysis gives an $A_{540\ nm}$ (0.1%) of 0.122 for the holoenzyme of D-amino acid oxidase. Under the same conditions the apoprotein gives an $A_{540\ nm}$ (0.1%) of 0.120. The holoenzyme–benzoate complex has an absorbance at 274 nm of 2.85 cm^{-1} for a solution of 1 mg/ml. Under the same conditions the apoprotein gives an absorbance of 1.95 cm^{-1} at 278 nm. The ratio of the absorbance for the holoenzyme–benzoate complex at 274 nm to that at 462 nm is 9.5.

Molecular weight

Determination of molecular weight carried out according to Weber and Osborn[8] gives a value of 38 000 ± 10% for the apoprotein monomer. The molecular weight of the holoenzyme calculated from the amino acid analysis and FAD content is 39 600. The flavin content (calculated on the basis of 39 600) is 25.3 nmoles/mg: similar results can be obtained by estimating the protein concentration by the biuret procedure and v.s.

DISCUSSION

Considerable discrepancies exist in the literature concerning the molecular weight and the amino acid composition of D-amino acid oxidase[22,23]. Values ranging from 35 000 to 55 000 have been reported for the monomer of this flavoprotein while the published amino acid analyses differ widely on the basis of the FAD content[1–5,8,21,24–26]. Moreover the attempts carried out in different laboratories in order to improve the already published purification procedures[2,25,27,28] seem to indicate some difficulties in obtaining a homogeneous protein.

The main advantage of our method consists in obtaining a homogeneous protein (as judged by disc electrophoresis) with an amino acid composition which remains constant throughout all the numerous analysis carried out in our laboratory. Furthermore the higher FAD content per mg of protein detectable in our preparation contributes to the higher specific activity as reported in Table I. The molecular weight (39 600) determined by amino acid analysis agrees well with that determined by gel electrophoresis and with the value reported by Weber *et al.*[29].

While $-NH_2$ and –COOH terminal amino acid analyses confirm the results of Kotaki *et al.*[4] but not the data of Mizon *et al.*[28], the amide content differs from the previous published values[4].

We hope that the present work will contribute to further studies on the structure and function of D-amino acid oxidase.

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