Biochem. J. (1987) 242, 603–606 (Printed in Great Britain)

Pea (*Pisum sativum*) diamine oxidase contains pyrroloquinoline quinone as a cofactor

Zdeněk GLATZ,* Jan KOVÁR,* Lumír MACHOLÁN* and Pavel PEČ†

*Department of Biochemistry, University of Brno, Kotlářská 2, 611 37 Brno, and †Department of Analytical and Organic Chemistry, University of Olomouc, Leninova 8, 771 46 Olomouc, Czechoslovakia

Diamine oxidase was prepared from pea (*Pisum sativum*) seedlings by a new purification procedure involving two h.p.l.c. steps. We studied the optical and electrochemical properties of the homogeneous enzyme and also analysed the hydrolysed protein by several methods. The data presented here suggest that the carbonyl cofactor of diamine oxidase is firmly bound pyrroloquinoline quinone.

INTRODUCTION

Pyrroloquinoline quinone (PQQ) is a recently revealed cofactor occurring in several oxidoreductases (in bacterial methanol, methylamine and glucose dehydrogenases and in bovine amine oxidase) [1–4]. This coenzyme is also supposed to be contained in pea (*Pisum sativum*) diamine oxidase (DAO, EC 1.4.3.6) [5]. Several indirect arguments can be adduced in favour of this hypothesis: (i) DAO has properties comparable with those of bovine amine oxidase [5]; (ii) it also contains copper and a carbonyl cofactor (different from pyridoxal 5-phosphate) [6,7]; (iii) its colour changes depending on its redox state, and the carbonyl groups of the cofactor can be titrated with phenylhydrazine with a 1:1 stoichiometry [8,9].

The objective of the present study was to collect other data which would confirm the presence of PQQ in DAO more convincingly. The enzyme was purified to homogeneity by using efficient fast protein liquid chromatography (f.p.l.c.), its optical and electrochemical properties were investigated, and an attempt to liberate and characterize the supposed cofactor was undertaken.

MATERIALS AND METHODS

Enzyme preparation

DAO was isolated from pea seedlings grown in the dark, and the crude enzyme (approx. 30 nkat/mg of protein) after rivanol and heat treatment [10] was purified to homogeneity using high-performance chromatographic steps; separations on an UltroPak TSK 3000 SWG column (LKB, Bromma, Sweden) and on a Mono S HR 5/5 column (Pharmacia, Uppsala, Sweden) were carried out. The columns were attached to the f.p.l.c. (Pharmacia) described in [11]. The active fractions were pooled and concentrated with an Amicon (Lexington, MA, U.S.A.) ultrafiltration cell equipped with a XM-50 membrane. The specific activity of the purified enzyme (determined as described in [10]) amounted to 650–680 nkat/mg of protein at 30 °C, pH 7, with putrescine as substrate. SDS/PAGE, which was performed as described previously [12], confirmed the homogeneity of the enzyme (the M_r of the subunit was in the range 90000–95000; cf. [5]). Chromatofocusing of purified DAO on a Mono P column (Pharmacia) [11] revealed the presence of one protein fraction with a pI of 7.35 ± 0.05 (cf. [9]).

Hydrolysis of pea seedling diamine oxidase

Non-enzymic hydrolysis was performed at acidic pH in the presence of methanol [13]. A 5 mg portion of DAO was incubated with 1 ml of a solution consisting of 0.1 M-H_3PO_4/KH_2PO_4, pH 1, and methanol in the ratio of 2:1 (v/v) at 25 °C for 1 h. Thereafter the reaction mixture was briefly heated to 100 °C. For the proteolytic digestion conditions similar to those described by Lobenstein-Verbeck *et al.* [4] were used. A 1.5 mg portion of DAO was incubated with 1.5 ml of 50 mM-sodium phosphate buffer, pH 7.5, in the presence of chymotrypsin (0.5 mg) at 37 °C for 48 h; thereafter Pronase (0.5 mg) was added and the incubation continued at 37 °C for 48 h. The precipitates formed in both cases were removed by centrifugation, and the supernatant was concentrated in a rotary evaporator and analysed either directly or after passage through a Sep-Pac C_{18} silica cartridge (Waters Associates, Framingham, MA, U.S.A.) by using the conditions described by Duine & Frank [13].

Analytical methods

H.p.l.c. separations of hydrolysates were carried out on a Silasorb C_{18} column (150 mm × 4 mm int. diam.) (Laboratorní přístroje, Prague, Czechoslovakia), the detection being done either spectrophotometrically by means of a variable-wavelength monitor equipped with a spectrocontroller (Knauer, Berlin, Germany) or fluorimetrically by using a RF-530 detector (Shimadzu, Kyoto, Japan). A Cary 118 (Varian Associates, Palo Alto, CA, U.S.A.) served for the spectrophotometric measurements, and a Dichrograph IV (Jobin Yvon, Longjumeau, France) was used for measuring c.d. spectra. Uncorrected fluorescence spectra were scanned in an Aminco–Bowman (SLM/Aminco, Urbana, IL, U.S.A.) spectrofluorimeter, and the fluorescence data

Abbreviations used: PQQ, pyrroloquinoline quinone; DAO, diamine oxidase; f.p.l.c., fast protein liquid chromatography; PAGE, polyacrylamide-gel electrophoresis.

were corrected by the method of Chen [14]. Differential pulse polarograms were measured in a PRG-4 apparatus (Tacussel, Villeurbanne, France) by using the conditions described by Kovář *et al.* [15], the given potentials being related to the saturated calomel electrode.

All chemicals used were of the highest grade commercially available.

RESULTS AND DISCUSSION

The high-performance chromatographic methods used for the purification of DAO proved to be very efficient. High-performance gel chromatography on a preparative TSK 3000 SWG column provided a suitable method for the separation of several contaminants (especially those of lower M_r); an approx. 5-fold increase in specific activity was attained (Fig. 1*a*). Moreover, the active fraction obtained could be applied to a cation-exchange column without dialysis or other desalting procedures. The second high-performance chromatographic step removed the remaining impurities (Fig. 1*b*). Most of them were eluted ahead of DAO. The combination of gel-permeation and cation-exchange chromatography yielded approx. 15 mg of homogeneous DAO from 300 mg of crude protein preparation (obtained after the removal of most of the protein by precipitation with rivanol [10]).

The physico-chemical properties of the purified enzyme were investigated by means of absorption spectrophotometry, c.d. spectroscopy, fluorimetry and differential pulse polarography.

The absorption spectrum of pea DAO (shown in Fig. 2*a*) is comparable with that reported by Kluetz *et al.* [16]. This spectrum reveals that, besides aromatic amino acid residues ($\lambda_{max.} \sim 280$ nm), another chromophore is present in this enzyme. The relatively weak absorption bands at approx. 290–300 nm, 320–370 nm and 390–410 nm coincide well with those of PQQ bound to proteins (cf. [17]). DAO also exhibits weak absorption bands at higher wavelengths (not shown in Fig. 2*a*) that can be attributed to the bound copper ions (cf. [18]). The c.d. spectrum of DAO (not shown) reveals the presence of large positive Cotton effects at 270–300 nm (aromatic amino acids and cofactor), smaller effects at 320–370 nm and 400–420 nm (cofactor) and weak effects at longer wavelengths (copper). The ratio of ellipticities at 340 nm is very close to that found for the typical quinoprotein, methanol dehydrogenase, from *Methylomonas* J [17]. The fluorescence spectra of the purified DAO are the most convincing arguments for the presence of PQQ in this enzyme. The enzyme shows an unusual fluorescence emission spectrum ($\lambda_{emission} = 335$ nm) when excited at 280 nm. However, it also fluoresces relatively strongly at longer wavelengths ($\lambda_{emission} = 460$ nm). The uncorrected excitation and emission spectra (Fig. 3) are very similar to those of quinoproteins containing PQQ as cofactor [17,19]. None of the other known prosthetic groups of oxidoreductases has comparable fluorescence properties.

The differential pulse polarograms of DAO (in 0.1 M-sodium phosphate buffer, pH 7.3) consisted of two smaller peaks at approx. −0.1 V and −0.6 V and a higher peak of about −1.1 V being approx. 2-fold in comparison with that at +0.1 V (not shown)]. The first signal corresponds to the reduction of the bound copper ions (it was also observed in the case of the other cuproproteins [20]); the second peak might reflect the reduction of disulphide bonds [21]. The third signal,

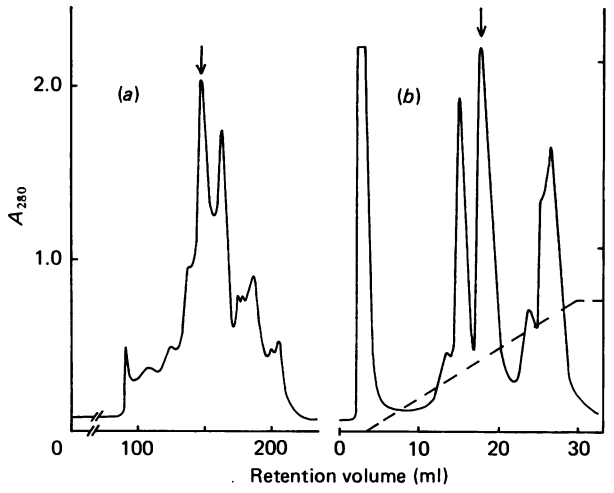

Fig. 1. High-performance chromatographic purification of pea DAO

——, A_{280}; – – – –, gradient; ↓, DAO activity; (*a*) Separation on a UltroPac TSK 3000 SWG column. Mobile phase, 20 mM-sodium phosphate buffer, pH 6; flow rate, 5 ml/min; approx. 100 mg of crude DAO preparation were injected. (*b*) Separation on a Mono S column. Buffer A, 20 mM-sodium phosphate buffer, pH 6; buffer B, the same with 0.3 M-NaCl; flow rate, 1.5 ml/min; approx. 25 mg of the protein fraction with DAO activity after gel chromatography were injected.

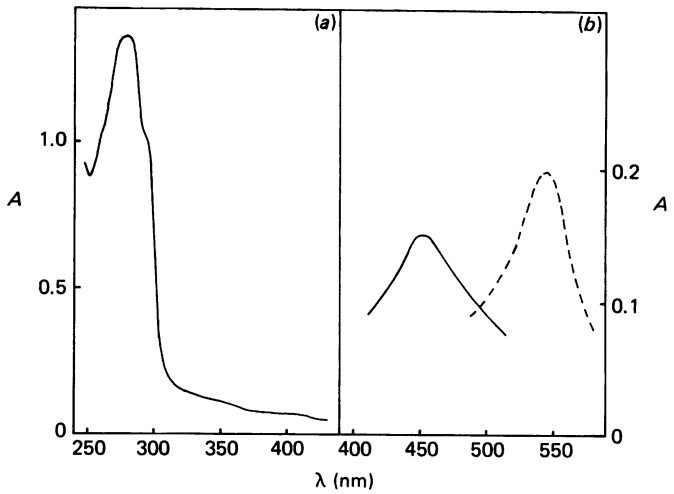

Fig. 2. Absorption spectra of purified DAO (*a*) and its adducts with reagents specific for carbonyl groups (*b*)

(*a*) The spectrum was obtained with 1 mg of DAO/ml in 0.1 M-sodium phosphate buffer, pH 7, at 25 °C. (*b*) ——, Absorption spectrum of 0.5 mg/ml solution of DAO in 0.1 M-sodium phosphate buffer, pH 7, after incubation with 0.05 mM-2,4-dinitrophenylhydrazine (16 h at 25 °C); – – – –, absorption spectrum of DAO after reaction with 3,4-dimethoxyaniline. A 1 ml portion of DAO solution (1 mg/ml) was mixed with 1 ml of reagent solution (1.4% in acetic acid); the mixture was incubated in a boiling-water bath for 20 min, cooled to 25 °C and scanned after 5-fold dilution with acetic acid.

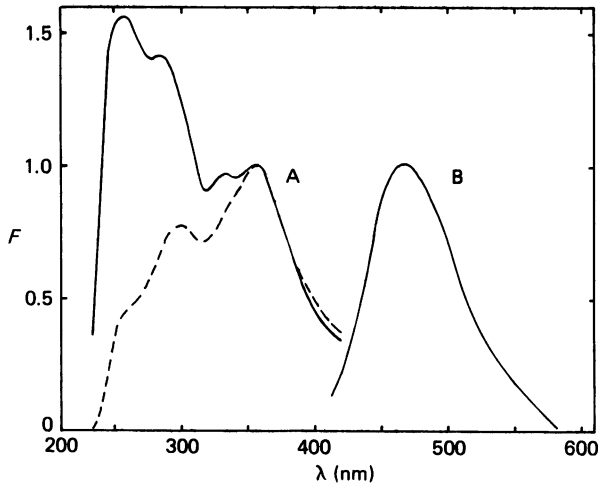

Fig. 3. Fluorescence spectra of purified DAO

The conditions were the same as those in Fig. 2(*a*); DAO at 0.1 mg/ml was used. Fluorescence intensity (*F*) is given in arbitrary units, the intensity at $\lambda_{excitation}$ = 365 nm and $\lambda_{emission}$ = 465 nm being regarded as unity. Trace A, Excitation spectra ($\lambda_{emission}$ = 465 nm); ----, uncorrected spectrum; ——, corrected spectrum; trace B, uncorrected emission spectrum ($\lambda_{excitation}$ = 365 nm).

which is absent in the other proteins containing Cu(II), shifts with pH changes and decreases in the presence of hydrazine. It might correspond to the reduction of bound PQQ. The optical and electrochemical properties of native pea DAO suggest that this enzyme contains PQQ as a tightly bound cofactor. This cofactor could not be removed by the gel-permeation and ion-exchange chromatography used in the isolation procedure.

The effects of known ligands forming more or less specific complexes with PQQ were also investigated. It is known that PQQ (hydrated form) is able to bind borate ions, the formation of the adduct being accompanied by an increase in the absorption at 340–350 nm and by changes in fluorescence spectra [19]. The addition of $Na_3B_4O_7$ (final concn. 0.1 M, pH 10) to the solution of the native enzyme resulted in an absorbance increase at these wavelengths, and the fluorescence excitation spectrum showed an increased maximum at about 350 nm. These observations are identical with those described for free PQQ [19]. The previously described experiments [8,9] proved that pea DAO forms a 1:1 complex (per active site) with phenylhydrazine that absorbs at approx. 450 nm. The adduct of the enzyme with this reagent has entirely different properties in comparison with those of pyridoxal 5-phosphate and is very similar to that observed by Suzuki *et al.* [22] in the case of bovine amine oxidase, which contains PQQ as a cofactor [4]. DAO also reacted with 2,4-dinitrophenylhydrazine, the observed spectral changes being nearly the same as those occurring in bovine amine oxidase on addition of this compound [4] (Fig. 2*b*). We also demonstrated the presence of inner-ring *o*-quinones in DAO with dimethoxyaniline, which is a more selective reagent. It forms Schiff bases absorbing at >500 nm with *o*-quinones whose carbonyl groups are located in inner rings of (hetero) aromatic compounds [23]. The addition of this reagent to the solution of pea DAO in sodium phosphate buffer, pH 7, resulted in an absorption maximum at 545 nm (Fig. 2*b*). The described interactions of DAO with the adduced ligands are also compatible with the assumption that this enzyme contains PQQ as a prosthetic group.

We also attempted to demonstrate the presence of free PQQ after the hydrolysis of the enzyme. Both hydrolytic procedures used (i.e. acid-methanol treatment and proteolysis; see the Materials and methods section) gave comparable results. The hydrolysates were chromatographed on a C_{18} reversed-phase column, the mobile phase used by us resulting in better separation than that achieved by Duine *et al.* [24] (see Fig. 4). The spectral characteristics of the pooled fluorescent peak (Fig. 4) at pH 7 were identical with those reported for PQQ [2,19] (absorption maxima at about 200 nm, 250–270 nm and two absorption bands in the range of 300–370 nm; excitation bands at 250–270 nm, 310–340 nm and 350–360 nm, and fluorescence emission maximum at 460 nm). The difference in the absorption spectra of free and bound cofactor at pH 7 (cf. Fig. 2) supports the assumption that some negatively charged groups are located in the vicinity of the bound cofactor (the spectrum of the bound cofactor resembles closely that of free PQQ in alkaline media [2]). This assumption is compatible with the fact that the active centre of DAO binds positively charged diamines. A slight red shift of the emission maximum and on additional excitation band at 290–300 nm are the main differences in the fluorescence spectra of the bound cofactor compared with that in the free form. The latter difference (see also [17]) might be attributable either to the influence of negatively charged groups in the vicinity of the bound cofactor (see above) or to the resonance energy transfer from excited tryptophan residues to the bound PQQ. The influence of temperature on the fluorescence properties of the liberated cofactor was the same as that described for PQQ [19]. The intensity decrease observed when temperature was elevated from 25 to 70 °C was nearly the same as that reported by Dekker *et al.* [19]. On the other hand, the influence of temperature on the cofactor bound to DAO was essentially smaller; this might be the result of a decreased mobility of the cofactor bound to the protein. An increase in pH (to approx. 11) brought about an essential enhancement in the fluorescence of the free cofactor, the same phenomenon also being observed in the case of PQQ [4]. The liberated cofactor formed the adducts with specific optical properties on addition of the abovementioned reagents for carbonyl compounds.

The crude hydrolysate of DAO was also analysed by differential pulse polarography. The polarograms revealed the presence of free (hydrated) Cu(II) ions (the potential of reduction at about 0.0 V) and of a component reducible at about −0.4 V at pH 8 (showing a potential shift of approx. 60 mV/pH unit). The current at −0.4 V decreased on addition of hydrazine (probably attributable to the reaction with reducible quinone groups). These data are in accord with those obtained for a analogue of PQQ with thin-layer cyclic voltammetry [2]. The more negative reduction potential of the cofactor bound to DAO (see above) in comparison with that of the free compound is usual with reducible groups bound to proteins [15]; the reduction at the electrode surface is more difficult when the reducible group is bound to the intact protein structure. The influence of negatively charged groups (supposedly in the active centre) on the reduction potential might also be involved. The results of the electrochemical measurements are in accord with the

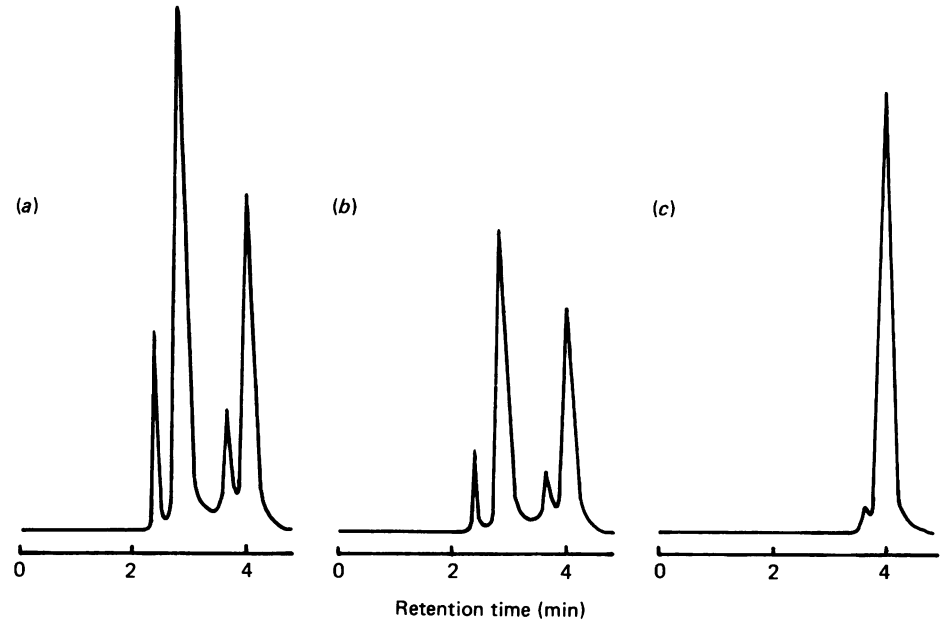

Fig. 4. H.p.lc. of hydrolysed pea DAO

The acid-methanol treatment was used (see the Materials and methods section); column, Silasorb C_{18} (150 mm × 4 mm int. diam.); mobile phase, 0.1 M-sodium phosphate buffer (pH 6)/4% (v/v) methanol; flow rate, 0.7 ml/min. (*a*) Photometric detection at 280 nm; (*b*) photometric detection at 315 nm; (*c*) fluorimetric detection at $\lambda_{excitation} = 365$ nm, $\lambda_{emission} = 460$ nm.

other data presented here and confirm the hypothesis that the carbonyl cofactor of pea DAO is identical with PQQ.

We thank Dr. B. Brzobohatý for his gift of Pronase and Dr. J. Jursa for the assistance in measuring c.d. spectra.

REFERENCES

1. Duine, J. A. & Frank, J. (1981) Trends Biochem. Sci. **6**, 278–280
2. Sleath, P. R., Noar, J. B., Eberlein, G. A. & Bruice, T. G. (1985) J. Am. Chem. Soc. **170**, 3328–3338
3. Vellieux, F. M. D., Frank, J., Swarte, M. B. A., Groendijk, H., Duine, J. A., Drenth, J. & Hol, W. G. J. (1985) Eur. J. Biochem. **154**, 383–386
4. Lobenstein-Verbeck, C. L., Jongejan, J. A., Frank, J. & Duine, J. A. (1984) FEBS Lett. **170**, 305–309
5. Smith, T. A. (1985) Biochem. Soc. Trans. **13**, 319–322
6. Werle, E., Trautschold, I. & Aures, D. (1961) Hoppe-Seyler's Z. Physiol. Chem. **326**, 200–211
7. Rinaldi, A., Floris, G., Sabatini, S., Finazzi-Agrò, A., Giartosio, A., Rotilio, G. & Mondovi, B. (1983) Biochem. Biophys. Res. Commun. **41**, 841–848
8. Michele, P. (1974) Thesis, Brno
9. Yanagisawa, H., Hirasawa, E. & Suzuki, Y. (1981) Phytochemistry **20**, 2105–2108
10. Macholàn, L. & Haubrová, J. (1976) Coll. Czech. Chem. Commun. **41**, 2987–2996
11. Kovář, J. & Stejskal, J. (1985) J. Chromatogr. **325**, 359–362
12. Matysková, I., Kovář, J. & Racek, P. (1985) Biochim. Biophys. Acta **839**, 300–306
13. Duine, J. A. & Frank, J. (1980) Biochem. J. **187**, 221–226
14. Chen, R. F. (1967) Anal. Biochem. **20**, 339–357
15. Kovář, J., Brzobohatý, P. & Studničková, M. (1982) Bioelectrochem. Bioenerg. **9**, 333–343
16. Kluetz, M. D., Adamsons, K. & Flynn, J. E. (1980) Biochemistry **19**, 1617–1621
17. Ohta, S., Fujita, T. & Tobari, J. (1981) J. Biochem. (Tokyo) **90**, 205–213
18. Dooley, D. M. & Coté, C. E. (1985) Inorg. Chem. **24**, 3996–4000
19. Dekker, R. H., Duine, J. A., Frank, J., Verwiel, P. E. J. & Westerling, J. (1982) Eur. J. Biochem. **125**, 69–73
20. Pitřincová, J. (1986) Thesis, Brno
21. Stanovich, M. T. & Bard, J. S. (1978) J. Electroanal. Chem. **86**, 189–195
22. Suzuki, S., Sakurai, T. & Nakahara, E. (1986) Biochemistry **25**, 338–341
23. Sawicki, E. & Elbert, W. (1960) Anal. Chim. Acta **22**, 448–451
24. Duine, J. A., Frank, J. & Verwiel, P. E. J. (1981) Eur. J. Biochem. **118**, 395–399

Received 5 November 1986/16 December 1986; accepted 22 December 1986