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

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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 Mr 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 H3PO4/KH2PO4, 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 C18 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 C18 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.

Vol. 242
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. 1a). 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. 1b). 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. 2a) 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. 2a) 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 Methylo-

domonas 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 mM-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,
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
Na2B4O7 (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. 2b). We also demonstrated the presence
of inner-ring o-quinones in DAO with dimethoxy-
aniline, 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. 2b). The described interactions of DAO with the
added 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 chromatog-
graphed on a C18 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
declined on addition of hydrazine (probably attribut-
able to the reaction with reducible quinone groups).
These data are in accord with those obtained for an
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
other data presented here and confirm the hypothesis that the carbonyl cofactor of pea DAO is identical with PQQ.

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REFERENCES


Fig. 4. H.p.l.c. of hydrolysed pea DAO

The acid-methanol treatment was used (see the Materials and methods section); column, Silasorb C18 (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 λex = 365 nm, λem = 460 nm.