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Soybean lipoxygenase-1 is not a quinoprotein

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Soybean lipoxygenase-1 was reinvestigated with respect to its quinoprotein nature. It has been reported previously that soybean lipoxygenase-1 contains pyrroloquinoline quinone as the organic cofactor [1]. Because spectroscopic data were found to be inconsistent [2] with the evidence presented in [1], we sought to reproduce the published data by carefully following the procedures described in [1] and supplementing them with new analytical results. The combined data lead us to conclude that soybean lipoxygenase-1 is not a quinoprotein.

Lipoxygenase; Pyrroloquinoline quinone; Quinoprotein; Redox-cycling; PQQ; Iron

1. INTRODUCTION

Native lipoxygenase-1 (EC 1.13.11.12), isolated from dry, mature soybeans is a colorless protein containing high-spin Fe(II) [3]. It catalyzes regio- and stereospecific hydroperoxide formation from natural poly-unsaturated fatty acids like linoleic and arachidonic acid. Based on an amino acid content of 838, a molecular mass of 94 038 [4] was calculated. Since the discovery of the presence of iron in a 1:1 stoichiometry [5,6], this was considered to be the only non-amino acid constituent. The functional role of iron in the catalytic cycle was demonstrated by EPR-spectroscopy [7]. Recently, Van der Meer and Duine reported on the presence of pyrroloquinoline quinone (PQQ) in soybean lipoxygenase-1 [1] and proposed a mechanistic scheme involving both an Fe(II)/Fe(III) shuttle and PQQ as participants in the electron transfer process. Besides, a model was presented for the iron-site in lipoxygenase. To us [2], it appeared difficult to reconcile the proposed model with EPR-, EXAFS- and other pertinent data.

According to Van der Meer and Duine, all quinoproteins from eukaryotic origin discovered sofar, contain covalently bound PQQ [1]. However, the very nature of PQQ in such proteins was recently scrutinized on the basis of X-ray diffraction studies on methylamine dehydrogenase [8,9] resulting in a novel two-ring structure, termed 'pro-PQQ', to be proposed for the genuine cofactor in eukaryotic quinoproteins. Another protein, dopamine β-hydroxylase, studied by Van der Meer et al. [10] and since designated as a 'quinoprotein', was found to contain neither PQQ nor a related cofactor [11]. The copper-containing amine oxidase from bovine serum, originally described as the first example of a eukaryotic quinoprotein [12], has recently been reinvestigated with respect to its cofactor content and was found to contain 6-hydroxy-DOPA [13] and not PQQ. Recent discussions on the methodology for detecting and quantitating PQQ (see e.g. [14,15]) merely emphasize the necessity to critically evaluate its adequacy, and to perform experiments independently. As for soybean lipoxygenase-1, we have repeated, modified and extended the procedures described by Van der Meer and Duine [1].

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2. MATERIALS AND METHODS

PQQ and porcine kidney diamine oxidase (PKDAO) were obtained from Sigma. Octadecyl silica (ODS) extraction cartridges were from Baker or from Waters (Seppak). Nitrobluetetrazolium (NBT) was from Janssen Chimica and hexanol (98%, product No. 804393) was from Merck. Soybean lipoxygenase-1 was isolated as described previously [16,17]. The specific activity was found to be 240 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$. A CP-Spher 8$\mu$-C18 HPLC-column (250 × 4.6 mm) was from Chrompack. The HPLC system consisted of a Hewlett-Packard 1090 solvent delivery system attached to a Hewlett-Packard 1040A diode-array detector. Data were processed on a Hewlett-Packard 310 SPU workstation. UV spectra were recorded on a Hewlett-Packard 8450A double-beam diode-array spectrophotometer.

Derivatization of soybean lipoxygenase-1 (31 mg protein) with phenylhydrazine or dinitrophenylhydrazine and subsequent pronase digestion were performed as described by Van der Meer for phenylhydrazine [1]. Extraction of the pronase digest on an ODS cartridge was performed according to Van der Meer et al. [18]. As the hydrazine adducts of PQQ appeared to elute from the ODS column in the 10% methanol washing step, this step was omitted. In order to wash the ODS column, 10 mM HCl was used instead of water.

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Derivatization of protein-bound PQQ, protein solvolysis with acidic hexanol, ODS extraction and RP-HPLC were performed as described by Van der Meer et al. [19] with 25 mg soybean lipoxygenase-1. 13*S*-hydroperoxo-octadecadienoate (13*S*-HPOD) was prepared by incubating 400 μM linoleic acid with 10 nM soybean lipoxygenase-1 in an oxygen-saturated 0.1 M borate buffer, pH 9.0, at 4°C. Product formation was monitored at 234 nm and by RP-HPLC analysis (flow: 1.0 ml/min; eluent: methanol/water/acetic acid 70:30:0.1). After completion of the reaction, the product was isolated with a Baker solid phase C18 extraction column, and collected in methanol.

Just before loading samples onto the gel, 13*S*-HPOD was added (molar ratio 13*S*-HPOD/protein: 1.2) to the native enzyme to prepare yellow Fe(III)-enzyme. The purple Fe(III)-enzyme species was prepared analogously, the initial ratio 13*S*-HPOD/protein being 20. The SDS-PAGE, electroblotting and staining with the NBT-glycinate redox cycling procedure was carried out (Paz, M.A., Flučkiger, R.A., Boak, A., Kagan, H.M. and Gallop, P.M., unpublished) as follows. SDS-PAGE (10%) was performed under reducing conditions according to Laemmli [20]. A Mini Protean II apparatus (BioRad) was used with 1.5 mm spacer arms. Before being loaded, all samples were heated at 95°C for 15 min. Runs were stopped as soon as the bromophenol blue dye reached the bottom of the gel. Immediately after electrophoresis, transfer to a nitrocellulose sheet was performed with a Mini Trans Blot apparatus (BioRad), cooled to 4°C in 25 mM Tris/192 mM glycine/20% methanol, pH 8.3, essentially as described by Towbin et al. [21]. The transfer was completed after 2 h at 110 V and 150 mA. After the transfer, the nitrocellulose sheet was subjected to NBT-staining by incubating the sheet for 45 min at room temperature in a 0.24 mM NBT/2 M K-glycinate solution, pH 10.0. The sheet was eventually dipped in 3 mM Na_3BO_3 and stored in this solution at 4°C in the dark. Proteins other than quinoproteins were visualized by staining with 0.1% Ponceau S in 5% acetic acid for about 15 min.

3. RESULTS

3.1. *Phenylhydrazine (PH) derivatization of native soybean lipoxygenase-1*

In repetitive experiments, purified soybean lipoxygenase-1 was reacted with phenylhydrazine with no significant UV-spectral changes of the reaction mixture as a result. In some instances, the mixture showed an absorption maximum at 340 nm, also reported by Van der Meer et al. [1] and assigned by these authors to a PQQ-phenylhydrazine adduct. When PKDAO, a putative quinoprotein, was treated with phenylhydrazine, the pronase digest showed two peaks in the HPLC pattern at 340 nm, one at 6.8 and one at 7.0 min. The UV spectra of both compounds were identical. It is not clear why derivatization of PKDAO yielded two peaks in the HPLC. On a molar basis, PKDAO yielded about 10 times more material absorbing at 340 nm than soybean lipoxygenase-1.

3.2. *Dinitrophenylhydrazine (DNPH) derivatization of native soybean lipoxygenase-1*

When soybean lipoxygenase-1 was incubated with DNPH, the absorption maximum of the mixture shifted from 365 to 375 nm. The HPLC pattern of the pronase digest of this material, detected at 350 nm, showed a peak eluting at 6.2 min with an absorption maximum at 375 nm. The retention time of DNPH proper was 9.3 min with an absorption maximum at 357 nm. However, the UV spectrum of the observed peak in the derivatization experiment did not match published spectra of DNPH adducts of PQQ [12]. In our experiments, the absorbance at 280 nm was very high compared to the absorbance at higher wavelengths and the maximum at higher wavelengths was 375 nm instead of the published 450 nm [12]. Therefore, we conclude that no PQQ-DNPH adduct has been formed.

3.3. *Hexanol derivatization of soybean lipoxygenase-1*

Solvolysis of the protein (25 mg) with acidic hexanol

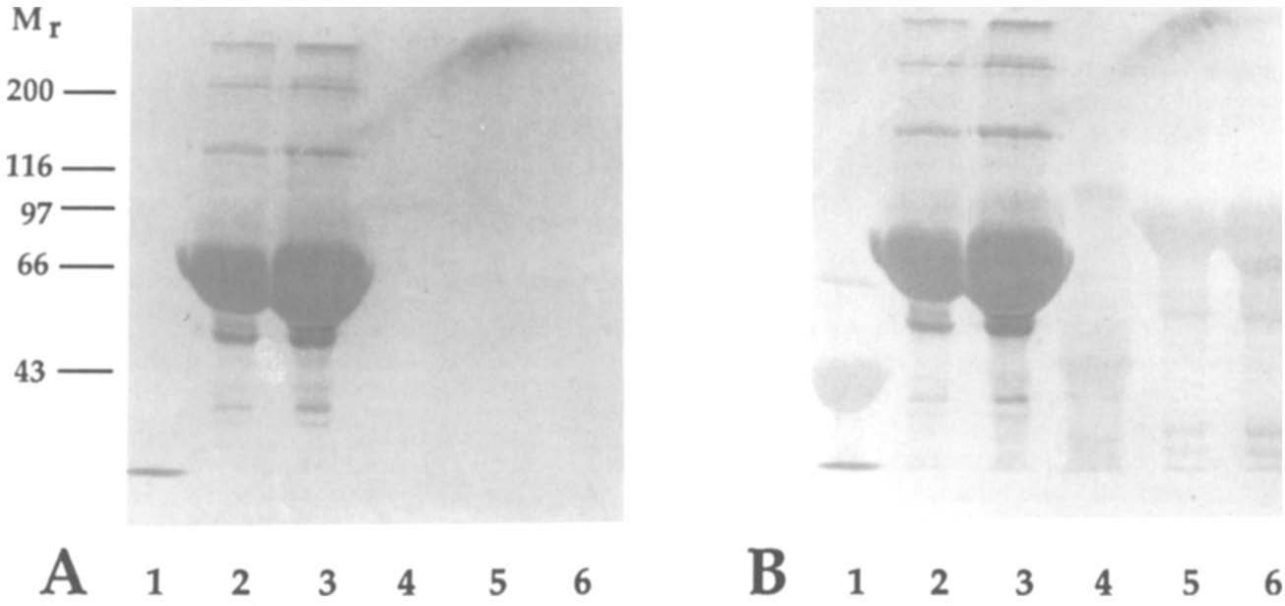

Fig. 1. (A) NBT staining of: 1, methylamine dehydrogenase (bacterium W3A1); 2,3, methylamine oxidase (Arthrobacter P1); 4, pig kidney diamine oxidase; 5,6 native lipoxygenase. (B) Ponceau S staining of the same gel (cf. A).

as described in [19] was used as an alternate method to determine PQQ in soybean lipoxygenase-1. The UV spectrum of the complete reaction mixture was not significantly different from that of untreated enzyme. Subsequent HPLC analysis of the ODS-extracted material did not reveal any compounds absorbing at 318 nm (cf. [19]). We used hexanol with the same product number from the same supplier as Van der Meer et al. [19].

3.4. *NBT-staining*

None of the samples of native lipoxygenase-1 showed formazan formation (purple blue) in the redox assay on the electroblot (Fig. 1A). Lane 1 shows a sample of methylamine dehydrogenase from bacterium W3A1 [22], which demonstrates a quinonoid component in its 15 kDa α-subunit; lanes 2 and 3 show two different concentrations of methylamine oxidase from Arthrobacter P1, which contains quinonoid components in its 80–90 kDa subunits. Lane 4 shows a weak positive reaction on a band of about 100 kDa of a crude preparation of PKDAO. Lanes 5 and 6 contain the lipoxygenase preparations, which show no staining at all. Fig. 1B shows the same electroblot stained with Ponceau S which demonstrates proteins other than the quinoproteins in the various samples staining red with Ponceau S. With the exception of lane 1, the lanes were overloaded to demonstrate the specificity of the NBT-potassium glycinate redox-cycling for quinoproteins. In addition to the lipoxygenase samples shown in Fig.1, the same procedure was applied to yellow an purple Fe(III)-lipoxygenase samples. Neither of these caused formazan formation. In fact, NBT-reduction was prevented by these enzymes as was apparent from the yellow bands against a bluish background, a result typical of the negative-staining procedure described by Leblanc and Cochrane [23] for protein detection in polyacrylamide gels.

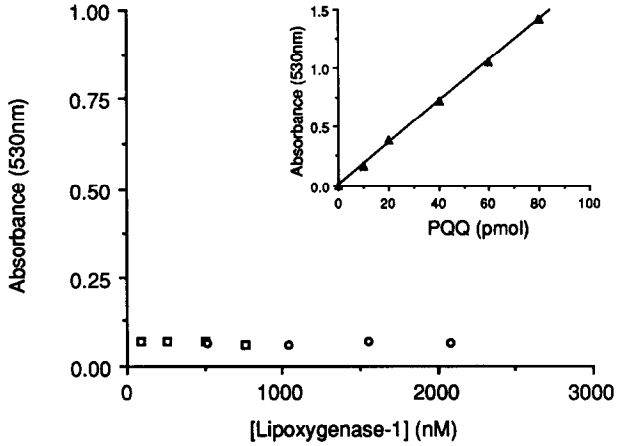

Fig. 2. Spectrophotometric assay of formazan formation. Circles, direct assay with linoleic acid as substrate. Reagents: BSAr (bovine serum albumin reduced under N_2 with 100-fold molar excess $NaBH_4$ and dialyzed overnight at 4°C against deionized water, previously degassed and flushed with N_2), 2 mg/ml, linoleic acid (200 mM), 0.1 M borate buffer, pH 9.0 and 0.24 mM NBT in the same buffer. Reaction mixture: 250 μl BSAr, 730 μl NBT-solution, 10 μl linoleate solution and varying amounts of lipoxygenase. Controls did not contain linoleate and/or lipoxygenase. Samples were incubated overnight at 37°C and the absorbance at 530 nm was then recorded. Squares, amplified detection. Native, yellow and purple enzyme samples were denatured by overnight incubation with 5% SDS at room temperature. Varying volumes of denatured lipoxygenase-1 solutions were then added to 0.24 mM NBT/2 M K-glycinate, pH 10.0 to make up a final volume of 1 ml. After 1 h at 37°C the absorbance at 530 nm was recorded. Samples with yellow and purple lipoxygenase-1 gave identical results, but have not been included in the graph for clarity. Controls did not contain lipoxygenase-1 and/or SDS. Inset: calibration line, obtained by incubating 0.5 mg BSAr, 500 μl 0.24 mM NBT/2 M K-glycinate, pH 10.0 and varying volumes of a solution of 2.5 μM PQQ/2 M K-glycinate, pH 10.0. Final volume 1.0 ml; PQQ-range: 0–80 pmol. In a separate set of experiments, the three forms of lipoxygenase-1 were treated with a 100-fold molar excess of $NaBH_4$ and dialyzed overnight at 4°C against deionized water, previously degassed and flushed with N_2. They were then subjected to the same treatment as the SDS-denatured samples. The results were the same as for the SDS-treated samples, but have been omitted from the graph for clarity.

3.5. *Spectrophotometric assay of formazan formation*

PQQ-detection in lipoxygenase-1 samples was further attempted by using the direct and amplified assay methods as described in [24,25]. Samples investigated included native, yellow and purple soybean lipoxygenase-1, as well as SDS-denatured and $NaBH_4$-reduced samples. None of these samples gave any indication for the presence of PQQ. For experimental details, see the legend to Fig. 2.

4. DISCUSSION

The rare experiments with phenylhydrazine derivatization described above that gave a partly positive indication of PQQ cannot possibly reflect the presence of PQQ as a cofactor covalently linked in a 1:1 stoichiometry to soybean lipoxygenase-1, because of the poor reproducibility and the considerable quantitative difference with PKDAO. Another carbonyl-derivatization technique with 2,4-dinitrophenylhydrazine, and an independent method involving solvolysis with hexanol/HCl failed to yield evidence for the presence of the cofactor. These published methods to detach covalently linked PQQ or a related cofactor from a protein, thus appeared ineffective in our hands towards soybean lipoxygenase-1. The redox-cycling assay, developed by Gallop et al. [24,25], and adapted in their laboratory to be used as a specific staining technique with electrophoresis/electroblotting was used here as an independent approach, with no evidence for formazan formation as a result. The conditions during electrophoresis may, however, be unfavorable to detect all PQQ-loci. Therefore, various soybean lipoxygenase-1 samples, i.e. native, yellow and purple, each of them also SDS-denatured or $NaBH_4$-reduced, were us-

ed to detect possible formazan formation in a way fully analogous to the methods described for the detection of formazan formation by PKDAO and PQQ [25] with putrescine and glycine as reductants, respectively (Fig. 2). In our experiments, the only formazan detected was in the calibration experiment with standard PQQ. Taken together, we find no evidence in the results presented here to sustain the quinoprotein nature of soybean lipoxygenase-1. A recent discussion in this journal [26,27] corroborates this conclusion. Paz et al. [26] have shown that the redox-cycling assay can be an efficient and specific tool in quantitating PQQ and other quinoid compounds. A negative result, as found with soybean lipoxygenase-1, thus demonstrates that this protein neither contains PQQ nor any other quinonoid compound that gives a positive response in the redox-cycling assay.

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