Photoaffinity labelling of plastoquinone binding sites in chloroplast cytochrome b_6/f-complex

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1. INTRODUCTION

The involvement of prenylquinones as electron and proton carriers in the mitochondrial respiratory chain and in photosynthetic electron-transport systems has been well established. The fact that prenylquinones are small and highly lipophilic molecules and abundant as compared to other redox carriers has led to the idea that they form a 'pool' within the membrane and shuttle between the other electron carriers. However, evidence for definite quinone-binding proteins is accumulating (review [1]). In the mitochondrial system, 3 ubiquinone-binding proteins have been verified [1]. The identification of one of them has been achieved by an ubiquinone–azide photoaffinity label [2].

In the photosynthetic electron-transport system of chloroplasts from higher plants the existence of 2 different herbicide-binding proteins at the reducing side of photosystem II has been shown which probably are also plastoquinone-binding proteins: a 32 000–34 000 M_r protein [3] and a 41 000 M_r photosystem II reaction center protein [4,5]. The identification of these proteins was possible by the use of photoaffinity labels derived chemically from photosystem II herbicides [3–5].

A plastoquinone-binding protein at the donor side of photosystem I so far has not been identified. In [6,7] a cytochrome b_6/f-complex has been isolated from spinach chloroplasts, which functions as a plastoquinol/plastocyanin oxidoreductase. Here, we report on plastoquinone-binding sites in this complex by use of a plastoquinone–azide photoaffinity label.

2. MATERIALS AND METHODS

2.1. *Synthesis of the plastoquinone–azide photoaffinity label*

2.1.1. 2,3-Dimethylphenol-5-butyric acid

β-(2-hydroxy-3,4-dimethylbenzoyl)Proprionic acid (2.08 g, 10 mmol) [8] in 10 ml abs. ether was added slowly under stirring to $LiAlH_4$ suspension (0.76 g, 20 mmol) in 10 ml abs. ether. The reaction mixure was stirred for 1 h at room temperature and then excess $LiAlH_4$ destroyed by addition of H_2O. H_2SO_4 (5 ml, 25%) was added and the mixtue extracted several times with ether. After drying over $MgSO_4$ the ether was evaporated in the vacuum; yield, 1.6 g (83%). Recrystallized from CCl_4; m.p. 86–87°C. Calc. %: C, 74.19; H, 9.34. Found %: C, 73.91; H, 9.34.

2.1.2. 2,3-Dimethyl-5-(4′-hydroxy-*n*-butyl)-1,4-benzoquinone

2,3-Dimethylphenol-5-butyric acid (0.78 g, 4 mmol) in 10 ml methanol and 4 ml 1 N Na-acetate were oxidized to the quinone by slow addition of 2.4 g K-nitrosodisulfonate in 120 ml H_2O [9]. After 20 min stirring at room temperature the mixture was extracted several times with ether, the ether dried over $MgSO_4$ and evaporated in the vacuum; yield, 0.80 g (96%). Recrystallized from petrol ether,

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m.p. 38.5°C. Calc. (%): C, 69.21; H 7.74. Found (%): C, 69.59; H, 7.88.

2.1.3. 2,3-Dimethyl-5-{4′-[3-(4-azido-3-nitro-anilino)-propionoxy]-*n*-butyl}-1,4-benzo-quinone

N-4-Azido-2-nitrophenyl-β-alanine [10] (100.4 mg, 0.4 mmol), 166.4 mg (0.8 mmol) 2,3-dimethyl-5-(4′-hydroxy-*n*-butyl)-1,4-benzoquinone, 10 mg 4-dimethylaminopyridine, 104 μl (3.2 mmol) triethylamine and 408 mg (1.6 mmol) 2-chloro-1-methyl-pyridiniumiodide as the condensing agent [11] in 15 ml abs. acetonitrile were refluxed for 10 h. Then 20 ml 0.01 N HCl were added and the reaction mixture extracted several times with ether. After drying over $MgSO_4$ and evaporation in the vacuum the residue was chromatographed twice on silica gel columns with petrol ether/ethyl acetate (5:2, v/v) as the eluent; yield 50 mg (28%); m.p. 56–58°C. Calc. (%): C, 57.13; H, 5.25; N, 15.87. Found (%): C, 57.94; H, 5.68; N, 14.83. UV/vis (methanol) 257 nm ($\varepsilon = 27\,448\ M^{-1} \cdot cm^{-1}$); 445 nm ($\varepsilon = 3\,626\ M^{-1} \cdot cm^{-1}$). The corresponding aminohydroquinone was obtained as in [12] by reduction with $NaBH_4$ in ethanolic solution.

2.1.4. 2,3-Dimethyl-5-{4′-[3-(4-azido-2-nitro-anilino)-[2′,3′-^{3}H]-propionoxy]-*n*-butyl}-1,4-benzoquinone (PQAz)

β-[2,3-^{3}H]Alanine (14 μg, 0.15 μmol) (Amersham Buchler, Braunschweig; spec. act. 32 Ci/mmol; total act. 5 mCi) were reacted with an excess of 4-fluoro-3-nitrophenyl azide as in [10]. The reaction product was diluted by addition of 8.4 mg (33 μmol) *N*-4-azido-2-nitrophenyl-β-alanine. Together with 15 mg (72 μmol) 2,3-dimethyl-5-(4′-hydroxy-*n*-butyl)-1,4-benzoquinone, 50 mg (196 μmol) 2-chloro-1-methyl-pyridiniumiodide, 5 mg (40 μmol) 4-dimethylaminopyridine and 60 μl (83 μmol) triethylamine in 2 ml acetonitrile it was stirred at 50°C for 45 min. Afterwards, 5 ml 0.01 N HCl were added and the reaction mixture extracted 5 times with ether. The ether phase was dried over $MgSO_4$ and the ether evaporated in the vacuum. The residue was taken up in a mixture of 350 μl methanol and 50 μl acetone and aliquots of ~ 70 μl chromatographed on silica gel-precoated plastic sheets 60 F-254 (Merck AG, Darmstadt) with petrol ether (boiling range 60–80°C)/ethyl acetate (5/2, v/v) as the eluent. The zone corresponding to the plastoquinone azide (R_F 0.40) was cut out and eluted with methanol. The concentration of azide was determined from the absorption at 257 nm. The compound was obtained in a yield of 15.3% and spec. act. 9.2 Ci/mol.

2.2. *Biochemical methods*

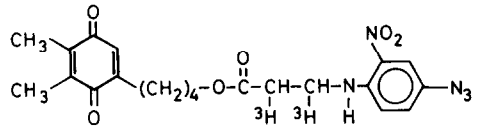
The cytochrome (cyt.) b_6/f-complex was prepared as in [6,7], but Triton X-100 treatment was omitted.

Plastoquinol/plastocyanin oxidoreductase activity was measured as in [6,7]. The activity was tested in 20 mM MES-buffer (pH 6.5) with cyt. 552 (8 μM) from *Euglena gracilis* as the electron acceptor. The purified cyt. b_6/f-complex was added to yield a final concentration of 105 nM cyt. *f*.

For the labelling experiments aliquots of the cyt. b_6/f-complex (corresponding to 800 pmol cytochrome *f*) in 40 μl 30 mM octyl-glycoside, 0.5% cholate, and 30 mM Tris/succinate buffer (pH 6.5) together with the azide were illuminated (light source 2000 W) in small glass vials under cooling and in a nitrogen atmosphere for 15 min. Solubilization, SDS–PAGE, and assay for radioactivity were performed as in [4]. Samples were counted for 5 min and corrected for the background.

3. RESULTS AND DISCUSSION

The chemical structure of the ^{3}H-labelled plastoquinone–azide photoaffinity reagent PQAz is shown in fig.1. The quinone part of the compound was synthesized by Friedel–Crafts acylation of 2,3-dimethyl-phenol with succinic anhydride [8], Clemmensen-reduction of the keto-carbonyl group [8], reduction by $LiAlH_4$ and oxidation with K-nitrosodisulfonate [9]. The quinone alcohol was condensed with *N*-4-azido-2-nitrophenyl-β-[2,3-^{3}H]-alanine [10] by means of 2-chloro-1-methyl-pyridinium iodide [11].



PQAz

Fig.1. Structure of photoaffinity label plastoquinone–azide (PQAz).

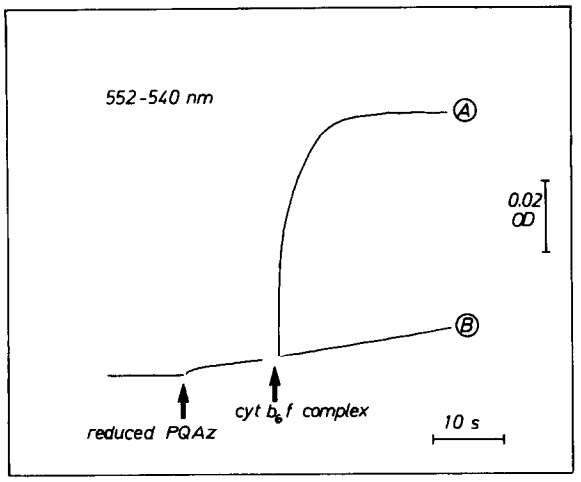


Fig.2. Reduced PQAz as electron donor to plastoquinol/plastocyanin oxidoreductase (cyt. b_6/f-complex). Where indicated, reduced PQAz (30 μM) and purified cyt. b_6/f-complex have been added. For conditions, see section 2. (A) Without inhibitor; (B) in the presence of 5 μM DNP–INT. The activity in (A) corresponded to ~40 μmol cyt. 552 reduced . nmol cyt. f^{-1} . h^{-1}.

Reduced PQAz, i.e., the corresponding amino-hydroquinone, is a good electron donor to isolated chloroplast cyt. b_6/f-complex (fig.2). The plastoquinol/plastocyanin oxidoreductase activity with reduced PQAz as the electron donor was found to be as high as performed with plastoquinol-1 (40 μmol cyt. 552 from *Euglena gracilis* reduced . h^{-1} . nmol cytochrome f^{-1}). The activity was completely inhibited by 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl-ether (DNP–INT) [13] which suggests that reduced PQAz is donating electrons to the cyt. b_6/f-complex via the same site as plastoquinol [6].

We have now investigated the labelling pattern of the cyt. b_6/f-complex by PQAz. The complex consists of 5 polypeptides with app. M_r = 34, 33, 23.5, 20, and 17.5 × 10^{-3} [6]. The 34 000 and 33 000 M_r protein doublet are both ascribed to cyt. f [7], the 23 500 M_r protein to cyt. b_6 [7] and the 20 000 M_r protein to the Rieske Fe–S center [14]. The function of the 17 500 M_r protein could not be identified yet. Photographs of part of a SDS–PAGE gel (11–15%) exhibiting the 5 polypeptides of the cyt. b_6/f-complex together with a typical labelling pattern in the absence and presence of 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB)

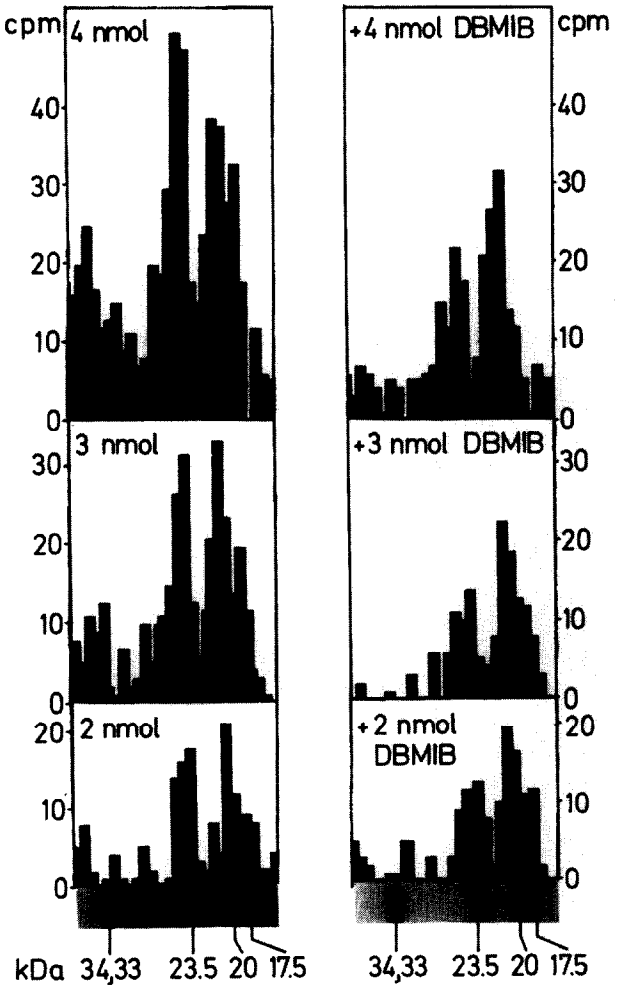


Fig.3. Photographs of part of SDS–PAGE gels of chloroplast cyt. b_6/f-complex and radioactivity distribution after labelling at different amounts of PQAz (2, 3 and 4 nmol) in the absence and presence of equimolar concentrations of DBMIB. The bars in the histogram correspond to the radioactivity count in 2 mm gel pieces. The amount of the complex was the same in all experiments and corresponded to 0.8 nmol cyt. f.

at various [PQAz] are shown in fig.3. The same patterns were observed in 3 different runs. In the absence of DBMIB (left, fig.3) at 2 nmol PQAz the highest amount of radioactivity is found on the Rieske Fe–S protein. A high degree of labelling is also observed in cyt. b_6. There is relatively little labelling on cyt. f and the 17 500 M_r protein. At higher concentrations of PQAz (at identical protein concentrations) the ratio of labelling in the Fe–S

center *vs* cyt. b_6 decreases (left, fig.3), suggesting a slightly higher affinity of PQAz towards the Fe–S center than to cyt. b_6. At higher concentrations of PQAz, radioactivity in the 17 500 M_r protein increases but remains small in the cyt. *f*. It seems reasonable to assume that the redox state of the plastoquinone may govern the tightness of its binding and its location on the subunits. Therefore, it would be of interest to examine the binding of PQAz in the quinol form. However, reduction of the quinone will also lead to a reduction of the azido- to the amino-function. Consequently, the properties of a photoaffinity label are lost.

Our label in its stretched form carries the azido group at a distance of ~ 10 Å from the quinone ring, which complicates the identification of quinone binding polypeptides in the cyt. b_6/f-complex with certainty. In this context it is of interest to compare the labelling pattern in chloroplast cyt. b_6/f-complex with the one in the mitochondrial cyt. b/c_1-complex obtained by a similar ubiquinone–azide label [2]. Labelling was reported in a 15 000–17 000 M_r and a 37 000 M_r protein [2], both of which are ascribed to *b*-type cytochromes. There is no labelling observed in the mitochondrial Rieske Fe–S center [2]. However, in [2] a ubiquinone derivative was used where the distance between the aromatic moiety bearing the azido group and the quinone part of the molecule is longer by 6 methylene groups as compared to our PQAz. If the quinone part of the molecule in fact binds to the Rieske Fe–S center the azido group may be adjacent to another neighbouring protein.

DBMIB has been introduced as an efficient inhibitor of plastohydroquinone oxidation [15]. Preincubation of chloroplast cyt. b_6/f-complex with DBMIB prior to addition of PQAz in general diminishes the radioactivity level in all proteins (right, fig.2). However, on a relative scale cyt. b_6 is affected more than the Fe–S center. At a threefold excess of DBMIB *vs* PQAz, the radioactivity in the cyt. b_6 is even further reduced (not shown).

DBMIB and analogous quinones [16] cause a shift in the *g*-value of the Rieske Fe–S center in isolated chloroplasts [17,18]. The redox titration of the EPR signal amplitudes indicated the formation of a strong complex between DBMIB and the Fe–S center which can undergo several redox stages [17]. From this it has been concluded that the plastoquinol oxidation site is at the Rieske Fe–S center. DBMIB also interacts with this center in the isolated cyt. b_6/f-complex, although it does not react with the separated Fe–S protein subunit [14]. This indicates that for binding of DBMIB and probably also plastoquinol to the cyt. b_6/f-complex ≥ 2 subunits are necessary. The photoaffinity labelling experiments reported here tentatively identify this second subunit as the cyt. b_6 peptide. In view of the decreased binding of PQAz to cyt. b_6 in the presence of DBMIB it might be feasible that DBMIB binds directly to the cyt. b_6. Then the shift in the *g*-value of the Fe–S protein would be caused indirectly via the cyt. b_6. These findings support the notion that plastoquinol oxidation appears to require 2 redox carriers for the 2 step oxidation via the semiquinone [19,20].

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