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Identification of ubiquinone-binding proteins in yeast mitochondrial ubiquinol-cytochrome *c* reductase using an azido-ubiquinone derivative *

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An azido-ubiquinone derivative, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone, was used to study the ubiquinone-protein interaction and to identify the ubiquinone-binding proteins in yeast mitochondrial ubiquinone-cytochrome *c* reductase. The phospholipids and Q_6 in purified reductase were removed by repeated ammonium sulfate precipitation in the presence of 0.5% sodium cholate. The resulting phospholipid- and ubiquinone-depleted reductase shows no enzymatic activity; activity can be completely restored by the addition of phospholipids and Q_6 or Q_2. The ubiquinone- and phospholipid-replenished ubiquinonol-cytochrome *c* reductase is also fully active upon reconstituting with bovine succinate-ubiquinone reductase to form succinate-cytochrome *c* reductase. When an azido-ubiquinone derivative was added to the ubiquinone and phospholipid-depleted reductase in the dark, followed by the addition of phospholipids, partial reconstitutive activity was restored, while full ubiquinol-cytochrome *c* reductase activity was observed when Q_2H_2 was used as substrate in the assay mixture. Apparently, the large amount of Q_2H_2 present in the assay mixture displaces the azido-ubiquinone in the system. Photolysis of the azido-Q-treated reductase with long-wavelength ultraviolet light abolishes about 70% of both the restored reconstitutive activity and Q_2H_2-cytochrome *c* reductase activity. The activity loss is directly proportional to the covalent binding of [^{3}H]azido-ubiquinone to the reductase protein. When the photolyzed, [^{3}H]azido-ubiquinone-treated sample was subjected to SDS-polyacrylamide gel electrophoresis followed by analysis of the distribution of radioactivity among the subunits, the cytochrome *b* protein and a protein with an apparent molecular weight of 14000 were heavily labeled. The amount of radioactive labeling in both these proteins was affected by the presence of phospholipids.

Introduction

Although the essential roles of ubiquinone (Q) in the mitochondrial, bacterial and photosynthetic electron-transfer systems have been established, the reaction mechanism of Q-mediated electron transfer and proton translocation is still a subject of intense investigation [1–6]. Ubiquinol-cytochrome *c* reductase complex (Complex III) catalyzes the antimycin A-sensitive electron transfer from ubiquinol to cytochrome *c*, generating both a membrane potential and a proton gradient, and has been used extensively for probing the physiological role of ubiquinone. The existence of specific Q-binding proteins in the ubiquinol-cytochrome *c*

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Abbreviation: Azido-Q, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone.

reductase region of the mitochondrial electron-transfer system was first suggested by Mitchell [1], and subsequently confirmed by the detection of the ubisemiquinone radicals in purified ubiquinol-cytochrome *c* reductase [7,8]; in the absence of specific binding, any ubisemiquinone radicals formed will undergo rapid and almost complete dismutation to ubiquinol plus ubiquinone at neutral pH and thus no radicals would be detected.

Recently, photoaffinity labeling studies using arylazido-Q [9] and azido-Q derivatives [10] have shown that two polypeptides with $M_r = 37000$ and 17000 in ubiquinol-cytochrome *c* reductase are responsible for Q-binding. Binding of azido-Q to the $M_r = 17000$ protein was directly proportional to the degree of inactivation and affected by the presence of phospholipids. Azido-Q binding to the $M_r = 37000$ protein was, however, not affected by phospholipids nor was it correlated to loss of activity. The nature of the binding of Q to the $M_r = 37000$ protein remains to be investigated. Further evidence for the existence of two distinct Q-binding sites in the ubiquinol-cytochrome *c* reductase region has been provided by different experiments [2,11,12].

The ubiquinol-cytochrome *c* reductase [13] obtained from yeast mitochondria (Complex III) has a biological function and a complement of redox components similar to those of the reductase obtained from bovine heart mitochondria, although the protein subunits have a slightly different molecular weight [13,14] and Q_6 is utilized in place of Q_{10}. It is, therefore, of considerable interest to compare the reactivity of the Q-site-directed photoaffinity label in the two enzyme complexes.

In this paper, we report the interaction and identification of the Q-binding proteins in yeast ubiquinol-cytochrome *c* reductase, using azido-Q derivatives.

Materials and Methods

Baker's yeast ubiquinol-cytochrome *c* reductase was prepared according to the method previously reported [13]. Enzyme depleted of Q_6 and phospholipid was prepared by repeated ammonium sulfate fractionation in the presence of sodium cholate and glycerol [15]. The Q- and phospholipid-depleted preparation was dissolved in 50 mM phosphate buffer (pH 7.5), divided into small portions, and stored in liquid nitrogen. Succinate-Q reductase from bovine heart mitochondria was prepared according to Yu and Yu [16]. The synthesis of 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl-[^{3}H]octyl)-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Q_{2s}), Q_1, Q_2, and other Q derivatives used in this study was carried out according to methods previously developed in this laboratory [10,17]. Cholate, octylglucoside and horse heart cytochrome *c*, type III, were purchased from Sigma. Other chemicals were of the highest purity commercially available.

Photolysis. The Q- and phospholipid-depleted ubiquinol-cytochrome *c* reductase was diluted to a protein concentration of about 2 mg/ml with 50 mM phosphate buffer (pH 7.4), containing 0.5% octylglucoside. 3-Azido-2-methyl-5-methoxy-Q_{2s} in 95% ethanol was added. The final concentration of ethanol in the mixture was kept lower than 5% to prevent denaturation of the enzyme. The azido-Q-treated sample was incubated at 0°C for 5 min in the dark, then illuminated [10]. The photolyzed samples were reconstituted with asolectin micellar solution (0.5 mg asolectin/mg protein) and incubated at 0°C for 30 min prior to activity assay for either ubiquinol-cytochrome *c* reductase using reduced Q_2 as substrate, or for succinate-cytochrome *c* reductase using succinate as substrate, after reconstitution with excess succinate-Q reductase from bovine heart mitochondria. The time-course study of the incorporation of radioactivity of azido-Q into protein was conducted as reported [10].

Determination of the distribution of ^{3}H radioactivity among the subunits of ubiquinol-cytochrome c reductase. The photolyzed samples were dialyzed against water, extracted with organic solvent, and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, essentially according to method described previously [10]. The SDS-polyacrylamide gel columns were prepared with a cleavable cross-linker, *N,N'*-diallyltartardiamide. Each gel was loaded with about 20 μg of protein. After electrophoresis, the gel was stained and destained and the individual protein bands isolated by slicing the gel. After the gel slices were completely dissolved by incubation in 0.7 ml of 3%

periodic acid at room temperature for 1 h, 10 ml of Insta-gel counting fluid was added, and the radioactivity was determined in a Beckman liquid-scintillation system, model LS-3150T, which has a counting efficiency of 64%.

Results and Discussion

Effect of azido-Q concentrations on activity inhibition after photolysis

Ubiquinol-cytochrome *c* reductase from yeast mitochondria contains 20% (by weight) phospholipid and 5.8 nmol Q_6/mg protein [13]. This enzyme complex catalyzes electron transfer from ubiquinol to cytochrome *c* with a specific activity of 19.3 μmol cytochrome *c* reduced/min per mg protein at 25°C using 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinone as the substrate [13]; V_{max} with Q_1H_2 is 24 μmol cytochrome *c* reduced/min per mg protein. The yeast enzyme can be reconstituted with succinate-Q reductase obtained both from yeast and from bovine heart mitochondria, to form succinate-cytochrome *c* reductase. Fig. 1 shows the reconstitution of succinate-cytochrome *c* reductase from bovine heart succinate-Q reductase with various amount of yeast ubiquinol-cytochrome *c* reductase. Maximal reconstitution could be obtained using two equivalents of ubiquinol-cytochrome *c* reductase to one equivalent of succinate-Q reductase. This ratio is similar to that obtained with the bovine heart system.

As with the bovine heart mitochondrial ubiquinol-cytochrome *c* reductase, the Q and phospholipids present in the yeast preparation can be removed by repeated ammonium sulfate fractionation in the presence of sodium cholate. The resulting Q- and phospholipid-depleted ubiquinol-cytochrome *c* reductase shows practically no enzymatic activity (in either the reconstitutive or quinol oxidation assays). However, activity can be completely restored by addition of a Q derivative (Q_2 or Q_6) and phospholipids.

We showed previously [10] that of all the synthetic azido-Q derivatives, 3-azido-2-methyl-5-methoxy-Q_{2s} (azido-Q) is the most suitable for studying Q-protein interactions and for the identification of Q-binding proteins in bovine heart mitochondrial ubiquinol-cytochrome *c* reductase.

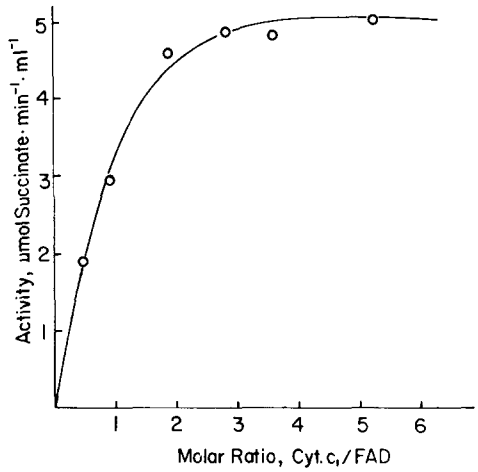

Fig. 1. Titration of the formation of succinate-cytochrome *c* reductase activity from bovine heart succinate-Q reductase and yeast ubiquinol-cytochrome *c* reductase. 0.1-ml aliquots of bovine succinate-Q reductase, 20 μM FAD, in 50 mM Tris-HCl (pH 7.8) containing 0.1% deoxycholate and 0.6 M sucrose, were added to 0.9 ml of 50 mM phosphate/0.5% octylglucoside (pH 7.4) containing varying amounts of yeast ubiquinol-cytochrome *c* reductase. Succinate-cytochrome *c* reductase activity was assayed after incubation for 20 min at 0°C.

Upon incubation in the dark, this azido-Q derivative restores about 12% of the reconstitutive activity to the Q- and phospholipid-depleted bovine heart ubiquinol-cytochrome *c* reductase. Photolysis abolishes both the restored activity and reactivation of the depleted enzyme complex by other active Q derivatives. To establish whether or not this azido-Q is also suitable for characterizing Q-protein interactions and identifying Q-binding proteins in the yeast ubiquinol-cytochrome *c* reductase, the reaction of this azido-Q with the yeast enzyme was investigated.

When Q- and phospholipid-depleted yeast reductase is treated with azido-Q, followed by phospholipid in the dark, partial restoration of reconstitutive activity (measured with bovine succinate-Q reductase) is observed; the extent of this reconstitution was 20% of that obtained with Q_2. However, before photolysis, this azido-Q-treated phospholipid-replenished reductase shows ubiquinol-cytochrome *c* reductase activity identical to that of the untreated phospholipid-replenished complex, both with and without Q_2 and Q_6, when Q_2H_2 (50 μM) is used as substrate in the assay mixture. It would appear that the large excess of Q_2H_2 present in the catalytic assay replaces azido-Q

bound to the enzyme. Photolysis of the azido-Q-treated, phospholipid-depleted reductase with long-wavelength ultraviolet light at 0°C abolishes both the restored reconstitutive activity and the Q_2H_2-cytochrome *c* reductase activity.

Since Q_2H_2 is used as substrate for the ubiquinol-cytochrome *c* reductase assay, the degree of inactivation after photolysis can be used to gauge the binding of azido-Q to the Q-binding sites of the enzyme complex. Fig. 2 shows the concentration-dependent azido-Q inactivation of yeast ubiquinol-cytochrome *c* reductase assayed with or without exposure to light after the addition of phospholipid. The photolysis-induced inhibition increased with the concentration of azido-Q. Maximal inactivation, about 70%, was observed when the molar ratio of azido-Q to cytochrome *b* was about 10, compared with 90% inhibition at a molar ratio of 6 found for the bovine enzyme [10]. The observations of a requirement for a higher concentration of azido-Q together with the smaller inactivation after photolysis suggest either the presence of residual endogenous Q in the depleted yeast enzyme or a lower affinity of the yeast enzyme for this azido-Q derivative. As HPLC analyses (Tsai, A.-L. and Palmer, G., unpublished data) consistently show the residual Q to be up to 0.05 equivalents, the latter alternative seems more reasonable. In this regard, it may be relevant that sodium cholate, which has been used successfully to disperse the Q- and phospholipid-depleted bovine heart ubiquinol-cytochrome *c* reductase, is not effective with the yeast enzyme; octylglucoside is the most effective detergent so far tested for dispersing the yeast enzyme complex.

Illumination time-dependent azido-Q uptake by protein and the enzymatic activity loss

When the azido-Q-treated and phospholipid-depleted ubiquinol-cytochrome *c* reductase is subjected to photolysis for varying times at 0°C, there is a decrease in the ubiquinol-cytochrome *c* reductase activity (measured after reconstitution with phospholipid) with a concomitant increase in [^{3}H]azido-Q uptake (Fig. 3). Maximum inactivation is observed when the sample is illuminated for 5 min, a time period shorter than that necessary to inactivate the bovine system. With smaller periods of illumination, the degree of inhibition of activity

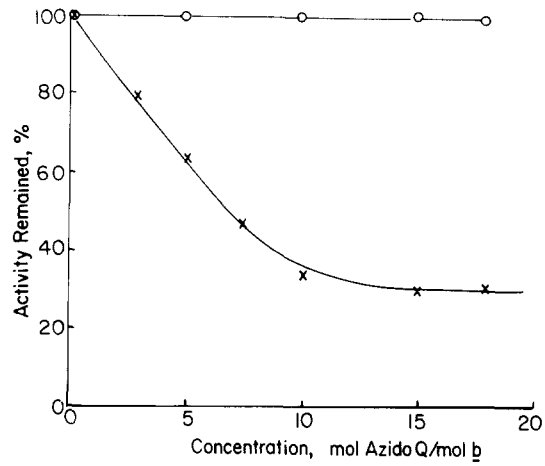

Fig. 2. Effect of azido-Q concentrations on the inactivation of ubiquinol-cytochrome *c* reductase activity after photolysis. 0.2-ml of the depleted ubiquinol-cytochrome *c* reductase, 9.7 μM cytochrome *b*, in 50 mM sodium/potassium phosphate buffer (pH 7.4) containing 0.5% octylglucoside, were mixed with the indicated concentrations of the azido-Q derivative. After incubation in the dark for 5 min at 0°C, each sample was divided into two equal portions. One was subjected to photolysis for 5 min at 0°C (× ——— ×) and the other was kept in the dark (○ ——— ○). To 50-μl portions of the azido-Q-treated samples, with or without light, were added 50 μl of 50 mM phosphate buffer (pH 7.4) containing 0.5% octylglucoside and 0.9 mg of asolectin. Ubiquinol-cytochrome *c* reductase activity was assayed using 50 μM of Q_2H_2 as the substrate. 100% activity equals 4.28 μmol cytochrome *c* reduced/min per nmol cytochrome *b* at 23°C.

is directly proportional to the amount of azido-Q taken up by the protein, a result indicating that the inactivation results from the formation of a covalent bond between azido-Q and one or more components of the enzyme. With further illumination, azido-Q uptake by the enzyme complex continues to increase, but at a slower rate. There is, however, no further inhibition of activity, suggesting that this slower uptake of azido-Q is due to non-specific binding. It should be noted that photolysis of the Q and phospholipid-depleted yeast ubiquinol-cytochrome *c* reductase in the absence of the azido-Q derivative leads to a 20% loss of activity over a 20 min period. This loss is small compared to that of the azido-Q-treated sample.

Identification of the Q-binding protein(s) in ubiquinol-cytochrome c reductase

To determine which subunit(s) of the yeast ubiquinol-cytochrome *c* reductase are responsible

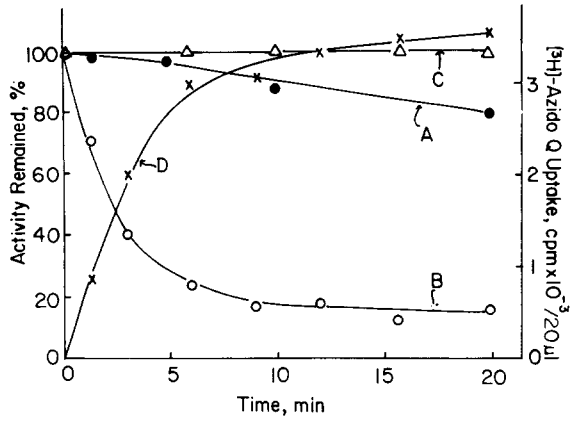

Fig. 3. Time-dependent photolysis-induced inactivation and [^{3}H]azido-Q uptake by ubiquinol-cytochrome *c* reductase. Three 0.4-ml aliquots of the cytochrome *b*-c_1 reductase complex containing 10.5 µM cytochrome *b* were mixed with 4 µl of 15.5 mM of [^{3}H]azido-Q, 10000 cpm/nmol (samples B and C), or with 4 µl of 95% ethanol (sample A) in 50 mM phosphate buffer (pH 7.4) containing 0.5% octylglucoside. After incubation at 0°C for 5 min in the dark, samples A and B were subjected to photolysis side by side, while sample C was wrapped with aluminum foil and then put into the photolysis apparatus. At the time indicated, aliquots of 20 µl were withdrawn from each sample and reconstituted with 0.9 mg asolectin in 100 µl of 50 mM phosphate buffer (pH 7.8) containing 0.5% octylglucoside. The reconstituted enzymes were assayed for ubiquinol-cytochrome *c* reductase activity using Q_2H_2 as the substrate. Activity of sample A before photolysis was used for 100%. 20-µl aliquots were also withdrawn from sample B at given time intervals and used to determine incorporation of radioactivity as described in curve D.

for binding coenzyme Q, the distribution of [^{3}H]azido-Q among the subunits has been determined. Before performing SDS-polyacrylamide gel electrophoresis, the photolyzed, [^{3}H]azido-Q-treated enzyme was extracted with chloroform/methanol [10] to remove Q, both free and that adventitiously bound to detergent and/or phospholipid. Fig. 4 shows the distribution of radioactivity among the subunits of ubiquinol-cytochrome *c* reductase. Two protein bands are heavily labeled; they have electrophoretic mobilities of 0.74 and 0.97 relative to cytochrome *c* (R_c). Under the particular experimental conditions, the band with $R_c = 0.74$ consists of both cytochromes *b* and c_1 *.

* It should be noted that the electrophoretic mobility of cytochrome *b* in yeast ubiquinol-cytochrome *c* reductase is very sensitive to the amount of detergent present in the sample. A similar abnormality in electrophoretic behavior of bovine cytochrome *b* protein has been well documented [3,18].

Although this band contains both cytochromes *b* and c_1, the radioactive component has been identified as cytochrome *b* by cleaving the labelled complex into cytochrome *b*- and cytochrome c_1-containing fractions prior to electrophoresis by treatment with 1% cholate/20% ammonium sulfate at room temperature for 4 h [19]; in such experiments, very little radioactivity is found in the cytochrome c_1-enriched fraction ($R_c = 0.74$). However, in the cytochrome *b*-enriched fraction, substantial radioactivity was located in bands due to cytochrome *b* ($R_c = 0.74$) and the $R_c = 0.97$ component; this latter species has an apparent molecular weight of 14000. The amino acid sequence of this protein has been determined by nucleotide sequencing [20]. Although it exhibits no visible redox prosthetic group, it is thought to be closely associated with cytochrome *b* protein [20]. It should be mentioned that the assessment of the photolabeled $M_r = 14000$ protein to that sequenced by De Haan et al. [20] was based only on the same apparent molecular weight estimated by

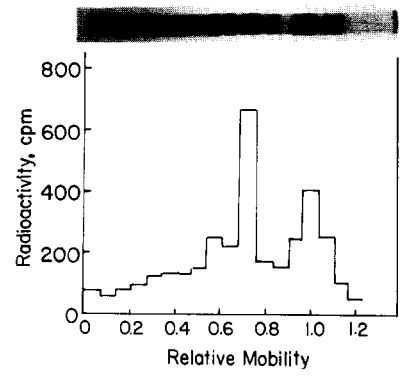

Fig. 4. Distribution of radioactivity among the subunits of azido-Q-treated ubiquinol-cytochrome *c* reductase after photolysis. 0.2-ml aliquots of the depleted ubiquinol-cytochrome *c* reductase containing 10.5 µM cytochrome *b*, were mixed with 2 µl of [^{3}H]azido-Q derivative (11 mM in absolute alcohol, 10000 cpm/nmol) in the dark in 50 mM phosphate buffer (pH 7.8) containing 0.5% octylglucoside. After incubation for 5 min, the azido-Q-treated sample was illuminated for 5 min at 0–2°C. The photolyzed sample was dialyzed against H_2O, overnight, with one change of buffer, extracted with organic solvent [10], dissociated with 1% SDS and 1% mercaptoethanol at 37°C for 2 h and subjected to SDS-polyacrylamide gel electrophoresis. About 20 µg of protein was loaded onto each gel. After staining and destaining, protein bands were recovered by slicing the gel and the radioactivity in each fraction was measured.

the electrophoretic mobility in the SDS-polyacrylamide gel electrophoresis. It is possible that they are actually not the same protein, because the electrophoretic conditions used were not identical. The identity of this photolabeled protein will not be revealed until, at least, a partial amino acid sequence is known. In bovine ubiquinol-cytochrome *c* reductase, a similar protein with a molecular weight of 17000 has been identified as a Q-binding protein [10].

Recently, a protein with a molecular weight of 13389 [21] has been isolated from bovine heart ubiquinol-cytochrome *c* reductase; it has been sequenced and postulated to be a Q-binding protein. However, its high hydrophilicity and the lack of accessibility to azido-Q binding [10] raises questions concerning its ability to bind Q, and further evidence for reconstitution, by this protein, of Q-binding protein-depleted reductase, is needed before its role as a Q-binding protein can be accepted. Unfortunately, the latter preparation is not yet available. Efforts to obtain such a Q-binding protein-depleted reductase are currently being made in several laboratories.

The effect of phospholipids on the binding of azido-Q to ubiquinol-cytochrome c reductase

It has been reported that prolonged incubation of the Q- and phospholipid-depleted bovine ubiquinol-cytochrome *c* reductase with phospholipids before the addition of azido-Q affects Q-binding sites of the 17000 subunit but not those on the 37000 subunit. It was thus of interest to see whether or not phospholipid has a similar effect on the yeast system. The addition of phospholipids, either before or after addition of the azido-Q derivative greatly decrease inactivation of the reductase by photolysis (Table I). These results indicate that phospholipids compete with azido-Q for both Q-binding sites in ubiquinol-cytochrome *c* reductase. When the photolyzed, phospholipid- and azido-Q-treated preparation was analyzed by SDS-polyacrylamide gel electrophoresis, the level of radioactivity in both the $R_c = 0.74$ and 0.97 subunits was greatly decreased. This observation contrasts with the results obtained with the bovine system, where only Q binding to the $M_r = 17000$ protein was seen to be affected by the presence of phospholipids. In this context, it may be relevant that binding of antimycin A to the yeast enzyme also requires phospholipid (Tsai, A.-L. and Palmer, G., unpublished data); no binding of antimycin A can be demonstrated to the phospholipid-depleted yeast complex.

TABLE I

EFFECT OF PHOSPHOLIPIDS ON INACTIVATION BY AZIDO-Q AFTER PHOTOLYSIS

Treatments	Percentage of inactivation
1 dQCR + AQ	64
2 (dQCR + PL) + AQ	20
3 (dQCR + AQ) + PL	21
4 dQCR + PL	4

Either AQ (experiments 1 and 3) or asolectin (experiments 2 and 4) was added to a solution of Q- and phospholipid-depleted yeast ubiquinol-cytochrome *c* reductase in the dark; the buffer was 0.05 M phosphate (pH 7.8) containing 0.5% octylglucoside. After incubation at 0°C for 5 min, sample 1 was photolyzed, asolectin was added to sample 3, and samples 2, 3 and 4 were incubated in the dark for an additional 20 min; then AQ was added to sample 2. Photolysis of 2, 3 and 4 was carried out during the further incubation of these samples for 5 min at 0°C. The final concentration of AQ was 10 nmol/nmol cytochrome *b* and that of asolectin was 0.5 mg/mg protein. Ubiquinol-cytochrome *c* reductase activity was assayed before and after photolysis. For sample 1, the activity was assayed after the addition of phospholipids. The activity of sample 4 measured before photolysis was taken as 100%. PL, phospholipids; AQ, 3-azido-2-methyl-5-methoxy-Q_{2s}; dQCR, Q- and phospholipid-depleted ubiquinol-cytochrome *c* reductase.

Acknowledgements

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