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THE EXISTENCE OF AN ANTIMYCIN A INSENSITIVE UBIQUINOL-CYTOCHROME C REDUCTASE ACTIVITY IN THE PHOTOSYNTHETIC APPARATUS

Linda Yu and Chang-An Yu

Department of Biochemistry, Oklahoma State University
Stillwater, Oklahoma 74078

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SUMMARY-- A nonproteinaceous, antimycin A insensitive ubiquinol-cytochrome c reductase activity is detected in and purified from chromatophores of Rhodopseudomonas sphaeroides, R-26. This activity is about 5 times the antimycin A sensitive reductase activity in chromatophores and the two are not interconvertable. The purification involved chloroform:methanol (2:1), and hexane extractions and florisil column chromatography. The purified preparation contains some bacteriochlorophyll-like pigments and phospholipids, and is stable in organic solvent. It catalyzes the oxidation of ubiquinol by cytochrome c with substrate specificity and pH optimum.

Participation of an antimycin sensitive, ubiquinol-cytochrome c reductase complex in photosynthetic electron transfer has been well documented (1, 2). The isolation and characterization of this enzyme complex has been reported from many laboratories including ours, using various photosynthetic organisms (3-5). In contrast to this well defined ubiquinol-cytochrome c reductase or the cytochrome b-c_1 complex of bacterial chromatophores, or the cytochrome b_6-f complex of chloroplasts, an antimycin insensitive ubiquinol-cytochrome c reductase activity has recently been observed in photosynthetic bacterial chromatophores and in chloroplasts upon treatment with sodium dodecylsulfate (SDS). This reductase activity can be extracted from the bacterial chromatophores by chloroform:methanol (2:1) and further purified by hexane extraction and florisil column chromatography. The purified preparation contains no protein or peptides yet catalyzes oxidation of ubiquinol by cytochrome c in a typical enzymatic

Abbreviations used: Q_2, 2,3-dimethoxy-5-isoprenyl-6-methyl-1,4-benzoquinone; Q_0C_{10}, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzoquinone; $Q_0C_{10}Br$, 2,3-dimethoxy-5-bromodecyl-6-methyl-1,4-benzoquinone; PQ_2, 2,3-dimethyl-5-isoprenyl-6-methyl-1,4-benzoquinone; SDS, sodium dodecylsulate.

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fashion. It shows rapid turnover, pH optimum, and substrate and acceptor specificity. In this communication we wish to report the purification procedure and some properties of this antimycin insensitive ubiquinol-cytochrome c reductase preparation.

MATERIALS AND METHODS

Cells and the reaction center of Rhodopseudomonas sphaeroides, R-26, were gifts from Drs. Okamura and Feher, Dept. of Physics, UCSD. Growth conditions for cells and the preparation procedure for the reaction center have been reported (6). The polyethylene glycol and KCl washed chromatophores were prepared according to the reported method (3). Ubiquinone derivatives: Q_2, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzoquinone (Q_0C_{10}), 2,3-dimethoxy-5-bromodecyl-6-methyl-1,4-benzoquinone ($Q_0C_{10}Br$) and 2,3-dimethyl-5-isoprenyl-6-methyl-1,4-benzoquinone (PQ_2) were prepared in this laboratory according to the reported method (7), and Q_6 and Q_{10} were purchased from Sigma. Reduced ubiquinone derivatives were prepared by dithionite reduction followed by cyclohexane extraction. The stock solutions of reduced ubiquinone derivatives (5 mM) were in 95% ethanol containing 2 mM HCl (3). Horse cytochrome c, type III, and florisil, 60-100 mesh were products of Sigma.

Enzymatic activity was determined spectrophotometrically (3). Protein concentration was determined by the Lowry method (8). Chlorophyll, ubiquinone and phospholipids were determined by the reported methods (9). Spectroscopic measurements were done in Cary spectrophotometers, models 219 and 14.

RESULTS AND DISCUSSIONS

Detection of an Antimycin A Insensitive Ubiquinol-Cytochrome c Reductase Activity in Chromatophores of Rhodopseudomonas sphaeroides, R-26-- The polyethylene glycol and KCl washed chromatophores catalyze antimycin A sensitive oxidation of ubiquinol by cytochrome c in the absence or presence of mild detergents such as Na-cholate, deoxycholate, Triton X-100 or octyl-glycoside. When the washed chromatophores were treated with SDS, the antimycin A sensitive ubiquinol-cytochrome c reductase activity gradually decreased as the detergent concentration increased and was finally abolished, while an antimycin A insensitive activity appeared. Fig. 1 shows the effect of SDS on both types of ubiquinol-cytochrome c reductase activities in chromatophores. The antimycin A insensitive activity is about five times greater than the antimycin A sensitive activity present in chromatophores. It is interesting that the antimycin A sensitive ubiquinol-cytochrome c reductase activity observed in the highly purified cytochrome b-c_1 complex (3) cannot be converted

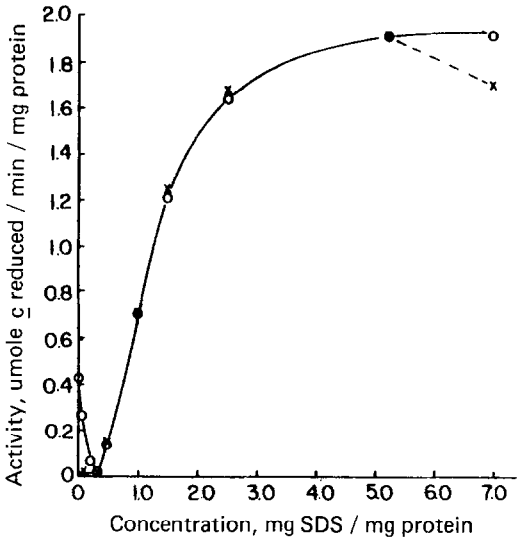


Fig. 1. Effect of SDS concentration on the antimycin A sensitive and Insensitive ubiquinol-cytochrome c reductase activities of chromatophores. Aliquots of polyethylene glycol and KCl washed chromatophores (7.7 mg/ml) in 10 mM Tris-acetate buffer, pH 7.8 containing 1 mM EDTA were treated with indicated amounts of SDS at 0° for 10 min. After incubation, the activities were assayed in the absence (-o-o-) and presence (-x-x-) of antimycin A.

to the antimycin A insensitive reductase activity by treatment with SDS. The purified reaction center (6), on the other hand, which shows no ubiquinol-cytochrome c reductase activity, is able to show a partial antimycin A-insensitive ubiquinol-cytochrome c reductase activity upon treatment with SDS. These results suggest that the antimycin A insensitive ubiquinol-cytochrome c reductase may co-exist with the antimycin-sensitive ubiquinol-cytochrome c reductase in the chromatophores. The former may be deeply buried in a hydrophobic region and thus, under usual experimental conditions, this activity is not detectable. This activity can only be observed when the hydrophobic environment is modified by SDS or extracted with organic solvent. The presence of an antimycin A insensitive ubiquinol-cytochrome c reductase activity in the purified reaction center suggests that this activity might be tightly associated with the pigments because the reaction center contains bacteriopheophytin and bacteriochlorophyll (6).

Extraction and Purification of Antimycin A Insensitive Ubiquinol-Cytochrome c Reductase Complex-- During the preparation of phospholipids from chromatophores, it was surprising to note that a very active antimycin A insensitive

ubiquinol-cytochrome c reductase activity was present in the chloroform: methanol (2:1) extract. The activity was expressed only when the extract was dried and redispersed in aqueous solution by sonication. The suspension was routinely made in 10 mM Tris-acetate, pH 7.8, containing 1 mM EDTA. When the organic solvent extract was directly added to the assay mixture, only a very low activity was observed. Since all the antimycin A insensitive ubiquinol-cytochrome c reductase activity observed in the SDS-treated chromatophores was recovered in the chloroform:methanol extract, it was a logical starting material for further purification and identification of the components responsible for this activity.

Thirty gm (wet weight) of washed chromatophores were extracted with 300 ml of chloroform:methanol (2:1) mixture. The extraction was repeated once and the combined extracts were dried under vacuum at a temperature $< 10^{o}$. The dried residues were re-extracted with 70 ml of chloroform:methanol (2:1). The extract was washed once with 14 ml of 0.034% of $MgCl_2$ and dried under vacuum. The residues were extracted with 32 ml of hexane and then chromatographed on a hexane-equilibrated florisil column (1.7 x 6.6 cm). The column was developed, stepwise, with 140 ml each of 3%, 5%, 10%, 20% and 25% ethanol in hexane, and ubiquinol-cytochrome c reductase activity was finally eluted from the column with a mixture of hexane : ethanol : H_2O (74:25:1). Those fractions containing activity were combined and dried. The residues were dissolved in 50 ml of hexane and partitioned with 40 ml of methanol and 10 ml of H_2O. The hexane layer was collected and the methanol layer re-extracted once with 30 ml of hexane. The combined hexane extracts were dried and dissolved in chloroform. The purification data is summarized in Table I. About 85% of the original activity and 20% of the pigments were recovered in the final step.

Properties of Purified Antimycin A Insensitive Ubiquinol-Cytochrome c Reductase Complex-- The isolated antimycin insensitive ubiquinol-cytochrome c reductase complex contains no detectable protein or peptides, yet catalyzes 12 μmoles cytochrome c reduced by ubiquinol per min per mg dry weight at 23^{o}. The preparation contains pigments with absorption peaks at 770, 680, 576,

Table I. Purification Data of Antimycin A Insensitive Ubiquinol-Cytochrome c Reductase.

Treatments	Dry Wt	Org. P	Activity			
			-SDS		+SDS	
			S.A	Recovery	S.A	Recovery
	mg	μmoles	μmol/min.mg	%	μmol/min.mg	%
Chromatophores	1,617	-	0	0	0.93	100
$CHCl_3$:CH_3OH Extract	453	278	3.15	95	4.02	121
$MgCl_2$ Washed $CHCl_3$:CH_3OH Extract	388	276	3.60	93	4.46	115
Hexane Extract	355	258	3.80	90	4.50	106
Florisil Column Eluate	129	114	10.30	88	12.10	104
Hexane Layer, Partition with CH_3OH & H_2O	106	98	11.91	84	14.01	99

424, 390 and 356 nm as indicated in Fig. 2. The role of the pigments in this ubiquinol-cytochrome c reductase activity has not yet been established. The pigments do not undergo a redox change upon the addition of substrate. Chemical analysis shows some amino groups but the amount did not increase upon hydroly-

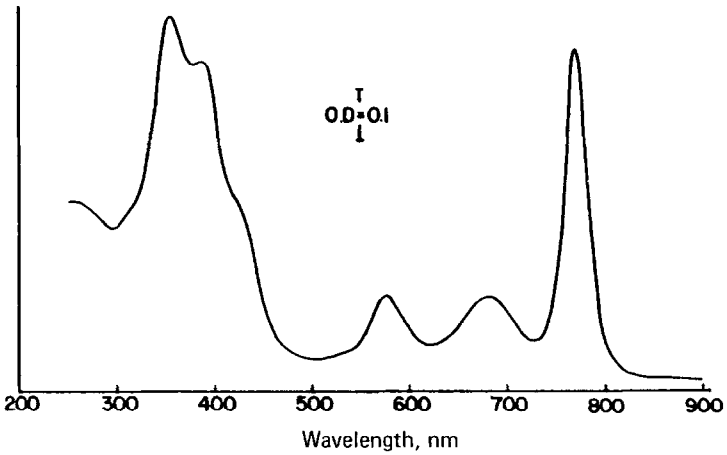
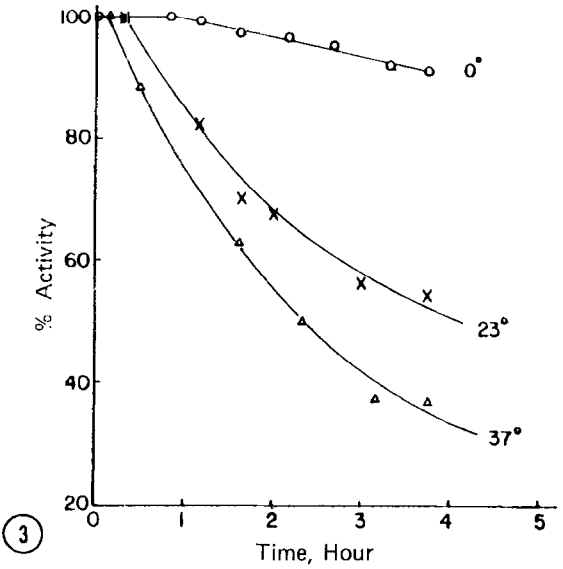


Fig. 2. Absorption spectra of purified antimycin A insensitive ubiquinol-cytochrome c reductase preparation. The spectrum was obtained in ether solution at room temperature. The preparation contains 0.16 mg dry weight per ml.

sis, suggesting that the amino groups are non-proteinaceous. Organic phosphate is present and a significant amount of phosphatidylethanolamine has been identified from thin layer chromatography.

The isolated ubiquinol-cytochrome c reductase is stable in organic solvent at - 20°. However, the stability decreased after the solvent was removed and the preparation redispersed in aqueous solution by sonication. Fig. 3 shows the stability of the sonication-dispersed preparation at various temperatures. The stability of the preparation decreased with increasing incubation temperature. The half life was about 12 hours at 0° in 10 mM Tris-acetate, pH 7.8. Introduction of SDS to the deactivated preparation did not restore its activity. The activity of the isolated ubiquinol-cytochrome c reductase preparation is pH-dependent, with a maximum at 8.5 (see Fig. 4). The activity decreased drastically when the pH of the assay mixture was higher than 9.0 or lower than 5.5. It should be mentioned that the pH dependency at higher pH may be somewhat



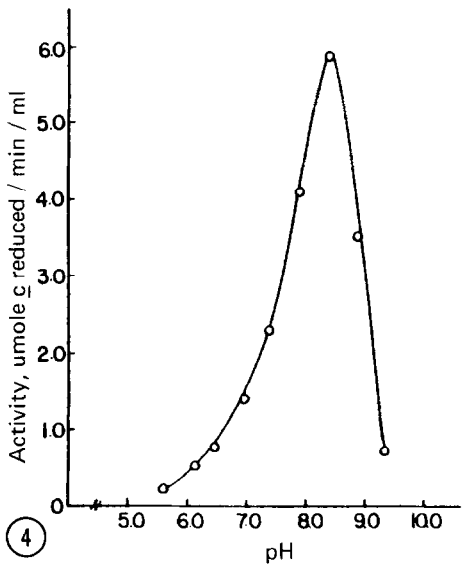


Fig. 3. Temperature dependent stability of antimycin A insensitive ubiquinol-cytochrome c reductase in aqueous solution. Aliquots of purified ubiquinol-cytochrome c reductase in chloroform were dried and dispersed in 10 mM Tris-acetate buffer, pH 7.8 containing 1 mM EDTA by sonication and incubated at 0° (-o-o-), 23° (-x-x-) and 37° (-Δ-Δ-). At indicated times, samples were withdrawn and activity assayed at room temperature.

Fig. 4. pH profile of purified antimycin A insensitive ubiquinol-cytochrome c reductase activity. The assay conditions were the same as described in Methods except the pH of the assay mixture was obtained by titrating a mixed buffer system containing 100 mM phosphate and glycine, with either NaOH or HCl.

uncertain, as at higher pH the autoxidation rate of ubiquinol became too fast to be followed with accuracy.

The efficiencies of reduced Q derivatives as electron donors for the isolated preparation are summarized in Table II. Among the reduced Q analogues used, it was found that Q_2H_2, $Q_0C_{10}H_2$ and $Q_0C_{10}BrH_2$ are the most effective and duroquinol is the least effective. Q_6H_2 and $Q_{10}H_2$ are less effective than Q_1H_2 and PQ_2H_2. Cytochrome c, but not succinylated cytochrome c, functions as electron acceptor, suggesting that no superoxide is involved in the reaction sequence. Ferricyanide has about 30% efficiency as an electron acceptor. Incorporating this antimycin A insensitive ubiquinol-cytochrome c reductase into lipid vesicles significantly decreased its activity. The activity was partially recovered upon addition of SDS, indicating that the active site is somewhat masked by phospholipids and is thus inaccessible to the acceptor.

The isolated reductase preparation can be further separated into two subfractions by chromatography on a second florisil column equilibrated with

Table II. Substrate Specificity of Antimycin A Insensitive Ubiquinol-Cytochrome c Reductase .

Q Analogs	Ubiquinol-Cytochrome c Activity	
	Regular Assay	*0.4% Na-Cholate
	%	%
Q_1H_2	35	-
Q_2H_2	100	65
$Q_0C_{10}H_2$	100	65
$Q_0C_{10}BrH_2$	100	65
PQ_2H_2	70	45
Duroquinol	2	0
Q_6H_2	0	20
$Q_{10}H_2$	0	6

* 0.4% Na-cholate is included in the regular assay mixture. 100% activity is 10 μmol c reduced per min per mg dry weight.

chloroform, and developed with chloroform and an increasing amount of methanol and 1 N ammonium hydroxide. The first fraction contained most of the pigment but no organic phosphorus, and the second fraction contained no pigment but most of the phosphorus. Neither fraction alone showed ubiquinol-cytochrome c reductase activity, but when they were combined the activity was restored. Diluted SDS solution can be substituted for the second fraction in the reconstitution of activity. This result suggests that the reductase activity requires both pigments and some phospholipid components. SDS may be able to mimic the phospholipid required for activity. Only two types of phospholipids are present in the second fraction: phosphatidylethanolamine and an unidentified phospholipid. The role of these phospholipids remains to be established. Further identification of the pigments is currently under investigation.

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