Identification of the Ubiquinone-Binding Site of NADH:Ubiquinone Oxidoreductase (Complex I) from *Neurospora crassa*†

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ABSTRACT: In order to localize the ubiquinone-binding site of complex I (NADH:ubiquinone oxidoreductase), a novel photoreactive ubiquinone analogue ($Q_0C_7ArN_3$) has been synthesized. It is shown that the direct chemical precursor of this analogue ($Q_0C_7ArNO_2$) and the analogue itself are accepted as substrates in an enzyme assay utilizing ubiquinone-depleted mitochondrial membranes of *Neurospora crassa*. The activity of the enzyme applying these derivatives is inhibited by 50% at a concentration of 9 and 20 μM rotenone. Photoaffinity labeling experiments were performed with both isolated complex I and whole mitochondrial membranes of *N. crassa* under various conditions. In each of these experiments a protein subunit with an apparent molecular mass of about 9.5 kDa was labeled with high specificity. Radioactive labeling was totally prevented by the addition of ubiquinone-2 at concentrations higher than 500 μM but was not affected by comparable concentrations of rotenone or other hydrophobic substances. In the labeling experiments using whole membranes, the labeling signal was dramatically increased in the presence of 1.5 mM NADH. These results strongly suggest that the ubiquinone analogue interacts specifically with the enzyme.

NADH:ubiquinone oxidoreductase (EC 1.6.5.3) (complex I) catalyzes the first step in the respiratory chain, the electron transfer from NADH to ubiquinone. This extraordinary large enzyme contains about 30 different polypeptide subunits (of mitochondrial and nuclear origin), several iron–sulfur clusters, flavin (FMN), and possibly an intrinsic ubiquinone [for reviews, see Weiss et al. (1991) and Ragan (1987)].

Among these polypeptide subunits a 51-kDa protein was identified in bovine heart functioning as the binding site for NADH and represents therefore the electron acceptor (Chen & Guillory, 1984). It also contains one iron–sulfur center and probably FMN (Pilkington et al., 1991).

Little is known about the electron transfer pathway within complex I and the electron output. On the basis of results of EPR studies, there is still a controversial discussion favoring different functional models (Krishnamoorthy & Hinkle, 1988; van Balzen & Albracht, 1989; Kotlyar et al., 1990). It seems to be generally accepted, however, that the iron–sulfur center with the most positive redox potential, namely N-2, serves as the electron donator for ubiquinone (Ragan, 1990). Nevertheless, the polypeptide containing the tetranuclear N-2 cluster, as well as the subunit(s) forming the binding site for ubiquinone, is not yet identified. EPR studies have shown (Ohnishi et al., 1985) that the signal of the N-2 cluster resides in the hydrophobic fragment (HP fraction). It has been further reported that this cluster exhibits a phospholipid-dependent midpoint potential (Ohnishi et al., 1974) and interacts most probably with a rotenone-sensitive ubisemiquinone radical (Kotlyar et al., 1990; Suzuki & King, 1983; Burbaev et al., 1989). On the other hand, Suzuki and King (1983) proposed a ubiquinone-binding protein and reported the isolation of a 14-kDa polypeptide from the "soluble" so-called IP fragment of bovine heart complex I (Suzuki & Ozawa, 1986), without giving more details on this subunit.

In order to answer the question about the particular binding site of ubiquinone, we decided to synthesize a ubiquinone analogue, which both is accepted as a substrate and reacts as a photoaffinity label. The experimental approach should consider the hydrophobic nature of the substrate and the significance of a bilayer environment for the NADH: ubiquinone reductase activity (Ragan, 1978). Thus, special attention was paid to the "physiological" activity of the derivative applying pentane-extracted mitochondrial membranes and to the potential effect of well-known inhibitors like rotenone. In any case, the specific labeling of isolated complex I, as well as that of mitochondrial membranes, needs to be demonstrated.

MATERIALS AND METHODS

Column chromatography was performed on silca gel 60 (Merck, Darmstadt, FRG) or on neutral aluminum oxide (Fluka, Buchs, Switzerland). Thin-layer chromatography (TLC)[1] was carried out on silica gel 60 plates (Merck), developed in CCl_4/ethyl acetate (9:1), and visualized by UV light or by staining with iodine. Organic solvents (analytical grade; Merck) were used without further purification. ^{1}H-NMR spectra were recorded on a Varian A-60 spectrometer.

Synthesis of the Chemically Reactive Ubiquinone Analogue

8-Phenyloctanoic Acid (**1**). 7-Benzoylheptanoic acid was prepared by the Grignard reaction using equimolar amounts of bromobenzene, magnesium, and cyclooctanone in dry ether and subsequent oxidation of the crude product by chromic anhydride treatment in glacial acetic acid as described by Fieser and Szmuskovicz (1948).

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[1] Abbreviations: ^{125}I-$Q_0C_7ArN_3$, 2,3-dimethoxy-5-methyl-6-[7-(4-azido-3-[^{125}I]iodophenyl)heptanyl]-*p*-benzoquinone; $Q_0C_7ArNO_2$, 2,3-dimethoxy-5-methyl-6-[7-(*p*-nitrophenyl)heptanyl]-*p*-benzoquinone; Q_0, 2,3-dimethoxy-5-methyl-*p*-benzoquinone; Q_2, ubiquinone-2; NaPP, sodium phosphate buffer; TLC, thin-layer chromatography; BSA, bovine serum albumin; DOC, desoxycholate.

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The arylcarbonyl compound was reduced to the 8-phenyloctanoic acid according to the method of West et al. (1973) using 7 equiv of trifluoroacetic acid and 2.4 equiv of triethylsilane. After a reaction time of 12 h the crude product was purified by following the isolation method Ia (West et al., 1973) and by a final distillation. A slightly yellow oil, boiling at 121–124 °C/0.075 mbar [lit. bp 206–208.5 °C/11 mm (Huisgen et al., 1954)] was obtained in a 77% yield.

^{1}H-NMR ($CDCl_3$): δ 1.33–1.60 (10 H, m, -CH_2-), 2.34 (2 H, t, -CH_2-COOH), 2.60 (2 H, t, Ar-CH_2-), 7.19 (5 H, m, Ar).

8-(p-Nitrophenyl)octanoic acid (**2**) was prepared according to van der Scheer (1934). A total of 7 g of 8-phenyloctanoic acid was slowly added to 55 mL of concentrated nitric acid (ρ 1.42) with intermittent ice cooling. The emulsion was kept for 24 h at room temperature with stirring and then poured on ice (about 200 mL) and extracted with ether. The organic layer was washed twice with water and dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. After recrystallization from a mixture of diethyl ether/petroleum ether (bp 40–60 °C) and vigorous drying, 5 g of yellow crystals was obtained (mp 52–55 °C; 72% yield).

^{1}H-NMR ($CDCl_3$): δ 1.30–1.54 (10 H, m, -CH_2-), 2.34 (2 H, t, -CH_2-COOH), 2.80 (2 H, t, Ar-CH_2-), 7.31 (2 H_{meta}, ψd, Ar), 8.16 (2 H_{ortho}, ψd, Ar).

2,3-Dimethoxy-5-methyl-6-[7-(p-nitrophenyl)heptanyl]-p-benzoquinone (**3**) ($Q_0C_7ArNO_2$). The acid chloride of **2** was obtained by treating the melted acid was freshly distilled thionyl chloride (1.8-fold molar excess) at 80–90 °C. After 2 h, when the reaction was finished, the excess of $SOCl_2$ was removed under reduced pressure. The crude acid chloride was converted to the diacyl peroxide by the pyridine acylation method described by Silbert and Swern (1958), using a molar ratio of acid chloride to pyridine to hydrogen peroxide of 1:1.2:0.75 in ether. The washed and dried ether solution was directly used for coupling the "side chain" to the quinone nucleus according to the syntheses reported by Wan et al. (1975). Therefore, the ether solution (about 8.6 mmol of the diacyl peroxide in 100 mL ether) was added to a solution of 2,3-dimethoxy-5-methyl-*p*-benzoquinone (Q_0) (4.3 mmol) in glacial acetic acid (40 mL), and then the ether was evaporated at 60–70 °C. The solution was kept refluxing for 20 h at 95 °C. Ether and water were added until two separated layers had formed. After a second extraction of the aqueous layer, the organic layers were pooled, washed subsequently with H_2O, 1 M HCl, H_2O, 0.5 M $NaHCO_3$, H_2O, and dried over anhydrous $MgSO_4$. After evaporation of the ether, the residue was taken up in ethyl acetate, applied to a short Al_2O_3 column (5 × 2.5 cm), and eluted with the same solvent. The crude product was further purified by chromatography on a silica gel column (3.5 × 20 cm) utilizing CCl_4/ethyl acetate (9:1). The elution profile was controlled by thin-layer chromatography. The product (orange oil; 35% yield) migrates with a R_f of 0.61 (corresponding value of Q_0 is 0.44).

^{1}H-NMR ($CDCl_3$): δ 1.35 (10 H, m, -CH_2-), 2.01 (3 H, s, -CH_3), 2.45 (2 H, m, quinone nucleus -CH_2-), 2.72 (2 H, t, Ar-CH_2-), 3.99 (6 H, s, -OCH_3), 7.30 (2 H_{meta}, ψd, Ar); 8.15 (2 H_{ortho}, ψd, Ar).

2,3-Dimethoxy-5-methyl-6-[7-(p-aminophenyl)heptanyl]-p-benzoquinone (**4**). The reduction of the nitro group was carried out according to Bell et al. (1989). **3** (0.35 mmol) was dissolved in hot methanol (15 mL), and $SnCl_2 \cdot 2H_2O$ (3.5 mmol) was added in small portions under nitrogen. The solution was held under reflux for 1 h at 65–70 °C turning from orange to pale yellow. A solution of 0.5 M $NaHCO_3$ was added until a pH value of 8–9 was reached. The colorless precipitation was removed by filtration, and the filtrate was extracted twice with ether. The ether layer was washed with water and stirred vigorously overnight with an aqueous solution of 25% $FeCl_3$. The organic layer was separated, washed with water, and dried over anhydrous $MgSO_4$. After the solvent was removed, the residue was dissolved in ethanol and was passed through a short silica gel column (42% yield; TLC R_f = 0.15).

IR (neat): 3445 cm^{-1} (R-NH_2). ^{1}H-NMR ($CDCl_3$): δ 1.39 (10 H, m, -CH_2-), 2.07 (3 H, s, -CH_3), 2.46 (2 H, m, quinone nucleus -CH_2-), 3.97 (6 H, s, -OCH_3), 6.81 (4 H, m, Ar).

2,3-Dimethoxy-5-methyl-6-[7-(4-azidophenyl)heptanyl]-p-benzoquinone ($Q_0C_7ArN_3$). A total of 34 mg of **4** (92 μmol) was dissolved in 7 mL of dimethylformamide, chilled to 0 °C, acidified by the addition of 150 μL of concentrated HCl, and treated with 1.2 mL of 0.1 M $NaNO_2$. After 20 min on ice and 20 min at room temperature, 1.4 mL of 0.1 M amidosulfonic acid, followed by 1.5 mL of 0.1 M NaN_3, was added to the sample under subdued light. The solution was stirred for 30 min on ice and 30 min at room temperature. After the addition of 3.2 mL of 1 M NaOH, the separated orange oil was extracted into ethyl acetate. The organic layer was washed with water and was vigorous stirred with a 25% solution of $FeCl_3$. The organic layer was separated, washed with water, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and 33 mg of a dark red oil was obtained (90% yield).

TLC: R_f = 0.72. UV λ_{max} (CH_2Cl_2): 276 nm, ε = 16.9 mM^{-1} cm^{-1} (quinone); 250 nm, ε = 15.0 mM^{-1} cm^{-1} (Ar-N_3) [cf. Q_2 λ_{max} (EtOH): 278 nm, ε = 14.5 mM^{-1} cm^{-1}]. IR (neat): 2118 cm^{-1} (Ar-N_3). ^{1}H-NMR ($CDCl_3$): δ 1.36 (10 H, m, -CH_2-), 2.00 (3 H, s, -CH_3), 2.46 (2 H, m, quinone nucleus -CH_2-), 3.98 (6 H, s, -OCH_3); 7.07 (4 H, m, Ar).

2,3-Dimethoxy-5-methyl-6-[7-(4-azido-3-[^{125}I]iodophenyl)-heptanyl]-p-benzoquinone (**5**) (^{125}I-$Q_0C_7ArN_3$). (*a*) *Radioactive Labeling with Na^{125}I.* A total of 5 μg of **4** (23 nmol) was taken up in 10 μL of concentrated acetic acid according to Kawada et al. (1989). At room temperature, 0.5 mCi of carrier-free Na^{125}I (2300 Ci/mmol) in dilute sodium hydroxide (pH 7–11) was added to the solution followed by 6 μL of chloramine T (1 mg/mL concentrated acetic acid). The mixture was vortexed briefly, and after a incubation period of 3 min the reaction was halted by the addition of 3 μL of 1 M $NaHSO_3$. Then, the solution was made basic by the addition of 112 μL of 2.5 M NaOH and extracted three times with ethyl acetate (500 μL each). The organic layer was dried over $MgSO_4$ and the solvent was removed under a stream of nitrogen (TLC R_f = 0.15).

(*b*) *Diazotation.* The residue was dissolved in 200 μL of dimethylformamide and chilled to 0 °C. A total of 6 μL of concentrated HCl was added, followed by 31 μL of 0.1 M $NaNO_2$. The mixture was kept on ice for 20 min, and the excess of $NaNO_2$ was destroyed by adding 37 μL of 0.1 M amidosulfonic acid. The following operations were performed under subdued light. A 0.1 M aqueous solution of NaN_3 (39 μL) was added to the mixture, and after 20 min the solution was made basic by the addition of 84 μL of 1 M NaOH. The mixture was extracted into ethyl acetate (three times, 0.5 mL each), and the combined organic layers were shaken with 300 μL of a solution of 25% $FeCl_3$ for 20 min. After separation of the two phases, the organic layer was washed with water and dried over $MgSO_4$ (yield of ^{125}I incorporation was 30% TLC R_f = 0.72; sp act. 6 μCi/nmol). Aliquots of the organic

solution were dried under a stream of nitrogen for the photoaffinity labeling experiments.

Nonradioactive iodo-$Q_0C_7ArN_3$ was synthesized as described above, applying an upscale preparation with 50 mg of (81% overall yield; TLC R_f = 0.72). ^{1}H-NMR data were identical to those obtained with $Q_0C_7ArN_3$.

Enzyme Assay

The NADH:ubiquinone reductase activity was measured at 25 °C following the absorbance at 340 nm. The assay medium contained 50 mM Tris, pH 7.5, 150 μM NADH, 2 mM NaN_3, and 100 μg of pentane-extracted mitochondrial membranes. The reaction was started by adding the ubiquinone analogues in various amounts. The analogues and the inhibitor rotenone (Sigma, Deisenhofen, FRG) were applied in methanolic or ethanolic solutions (final concentration of alcohol in the assay did not exceed 2%). $Q_0C_7ArN_3$ was dissolved in ethanol/tetrahydrofuran (9:1).

Photoaffinity Labeling

All experiments were carried out at 4 °C and under subdued light.

Isolated Complex I. A total of 100 μg of isolated complex I was finely dispersed in 0.5 mL of phosphate buffer (0.1 M, pH 8.0) by a brief sonication step. Cholate and desoxycholate (standard procedure), or other detergents, were added to a final concentration of 0.25% each. When indicated, the mixture was preincubated under conditions specified in the legend of Figure 7. Then, the solution was applied either directly to the dried ubiquinone analogue ^{125}I-$Q_0C_7ArN_3$ (about 0.15 nmol; 6 μCi/nmol) or to the solution in an appropriate organic solvent. After an incubation period of 60 min, the mixture was transferred into a semimicro quartz cuvette and illuminated for 4 min with a UV lamp (Model XX-15; Ultra-Violet Products Inc., San Gabriel, CA.). Finally, the protein was precipitated by the addition of a saturated solution of $(NH_4)_2SO_4$ (300 μL) and collected by a centrifugation step. The pellet was washed with 1 mL of 0.1 M NaPP, pH 8, and was subjected to SDS gel electrophoresis.

Mitochondrial Membranes. A total of 1 mg of mitochondrial membranes was incubated in 1 mL of phosphate buffer (0.1 M, pH 8.0) containing either detergent or organic solvent (see legend to Figure 6) and the radioactive ubiquinone analogue (about 0.5 nmol; 6 μCi/nmol). The samples were kept for 4 h at 4 °C and then illuminated with UV light for 10 min and finally solubilized in 1% Triton X-100. After a clarifying spin (20 min, 48000*g*), complex I was immunoprecipitated as described (Werner & Sebald, 1981; Zauner et al., 1985).

Miscellaneous Methods

Preparation of submitochondrial particles was carried out as described previously (Werner, 1977). Complex I was isolated by the method of Ise et al. (1985) with minor modifications (Filser & Werner, 1988). Ubiquinone-depleted mitochondrial particles were prepared by extracting lyophilized mitochondrial membranes with dry *n*-pentane according to Gutman et al. (1971). The pentane-extracted membranes were taken up in 0.1 M sodium phosphate buffer, pH 8.0, to a protein concentration of about 10 mg/mL and used immediately in the enzyme assay.

SDS gel electrophoresis (18.5% polyacrylamide) according to Laemmli (1970) was used to analyze the photoaffinity-labeling products.

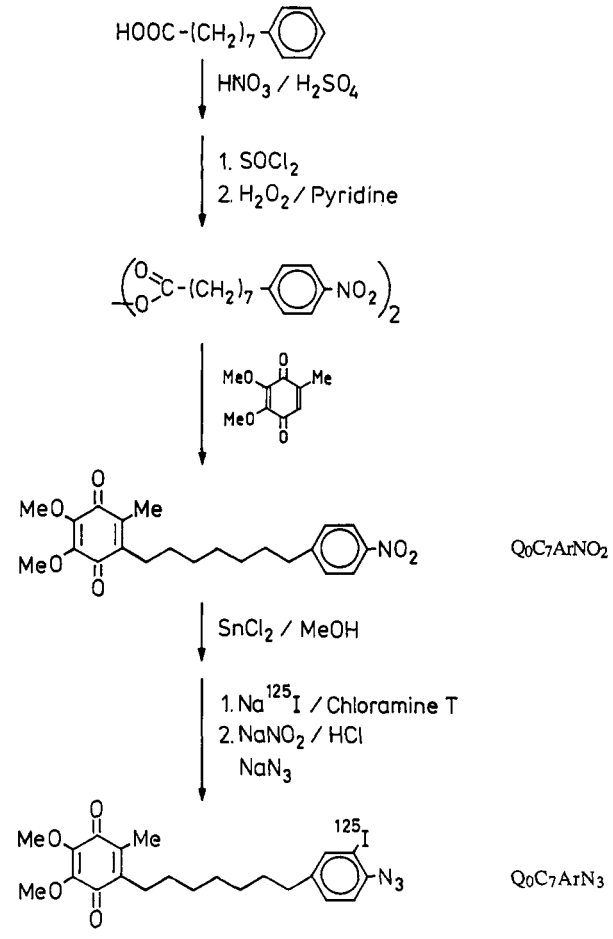

FIGURE 1: Synthesis and structure of the precursor compound $Q_0C_7ArNO_2$ and of the chemically reactive ubiquinone analogue $Q_0C_7ArN_3$.

The incorporation of isolated complex I into phospholipid vesicles and mixed micelles was performed according to Linke et al. (1986). A solution of L-α-phosphatidylcholine (from soybeans, Sigma, type II-S; 400 μg in 400 μL of phosphate buffer, pH 8.0) containing 0.25% of detergents (see legend to Figure 4), was sonicated after addition of 100 μg of isolated complex I. The mixture was applied to the dried analogue ^{125}I-$Q_0C_7ArN_3$, incubated for 4 h at 4 °C, and processed as detailed above (section Isolated Complex I under Photoaffinity Labeling).

RESULTS

Chemical Synthesis of the Ubiquinone Derivative $Q_0C_7ArN_3$

A strategy for the synthesis of a substrate analogue designed according to criteria discussed below was elaborated. Figure 1 depicts the synthetic approach of the photoreactive ubiquinone analogue $Q_0C_7ArN_3$ and its direct chemical precursor $Q_0C_7ArNO_2$. The preparation was started by the assembly of the side chain. For this purpose, the 8-phenyloctanoic acid was nitrated and converted into the diacyl peroxide via the acid chloride. The accomplished side chain was then attached to 2,3-dimethoxy-5-methyl-*p*-benzoquinone (Q_0) by a radical reaction in glacial acetic acid as described by Wan et al. (1975), and the nitro group was subsequently reduced using $SnCl_2 \cdot 2H_2O$ in methanol. The compound was radioactively labeled with carrier-free $Na^{125}I$ by the chloramine T method, and the amino group was finally converted into the arylazido moiety.

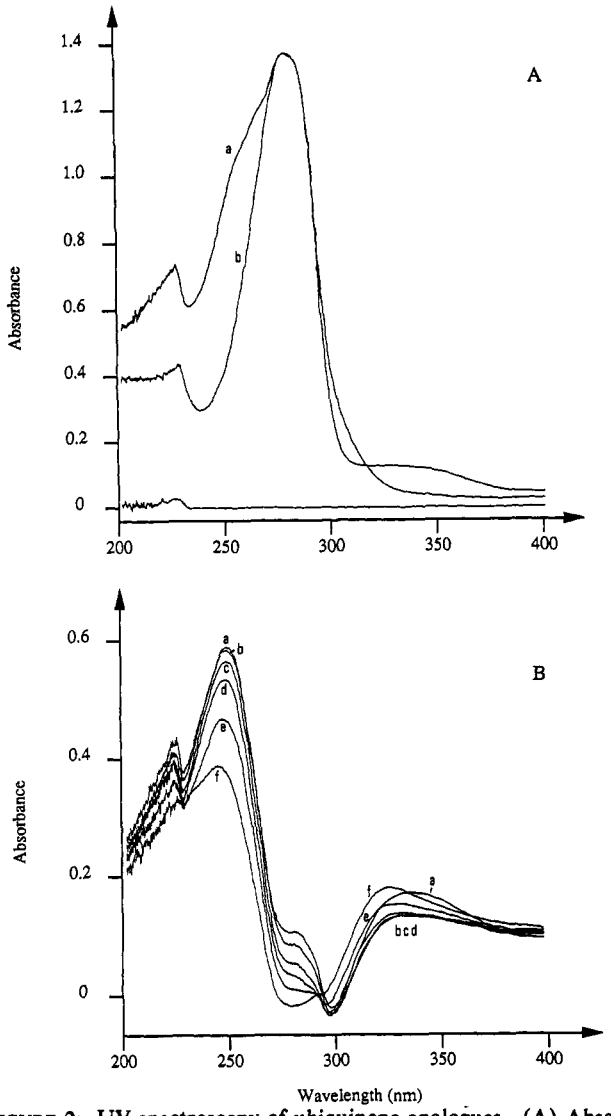

FIGURE 2: UV spectroscopy of ubiquinone analogues. (A) Absolute UV spectra of $Q_0C_7ArN_3$ (a) and $Q_0C_7ArNO_2$ (b) are shown (concentrations were 82 μM and 88 μM, respectively, in dichloromethane). (B) UV difference spectra ($Q_0C_7ArN_3$ versus $Q_0C_7ArNO_2$) were recorded after various periods of UV irradiation of both samples: control (a), 1 min (b), 3 min (c), 5 min (d), 10 min (e), 20 min (f).

The azido compound was characterized by its IR and UV data. The photolytic behavior of $Q_0C_7ArN_3$ was monitored by UV spectroscopy after various periods of irradiation. Due to the strong absorption of the quinone nucleus (λ_{max} 278 nm), the photodecomposition of the arylazido moiety (λ_{max} 250 nm) reveals only small spectral changes: disappearance of the shoulder at 265 nm (compare absolute spectra of the two derivatives $Q_0C_7ArN_3$ and $Q_0C_7ArNO_2$ depicted in Figure 2A). To illustrate the typical behavior of the arylazido compound upon illumination, a set of difference spectra was recorded, exhibiting a marked decrease of the absorbance at 250 nm (Figure 2B). The radioactive product was identified by thin-layer chromatography, exhibiting an identical R_f value with the nonradioactive counterpart. The latter compound, as well as the intermediate, was further characterized by ^{1}H-NMR spectroscopy.

Enzymatic Properties of the Ubiquinone Derivatives $Q_0C_7ArNO_2$ and $Q_0C_7ArN_3$

In order to assess the biological acceptance of the novel derivative by the enzyme, the chemical precursor $Q_0C_7ArNO_2$ as well as the label $Q_0C_7ArN_3$ was applied to an enzyme assay exploiting ubiquinone-depleted mitochondrial membranes of *N. crassa*. Ubiquinone-depleted membranes were chosen for several reasons: (1) interference of competing endogenous ubiquinone is avoided or minimized (see below); (2) measured values become directly comparable with those obtained in the presence of other ubiquinone analogues, for instance Q_2; (3) the enzymatic reaction can be initiated by the addition of the derivative and hence allows a better evaluation of the kinetics. The pentane-extracted membranes exhibit only a negligible small NADH:ubiquinone reductase activity (<5%) without supplemented exogenous ubiquinone. By adding the ethanolic solution of the Q derivatives, activity was restored by $Q_0C_7ArNO_2$ to approximately 60%, compared with Q_2 (concentrations of 39 μM each).

The following kinetic properties were calculated by an Eadie-Hofstee plot:

$Q_0C_7ArNO_2$	V_{max} = 0.55 unit/mg	K_m = 67 μM
$Q_0C_7ArN_3$	V_{max} = 0.23 unit/mg	K_m = 83 μM
Q_2	V_{max} = 1.12 unit/mg	K_m = 56 μM

Data of the iodo derivative showed no significant difference to those obtained with $Q_0C_7ArN_3$. The activity of the enzyme using the derivatives $Q_0C_7ArNO_2$ and $Q_0C_7ArN_3$ is highly sensitive to the specific inhibitor rotenone. We obtained IC_{50} values (inhibitor concentration resulting in a 50% loss of enzyme activity) of about 9 and 25 μM, respectively, that is, on the same order of magnitude as those of Q_2 and decylubiquinone [IC_{50} values about 5 μM (Filser & Werner, 1988)]. The enzymatic activity is almost completely inhibited at rotenone concentrations as high as 100 μM.

Photoaffinity Labeling

Photoincorporation into Isolated Complex I. In a first set of experiments, we tried to define optimum conditions for the incorporation of $Q_0C_7ArN_3$, including also the application of a suitable "solvent" for the very hydrophobic ubiquinone label. Therefore, an enzyme preparation of isolated complex I from *Neurospora crassa* was incubated with the ^{125}I-labeled photoreactive ubiquinone analogue $Q_0C_7ArN_3$ in the presence of different organic solvents and detergents, respectively. After photoactivation, the samples were collected by $(NH_4)_2SO_4$ precipitation, washed, and subjected to SDS gel electrophoresis. The fluorographic resolution is shown in Figure 3. It is evident that without an appropriate "solvent" no covalent labeling could take place (Figure 3, lane f). By applying various organic solvents (lanes a–c), a fair portion of the offered radioactivity was incorporated into complex I, but at the same time an unfavorable ratio between specific labeling of distinct polypeptide subunits and background noise was obtained. Moreover, the radioactive material migrating with the dye front represents label not linked to protein. By adding detergents (lanes d and e), instead of organic solvents, the amount of total incorporation was somewhat smaller, yet the distribution of the radioactivity on the gel was truly distinct. In all these experiments allowing covalent binding of the analogue ^{125}I-$Q_0C_7ArN_3$, a small polypeptide subunit of complex I with an apparent molecular mass of about 9.5 kDa became labeled significantly. A comparison between Triton X-100 (lane d) and cholate/desoxycholate (lane e) exhibits a rather "protective" effect of Triton in terms of labeling of the 9.5-kDa polypeptide subunits. This behavior has been demonstrated in a more impressive way using complex I

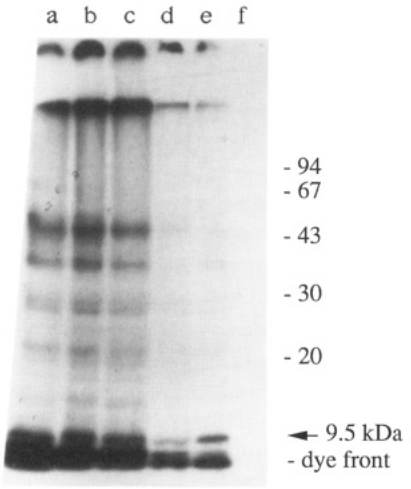

FIGURE 3: Covalent photoincorporation of the arylazido Q derivative $Q_0C_7ArN_3$ into isolated *N. crassa* complex I. A total of 100 μg of complex I was incubated (see Materials and Methods) in the presence of the following additives: (a) 5% dimethyl sulfoxide; (b) 5% dimethoxyethane; (c) 5% ethanol; (d) 0.5% Triton X-100; (e) 0.25% cholate/0.25% desoxycholate; (f) 0.1 M sodium phosphate buffer, only. After illumination, all samples were analyzed by SDS gel electrophoresis and the gel was subsequently fluorographed.

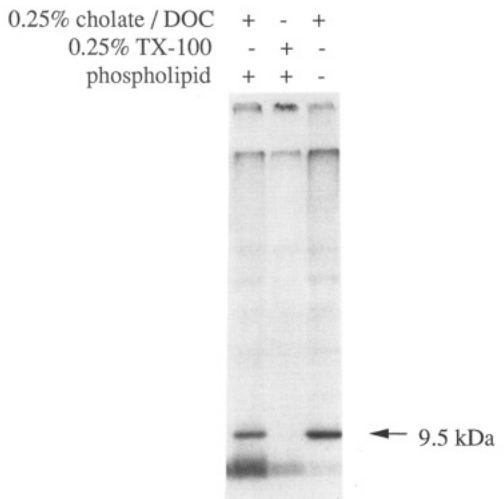

FIGURE 4: Effect of cholate/desoxycholate and Triton X-100, respectively, on the labeling behavior of complex I embedded in mixed micelles prepared from phosphatidylcholine and detergent. A total of 100 μg of the isolated enzyme was incorporated into the micelles (protein/phospholipid/detergent ratio was 1:4:10) by an intermittent sonication procedure (Linke et al., 1986), and afterward the mixture was incubated with the radioactive $Q_0C_7ArN_3$ analogue for 60 min. After illumination, complex I was collected by precipitation with $(NH_4)_2SO_4$, subjected to SDS gel electrophoresis, and fluorographed.

embedded in mixed micelles received from detergent and phospholipids (Figure 4). In the presence of Triton, labeling was completely repressed. The same results have been obtained using whole mitochondrial membranes (data not shown). These findings are also reflected by the different enzymatic activities persisting in complex I after applying various concentrations of both groups of detergents to the enzyme assay (Figure 5).

Photoincorporation into Whole Mitochondrial Membranes. A more physiological situation was studied by exploiting whole mitochondrial membranes as a target for the photoreactive compound. Mitochondrial membrane preparations were incubated under various conditions with the substrate analogue ^{125}I-$Q_0C_7ArN_3$ and after illumination, complex I was isolated by immunoprecipitation. Figure 6 shows the autoradiograph of the samples after gel electrophoretic analysis. Two different antibodies have been applied: one against the whole complex

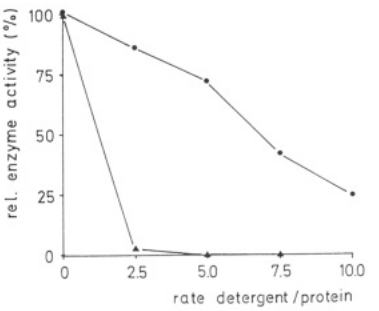

FIGURE 5: Inhibitory effect of cholate/desoxycholate (●) and Triton X-100 (▲), respectively, on the enzyme activity. Various amounts of detergents were added to the standard enzyme assay monitoring the NADH:Q_2 reductase activity (concentration of Q_2 was 39 μM).

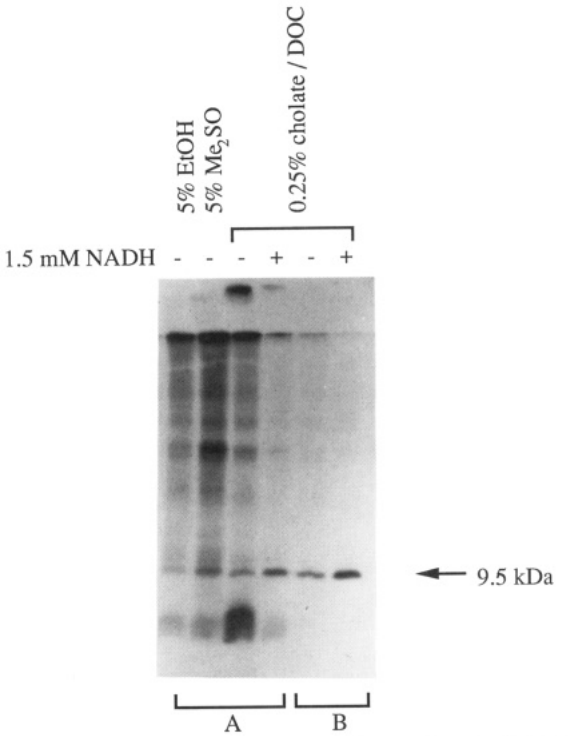

FIGURE 6: Covalent photoincorporation of the radioactive ^{125}I-$Q_0C_7ArN_3$ analogue into whole mitochondrial membranes of *N. crassa*. One milligram of membranes was incubated in the presence or absence of NADH under conditions depicted in the figure. Complex I was isolated by immunoprecipitation applying two different antibodies: (A) against whole complex I or (B) against an individual subunit (22 kDa); the latter serum is also able to precipitate whole complex I.

(panel A) and a second one against an individual subunit (22-kDa polypeptide) (see panel B). The latter antiserum is also capable to precipitate whole complex I. This approach clearly indicates that the radioactive labeled 9.5-kDa subunit is indeed a component of this enzyme complex.

The results of photoaffinity labeling using whole membranes confirm those described above with isolated complex I. They differ only in the amount of incorporated radioactivity: while 4–8% of the added radioactive analogue was found covalently linked to the protein applying isolated complex I, still one-tenth of this amount could be detected in the experiments with whole membranes. In this context, we want to point out that pentane extracted membranes also showed the same labeling behavior as untreated, intact particles.

Of special interest is the influence of NADH in the experiments using whole membranes. In the presence of 1.5 mM NADH, the signal of the 9.5-kDa polypeptide is enhanced (Figure 6).

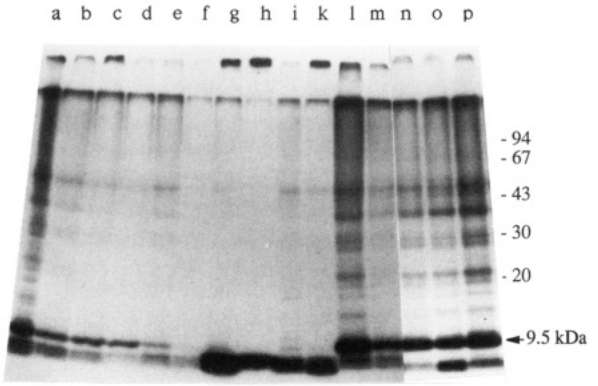

FIGURE 7: Effect on the labeling of the 9.5-kDa polypeptide subunit of complex I by preincubation of the isolated enzyme with various compounds. A total of 100 μg of complex I was preincubated for 60 min under standard conditions with different amounts of the substances listed below. These samples were then added to the radioactive Q analogue (^{125}I-$Q_0C_7ArN_3$). The mixture was incubated for a further 45 min and processed (illumination, precipitation, gel electrophoresis, fluorography) as described in Materials and Methods (section Isolated Complex I under Photoaffinity Labeling): (a) control; (b) 100 μM rotenone; (c) 500 μM rotenone; (d) 1 mM rotenone; (e) 100 μM Q_2; (f) 500 μM Q_2; (g) 1 mM Q_2; (h) 5 mM Q_2; (i) 500 μM $Q_0C_7ArNO_2$; (k) 1 mM $Q_0C_7ArNO_2$; (l) 1% BSA; (m) 5% BSA; (n) 500 μM vitamin A; (o) 500 μM vitamin E; (p) 500 μM Q_0.

Competition Experiments. In order to make sure that the labeling of the 9.5-kDa polypeptide subunit is the result of a specific interaction of the substrate analogue with the NADH: ubiquinone reductase, complex I was incubated with various substrates or the inhibitor prior to photolysis. Then, these samples were processed as described in the preceding section. An autoradiograph of samples exposed to different concentrations of competitors is shown in Figure 7. Remarkably, the inhibitor rotenone, even high amounts (lanes b–d), was not able to prevent radioactive labeling of the 9.5-kDa polypeptide. On the other hand, equivalent concentrations of the substrate ubiquinone-2, and of $Q_0C_7ArNO_2$ as well (the chemical precursor of the affinity label) (compare lanes f–k), did not allow the covalent attachment of the label to this subunit. At about 100 μM Q_2 (lane e), the signal was already significantly attenuated.

Neither various other hydrophobic substances like vitamin A and E (see Figure 7; lanes n and o) nor the hydrophilic substrate analogue Q_0 (lane p) nor the presence of 5% bovine serum albumin (lane m), which is known to prevent unspecific incorporation, had a protective effect upon labeling.

DISCUSSION

Several approaches have been reported to identify polypeptide subunits, which may be involved in the ubiquinone binding sites of protein complexes within the respiratory chain. Most studies have been preformed on complex III, describing mainly two techniques: (1) the isolation of "hydrophobic" subunits, which can be "recharged" with ubiquinone after a chromatographic separation procedure (Wang & King, 1982; Suzuki & Ozawa, 1984) and (2) the application of arylazido and azido-Q derivatives, respectively, to achieve a photoaffinity labeling of the participating subunit(s) (Yu & Yu, 1982; Yu et al., 1985; Usui et al., 1990). Investing complex I, Suzuki and Ozawa (1986) have claimed the isolation of a Q-binding protein from the soluble so-called IP subfraction of the enzyme, without giving a further characterization of this polypeptide. The more specific approach applying photoreactive ubiquinone analogues to complex I has not yet been published. Ubiquinone derivatives exploited so far have suffered from several handicaps. The azido-Q derivatives reported show a relative poor reconstitutive activity (in the best case 12%) (Yu et al., 1985). On the other hand, the utilized arylazido-Q compounds exhibit a rather unspecific labeling behavior or/and do not compete with added substrate analogues (Campbell et al., 1986). This prompted us to design a novel photoreactive analogue of ubiquinone, which may mimic the essential structural and hence functional requirements of the naturally occurring substrate.

In this study, synthesis and properties of the novel arylazido-Q derivative ($Q_0C_7ArN_3$) are presented. The following criteria were set up: (1) strict observation of the explicit hydrophobic character of the whole molecule; (2) preservation of the authentic quinone nucleus; (3) utilization of a discrete length of the side chain; and (4) attachment of the photochemical group without impairing the biological activity. A relative short alkyl homologue of ubiquinone (the side chain is composed of seven C-atoms plus the phenyl moiety) was chosen in analogy to the practical knowledge acquired with the decyl-Q derivative. In order to couple the phenylazide to the end of the chain, arrangements with functional groups (esters, amides, etc.), which may cause a restriction in the flexibility of this part of the molecule, were avoided. Furthermore, the selected strategy for the synthesis allows the insertion of the [^{125}I]iodine in the almost last step. The application of this isotope results in a high specific radioactivity of the affinty compound (6 mCi/μmol) and helps considerably to align the signals to distinct polypeptides of a multisubunit complex.

Enzyme kinetics carried out with the direct chemical precursor $Q_0C_7ArNO_2$ and $Q_0C_7ArN_3$, measuring the NADH: ubiquinone reductase activity, demonstrate the excellent acceptance of the derivatives as substrate and the high reconstitutional potency in pentane-extracted mitochondrial membranes. A further important aspect is the sensitivity of the system concerning the specific inhibitor rotenone. The observed inhibitory effect was on the same order of magnitude as that found with Q_2 and thus makes it likely that these two analogues ($Q_0C_7ArN_3$ and Q_2) act at the same catalytic site. This view is also confirmed by results obtained from the substrate competition experiments, where the radioactive labeling is totally prevented in the presence of 500 μM Q_2 (equivalent to a 2000-fold molar excess). On the other hand, the studies with rotenone lead to the conclusion that this compound acts as a noncompetitive inhibitor with regard to ubiquinone, because the drug had no influence on the labeling of the 9.5-kDa polypeptide subunit, even at excessive concentrations (1 mM, corresponding to a 4000-fold molar excess). The mechanism of how this substance interferes with complex I is still not understood. For this purpose, no kinetic data are available, showing clear-cut results.

Furthermore, the labeling pattern is not influenced by the addition of other hydrophobic substances, by bovine serum albumin, or by the low-molecular-weight homologue Q_0, which is known to cause a rotenone-insensitive substrate reaction, like that produced by artificial electron acceptors, such as ferricyanide. The "protective" effect of Triton X-100 observed in the labeling experiments (Figure 4) has to be seen in close correlation with the results of the enzyme assay (Figure 5). It has been already reported (Ise et al., 1985; Suzuki & King, 1983) that this detergent has a negative influence both on enzyme activity and on the intensity of EPR signals obtained from ubisemiquinone radicals.

The fact that no significant differences in labeling were found comparing pentane-extracted with intact membranes could have different reasons: (1) The portion of the endogenous ubiquinone [4–6 nmol/mg of protein in mitochondrial membranes (Norling et al., 1974)] could be too small to compete successfully with the label applied (about 0.6 nmol of arylazido analogue/mg of membrane protein). This may be rationalized be the fact that the covalently blocked binding sites are no longer "free for competiton". (2) Part of the ubiquinone (the so-called "tightly bound" species) could resist the pentane extraction, as already reported by Norling et al. (1974).

A very interesting aspect, which will be investigated in more detail, is the role of the redox state of complex I during labeling. The radioactive incorporation into the 9.5-kDa subunit is increased in the presence of 1.5 mM NADH using whole mitochondrial membranes. With an equal concentration of NAD^+, or when isolated complex I is applied instead of whole membranes, labeling is not affected or even decreased. This finding may be explained by the assumption that ubiquinone bound to the protein, prior to the addition of the radioactive analogue, is able to dissociate from complex I only in the reduced state and with the aid of the phospholipid bilayer, resulting in a better accessibility of the binding site.

In summary, the obtained data strongly indicate that the 9.5-kDa polypeptide, which is specifically labeled using both isolated complex I and mitochondrial membranes of *N. crassa*, represents the putative ubiquinone-binding site. This protein has been further characterized by its primary structure, its predicted secondary structure, and by its rough localization within complex I (Heinrich et al., 1992).

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