Interaction and Identification of Ubiquinone-binding Proteins in Ubiquinol-Cytochrome c Reductase by Azido-ubiquinone Derivatives*

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Various azido-ubiquinone derivatives were synthesized and characterized. 3-Azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone was found to be suitable for the study of specific interaction between ubiquinone (Q) and protein. It was synthesized with high specific radioactivity and used to identify the Q-binding proteins in purified ubiquinol-cytochrome c reductase. This azido-Q derivative showed partial efficiency in restoring activity to the Q- and phospholipids-depleted ubiquinol-cytochrome c reductase in the absence of light. Azido-Q derivative treated samples, however, became completely inactivated upon photolysis, and the inactivation was not reversed by addition of Q derivatives. The redox state of the azido-Q derivative has little effect on the Q-binding affinity. Two protein subunits with \( M_r = 37,000 \) and 17,000 were found to be heavily labeled when depleted ubiquinol-cytochrome c reductase was treated with \(^{3}H\) azido-Q derivative followed by photolysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amount of radioactive labeling of the \( M_r = 17,000 \) protein was proportional to the degree of inactivation and affected by the presence of phospholipids. The radioactive labeling of the \( M_r = 37,000 \) protein subunit, however, showed no correlation with degree of inactivation and affected by the presence of phospholipids. Since the radiolabeling at the \( M_r = 17,000 \) protein subunit was affected by phospholipids and correlated with the enzymatic activity, this subunit is probably the Q-binding protein in this enzyme complex (QPC). The inhibition of enzymatic activity by \( n \)-heptyl-4-hydroxyquinoline-N-oxide was easily reversed by addition of the azido-Q derivative. The distribution of radioactivity among the subunits of ubiquinol-cytochrome c reductase was not affected by the presence of antimycin A, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole or \( n \)-heptyl-4-hydroxyquinoline-N-oxide, suggesting that the binding site(s) of these inhibitors are not the Q-binding site.

The existence of specific ubiquinone (Q)-binding proteins in the mitochondrial and photosynthetic electron transfer systems has been established through biochemical and biophysical studies (1). The Q-binding proteins in the mitochondrial cytochrome b-c1 region have been detected by epr measurement, using the ability to stabilize ubisemiquinone radicals (2-5). Recently, a photoaffinity labeling study using an arylazido-Q derivative has identified two polypeptides with \( M_r = 37,000 \) and 17,000 (6) in the highly purified ubiquinol-cytochrome c reductase that are photolabeled by the reagent. Whether both proteins have individual Q-binding sites or both form a common Q-binding site remains to be elucidated. Evidence for the existence of two distinct Q-binding sites in the cytochrome b-c1 region is available (5, 7).

One ambiguity of studies using an arylazido-Q derivative to identify the Q-binding protein is due to the location of the photoactivatable group on the Q molecule. In the arylazido-Q molecule, the photoactivatable group is located on the part of the Q molecule where structural requirements for the electron transfer reaction are much less specific (8). A nonspecific labeling of Complex III (9) with azido-phenolphospholipid has been reported (10). This problem can now be resolved through the use of azido-Q derivatives. Among various azido-Q derivatives (11) synthesized for this work, 3-azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Q) and its dehydrogenated derivative, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone (3-azido-2-methyl-5-methoxy-Qa) possess the best properties for use as photoaffinity labels in identification of those Q-binding protein(s) for which no isolation procedure has yet been developed, and for study of the interaction between Q and protein and/or between Q and phospholipids. These azido-Q derivatives have the ability to restore partial (12%) succinate-cytochrome c reductase activity to the Q- and PL-depleted succinate-cytochrome c reductase in the absence of light, and show complete binding to the Q-binding sites after photolysis (12). The relative ease of preparation of these azido-Q derivatives in radioactive form greatly aided our use of these azido-Q derivatives to unambiguously identify the Q-binding proteins in this segment of the electron transfer chain.

In this paper we report the detailed photoaffinity labeling conditions, identification of the Q-binding proteins in the cytochrome b-c1 region, and the effect of phospholipids and inhibitors on the Q-binding and Q-protein interaction. Synthesis and characterization of various azido-Q derivatives are given in the Miniprint.
EXPERIMENTAL PROCEDURES

Beef heart submitochondriol particles (13), succinate-Q reductase (13), and a highly purified ubiquinol-cytochrome c reductase (the cytochrome b-5, III complex) (14) were prepared and assayed according to the reported methods. The Q- and PL-depleted ubiquinol-cytochrome c reductase was prepared by repeated ammonium sulfate precipitation in the presence of sodium chloride and glycerol (15). The depleted enzyme was dissolved in 50 mM phosphate buffer, pH 7.4, containing 10% glycerol and 1 mM EDTA. Determination of protein concentration (16) and quantitative analysis of essential components, such as cytochrome b (14), cytochrome c (14), phospholipids (17), and Q (18) were carried out according to the reported methods.

Synthesis of oxidized and reduced forms of Bligh and Dyer (20). The protein solution (0.8 ml) was mixed with 0.25% sodium cholate and phospholipids and incubated at 0°C for 30 min with occasional shaking. After incubation, 1 ml of H2O and 1 ml of chloroform were added. The final concentration of ethanol in the mixture was kept lower than 5% to prevent denaturation of the enzyme by high solvent concentration. The azido Q-treated sample was incubated at 0°C for 20 min. After incubation, the previous work (6). For activity assays, the photolyzed samples were reconstituted with phospholipids and incubated at 0°C for 1 h prior to activity determination.

The time course study of the incorporation of radioactivity of azido-Q into protein was conducted as reported (6).

RESULTS AND DISCUSSION

Synthesis, Structure, Spectral Properties, Biological Activity, and Radioactivity of 3-Azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone(3-azido-2-methyl-5-methoxy-Q6)—Preliminary studies showed that the azido-Q derivatives synthesized by us all have partial activity in restoration of enzymatic activity to the Q- and PL-depleted succinate-cytochrome c reductase in the dark and all abolish the restored activity upon photolysis (see Table I of the Miniprint). 3-Azido-2-methyl-5-methoxy-Q6 is superior to the other azido-Q derivatives for study of the Q-protein interaction and identification of the Q-binding proteins because of its ability to allow reconstitution of these Q derivatives to the Q-binding proteins to the Q-protein interaction and identification of the Q-binding proteins because of its ability to allow reconstitution of these Q derivatives to the Q-binding proteins.
The Ubiquinone-binding Proteins

TABLE I

Biological activity of 3-azido-2-methyl-5-methoxy-Q2 and 3-azido-2-methyl-5-methoxy-Q2

Two-tenth-ml aliquots of the Q- and PL-depleted ubiquinol-cytochrome c reductase in 50 mM phosphate buffer, pH 7.4, containing 0.25% sodium cholate and 1 mM EDTA were mixed with 5 μl of 5 mM alcoholic solution of Q analogues and incubated at 0 °C for 10 min in the dark. Fifty-μl aliquots were withdrawn and mixed with 5 μl of asolectin micellar solution (10 mg/ml in H2O) and reconstituted with 80 μl of succinate-Q reductase (3 mg/ml) in 20 mM Tris-succinate, pH 8.0, containing 0.1% deoxycholate to form succinate-cytochrome c reductase. After 1 h incubation, the mixture was diluted with 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and the succinate-cytochrome c and ubiquinol-cytochrome c reductase activities were assayed. The remaining enzyme-Q mixture were subjected to photolysis at 0 °C for 20 min. After photolysis, aliquots were withdrawn and reconstituted with asolectin and succinate-Q reductase and the activities of both reductases were assayed. Reduced QH2, 50 μM, was used as substrate for the ubiquinol-cytochrome c reductase activity assay.

<table>
<thead>
<tr>
<th>Q derivatives</th>
<th>Succinate-cytochrome c (reconstituted)</th>
<th>Ubiquinol-cytochrome c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before hr</td>
<td>After hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.96</td>
<td>0.89</td>
</tr>
<tr>
<td>Q2C2Br</td>
<td>17.51</td>
<td>16.28</td>
</tr>
<tr>
<td>3-Azido-2-methyl-5-methoxy-Q2</td>
<td>3.05</td>
<td>0.61</td>
</tr>
<tr>
<td>3-Azido-2-methyl-5-methoxy-Q2a</td>
<td>3.10</td>
<td>0.58</td>
</tr>
</tbody>
</table>

The absorption spectra and photodecomposition behavior of 3-azido-2-methyl-5-methoxy-Q2 are very similar to those of 3-azido-2-methyl-5-methoxy-Q2 (see Fig. 2 of the Mini-Print).

Illumination Time-dependent Azido-Q Uptake by Protein and Loss of Enzymatic Activity—Fig. 1 shows the photolysis time-dependent binding of the radioactive [3H]-azido-Q derivative to the ubiquinol-cytochrome c reductase and inactivation of the azido-Q-treated ubiquinol-cytochrome c reductase. When the azido-Q-treated and PL-depleted ubiquinol-cytochrome c reductase was subjected to varying times of photolysis at 0-2 °C with long wavelength UV light, the ubiquinol-cytochrome c reductase activity, measured after reconstitution with phospholipids, decreased concurrently with increased [3H]-azido-Q derivative uptake by protein over time. The maximal inactivation was observed when the azido-Q-treated sample was illuminated for 20 min. Illumination beyond 20 min did not further inhibit the activity, although the azido-Q uptake by the enzyme complex continued to increase, at a slower rate. A control sample, treated with the same amounts of solvent but no azido-Q derivative, was placed beside the azido-Q-treated sample during photolysis. Little loss of activity occurred in the illuminated control sample. A plot of the logarithm of inactivation versus illumination time...
The Ubiquinone-binding Proteins (not shown) yields a nearly linear curve, indicating that the inactivation resulting from photolysis follows first order kinetics. The kinetics of azido-Q uptake by ubiquinol-cytochrome c reductase, however, was more complex, as two different rates were involved, one of which was 10 times faster than the other. The rapid uptake seemed to correlate with the inactivation and was complete in 20 min of photolysis. The slower uptake was probably due to nonspecific binding of azido-Q derivative to protein. Thus, a 20-min illumination time was chosen for the identification of the Q-binding protein and study of the Q:protein interaction in ubiquinol-cytochrome c reductase. The kinetics of azido-Q uptake by protein and inactivation are quite similar to those observed with the arylazido-Q derivative (6), although the inactivation was more complete with the azido-Q derivative.

Identification of the Q-binding Protein(s) in Ubiquinol-Cytochrome c Reductase—Since the uptake of azido-Q derivative by ubiquinol-cytochrome c reductase upon photolysis was correlated to the enzymatic inactivation, it is reasonable to assume that the azido-Q derivative is bound specifically to the Q-binding site(s). Thus, the distribution of the covalently bound azido-Q among the subunits of ubiquinol-cytochrome c reductase after SDS-PAGE indicates the specific Q-binding protein in this enzyme complex. Fig. 2 shows the $^3$H-radioactivity distribution among subunits of ubiquinol-cytochrome c reductase under various conditions. Two SDS-polyacrylamide gel systems were used: System A, the Weber-Osborn gel system, exactly as described for identification of the Q-binding proteins in ubiquinol-cytochrome c reductase with the arylazido-Q derivative (Q$_6$C$_{10}$NAPA) (6); and System B, the SDS-DATA gel system, which is a modification of the Weber-Osborn gel system. The Weber-Osborn cross-linker, bisacrylamide, was replaced with a cleavable linker, DATA (23). The advantage of using the SDS-DATA gel system is that the gel slices can be completely dissolved in 3% periodic acid and directly used for radioactivity determination without further treatment. The SDS-DATA gel system has been successfully used for identification of the Q-binding protein in a bacterial reaction center (22). As shown in Fig. 2, the electrophoretic pattern of ubiquinol-cytochrome c reductase obtained from the SDS-DATA gel system was similar to that obtained from the Weber-Osborn gel system, with seven major protein bands observed in each. Although the electrophoretic mobility of each subunit, relative to cytochrome c, in the SDS-DATA gel system was different from that in the Weber-Osborn gel system, the distribution of radioactivity among the subunits of ubiquinol-cytochrome c reductase was the same in these two gel systems (see panels i and ii of Fig. 2, A and B). One can assume that the protein subunits of ubiquinol-cytochrome c reductase revealed in the SDS-DATA gel correspond to those observed in the Weber-Osborn gel system, and the former is preferred for study of the Q-binding proteins in ubiquinol-cytochrome c reductase.

Results of photoaffinity labeling experiments are often complicated by the radioactivity observed near the dye front of the gel column resulting from free Q, phospholipid-Q, or

![Fig. 2](image-url) Effect of organic solvent extraction of the azido-Q derivative treated sample on the radioactivity distribution among the subunits of ubiquinol-cytochrome c reductase in the Weber-Osborn gel and the SDS-DATA gel systems. Four-tenth ml of the Q- and PL-depleted ubiquinol-cytochrome c reductase (3.1 mg/ml) was treated with 10 μl of 6.3 mM ethanolic solution of $[^3$H]3-azido-2-methyl-5-methoxy-Q$_6$ (4000 cpm/nmol). The mixture was photolyzed and diluted to 10 ml with 50 mM phosphate buffer, pH 7.4. The protein was collected by centrifugation and suspended with 0.5 ml of H$_2$O. The sample was used for the SDS-PAGE with or without subject to organic solvent extraction step according to the procedure described under "Experimental Procedures," using Weber-Osborn gel (A) and SDS-DATA gel (B) systems. Panels i and ii represent sample with and without organic solvent extraction, respectively, and panel iii represents a mixture of untreated ubiquinol-cytochrome c reductase and the organic extract of azido-Q-treated sample.
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detergent-Q adducts. It is especially important that the
electrophoresis data be completely devoid of this radioactivity
interference when the Q-binding site is in a small molecular
weight protein. In our previous study of interaction of an
arylazido-Q derivative with ubiquinol-cytochrome c reductase
(6), we observed that the radioactivity interference resulting
from free Q and PL-bound Q in the gel column could be removed
by prolonged destaining of the gels in the acetic acid-methanol
solution. However, this usually required more than 2 weeks,
with constant changes of destaining solution, to completely
eliminate the interference. Introduction of an organic solvent
extraction step successfully eliminated this interference. Fig.
2 shows the distribution patterns of radioactivity after (i) and
before (ii) the extraction step and in the extract (iii) in SDS-
bisacrylamide and SDS-DATA gel systems.

To ensure that the organic solvent extraction of the photo-
ylzed sample removed no protein-Q complex but only free Q,
Q-PL, or Q-detergent adducts, the chloroform extracts of the
photoylzed samples were collected, dried, redissolved in a
small amount of alcohol and assayed for protein. When the
alcohol solution was mixed with the untreated, depleted ubi-
quinol-cytochrome c reductase and subjected to SDS-PAGE,
the radioactivity was found mainly on the dye front of the gel
column (see panel i of Fig. 2, A and B). The very low amount
of radioactivity found with the proteins confirmed that all the
[3H]azido-Q in the chloroform extract had been photoacti-

dated and covalently linked to some small molecules in the
system and was no longer able to react with protein. This
result also indirectly substantiated the observation that the
radioactivity located in the protein bands of panels i and ii
resulted from the covalent linkage between azido-Q and pro-
tein subunits and not a nonspecific hydrophobic interaction
between the photolyzed Q molecule and protein during SDS-
PAGE. It should be mentioned that the extraction method
described under "Experimental Procedures" for the removal of the
non-protein bound azido-Q derivative does not cause protein
aggregation, a common phenomenon after organic solvent
extraction, as no protein remained on the top of the
gel column after SDS-PAGE.

Panels i and ii of Fig. 2 clearly show that the two proteins
with $M_r = 37,000$ and 17,000 are responsible for specific Q-
binding in ubiquinol-cytochrome c reductase. These results are
identical to those obtained from our previous study, using the
arylazido-Q derivative, Q$_b$C$_{10}$NAPA, as the labeling re-
agent. In addition to the $M_r = 17,000$ and 37,000 subunits, a
significant amount of radioactivity was also found in the $M_r$
= 30,000 protein, which was not labeled when Q$_b$C$_{10}$NAPA
was used. Whether the observed radioactivity on the $M_r$
= 30,000 subunit results from an abnormal electrophoretic
mobility of the $M_r = 37,000$ protein or from an actual partial
labeling of the $M_r = 30,000$ protein remains to be verified
experimentally. The electrophoretic abnormality of the $M_r$
= 37,000 protein in ubiquinol-cytochrome c reductase has been
documented (1, 24). In fact, in some reports, the $M_r = 37,000$
subunit was completely absent from the subunit structure of
ubiquinol-cytochrome c reductase or Complex III on SDS-
PAGE. The $M_r = 37,000$ subunit of ubiquinol-cytochrome c
reductase has been purified and identified as one of the b
cytochrome proteins (25). The $M_r = 17,000$ protein has been
identified as another b cytochrome protein (25). The $M_r$
= 17,000 protein has an electromobility faster than that of the Rieske's
iron-sulfur protein but slower than the cytochrome c-associated
protein in the SDS-PAGE. It is not the same protein
recently isolated and assumed by Wang and King as QPC
(26). The reason they assumed to be the Q-binding protein
in the cytochrome b-c$_1$ region has the same electrophoretic

mobility as the cytochrome c, associated small molecular
weight polypeptide ($M_r = 15,000$ or less), and was not labeled
either with the radioactive azido-Q or arylazido-Q derivative.

The nature of this protein (26) remains unclear, but is highly
likely to be a Q:PL:protein mixture rather than a specific Q-
binding protein.

Effect of the Redox State of the Azido-Q Derivative on the
Binding to Ubiquinol-Cytochrome c Reductase—It has been
suggested that the binding affinity of reduced Q to ubiquinol-
cytochrome c reductase is stronger than that of the oxidized
form of Q (27). It is, therefore, of interest to see whether the
reduced azido-Q derivative binds ubiquinol-cytochrome c re-
ductase better than does the oxidized azido-Q derivative.

When the depleted ubiquinol-cytochrome c reductase was


treated with the different redox forms of azido-Q derivative

at various concentrations, a 50% inactivation after photolysis
was observed when 2 mol of azido-Q/mol of cytochrome b in
the depleted enzyme complex were used, regardless of the
redox state of the azido-Q derivative (see Fig. 3). The maximal
inactivation by either redox state of the azido-Q derivative
was almost the same. The results indicate that no significant
difference in the Q-binding affinity between the oxidized and
reduced forms of azido-Q exists in the cytochrome b-c$_1$ region
of the electron transfer chain.

In the above experiment, the reduced form of the azido-Q

derivative was generated by reduction of the oxidized azido-

Q derivative with a catalytic amount of succinate-Q reductase

and succinate. The formation of the reduced azido-Q was

confirmed by thin layer chromatography after extraction of
the azido-Q from the system. After the reduction was com-
plete, the enzymatic activity of succinate-Q reductase was

stopped by the addition of oxalaceta before mixing with


\[
\text{Activity Remaining}\% = \frac{\text{Activity after reduction}}{\text{Activity before reduction}} \times 100
\]

Fig. 3. Effect of the redox state of the azido-Q derivative
on the binding to ubiquinol-cytochrome c reductase. Indicated

amounts of 3-azido-2-methyl-5-methoxy-Q$_b$, in 0.2 ml of 55 mm

phosphate buffer, pH 7.4, containing 0.25% cholate and 1 mM EDTA

were reduced by 0.43 mM succinate, in the presence of succinate-Q

reductase (10 μl, 1.6 mg/ml) in the dark at room temperature. After

reduction, the activity of succinate dehydrogenase was inhibited by

the addition of oxalaceta (5 μl, 0.5 M) and the mixtures were placed

in the ice bath and mixed with 60 μl of the Q- and PL-depleted

ubiquinol-cytochrome c reductase (O). When the oxidized azido-Q

($\times$) was used, 0.2-ml aliquots of buffer containing the indicated

amounts of azido-Q were mixed with the depleted enzyme complex

without pretreatment. Ten-μl aliquots were withdrawn from each

sample before and after photolysis, reconstituted with asolectin, and

assayed for ubiquinol-cytochrome c reductase activity. The per cent
activity remaining after photolysis was based on the activity obtained
before photolysis of each sample.

\[
\text{Activity Remaining}\% = \frac{\text{Activity after reduction}}{\text{Activity before reduction}} \times 100
\]
depleted ubiquinol-cytochrome c reductase, to prevent a slow reduction of the depleted enzyme by the added succinate-Q reductase. To control for possible complications resulting from the addition of succinate and oxalacetaate to the system, succinate and oxalacetaate were also added to the system with oxidized azido-Q derivative. No difference in the inactivation effect of the oxidized azido-Q derivative after photolysis was found between the systems with and without succinate and oxalacetaate.

Since more than one specific Q-binding subunit was detected in ubiquinol-cytochrome c reductase by the azido-Q derivative, the observed equal degree of inactivation by the oxidized and reduced forms of the azido-Q derivative after photolysis might result from the different contributions of the Q-binding proteins to the azido-Q binding. To clarify this point, the radioactivity distribution among subunits of the enzyme complex treated with the oxidized and reduced forms of the azido-Q derivative was investigated, and the results were compared. An identical distribution of H-radioactivity among the subunits of ubiquinol-cytochrome c reductase was obtained for the reduced and oxidized azido-Q derivative treated samples.

Effect of Phospholipids on the Inactivation of Ubiquinol-Cytochrome c Reductase by Azido-Q Derivative after Photolysis—The requirement for phospholipids, in addition to Q, for restoration of enzymatic activity and stabilization of the ubisemiquinone radical (1, 2) in ubiquinol-cytochrome c reductase, suggests an intimate relationship between the phospholipids and Q in this segment of the electron transfer chain. The reported observation (28) that only by adding Q prior to addition of phospholipids to the Q- and PL-depleted succinate-cytochrome c reductase can the enzymatic activity be completely restored, further confirms the role of phospholipids in the Q-protein interaction. A similar phospholipid effect was observed with azido-Q derivative binding to ubiquinol-cytochrome c reductase. As indicated in Table II, when the azido-Q derivative was added to the Q- and PL-depleted ubiquinol-cytochrome c reductase prior to the addition of phospholipids, a greater inactivation after photolysis was observed than in the sample mixed with phospholipids before addition of azido-Q derivative. This result suggests that at least one of the Q-binding sites can be easily masked or modified by phospholipids when Q is absent. It is possible that when photolysis of the azido-Q-treated enzyme complex is carried out in the presence of phospholipids, some of the photoactive nitrenes may covalently link to phospholipids close to the Q-binding site, resulting in less inactivation of ubiquinol-cytochrome c reductase. When phospholipids and azido-Q were premixed and then added to the depleted enzyme, only slightly less inactivation was observed after photolysis than with addition of azido-Q prior to phospholipids. This suggests that the binding of azido-Q to the Q-binding site is somewhat stronger than the binding of phospholipids to the Q-binding site. These results differ from those obtained by activity restoration using Q6 mixed first with phospholipids, then added to the depleted enzyme complex (28). In the latter case, only 30% of the activity was restored when Q6 and phospholipids were mixed before being added to the depleted enzyme, compared to the activity restored by addition of Q6 prior to the addition of phospholipids to the depleted enzyme. One explanation for this difference is that the photoactive nitrene labeled Q, once activated by photolysis, become covalently linked to the Q-binding protein and could not be displaced by the competing phospholipids. Thus the binding competition during photolysis favors azido-Q against phospholipids.

The phospholipid masking or competition at the Q-binding site can be overcome by excess (substrate level) QH2 as the Q-depleted but phospholipid-sufficient enzyme showed full activity when assayed with QH2.

Effect of Phospholipids on the Distribution of Azido-Q Binding among Subunits of Ubiquinol-Cytochrome c Reductase—The Q- and PL-depleted ubiquinol-cytochrome c reductase was reconstituted with phospholipids in the absence of azido-Q derivative and subsequently replenished with the azido-Q derivative at various time intervals. The amount of azido-Q derivative binding to the Q-binding sites decreased, as indicated by the decreased inactivation upon photolysis, as the time of phospholipids incubation with the depleted enzyme increased (see Fig. 4A). The decreased azido-Q derivative binding at the Q-binding sites, or the decreased inactivation upon photolysis, resulting from prolonged incubation of phospholipids with the depleted enzyme before addition of azido-Q, could be explained as either an increase in enzyme aggregation by phospholipids upon incubation or, more probably, a gradually increasing occupation (masking) of the Q-binding sites by phospholipids. This masking effect could also result from a protein conformational change upon incubation with phospholipids in the absence of Q, thus changing the affinity of Q-binding. The fact that the Q-depleted but PL-sufficient sample shows full ubiquinol-cytochrome c reductase activity when QH2 is used as substrate in the assay mixture suggests this deduction. As the amount of azido-Q added to the Q-depleted but PL-sufficient ubiquinol-cytochrome c reductase was increased, an increase in inactivation upon photolysis was observed. This also supports the observation that replenishing Q to the Q-depleted, PL-containing ubiquinol-cytochrome c reductase requires a higher concentration of Q than in the absence of phospholipids.

When the azido-Q derivative was added to the Q- and PL-depleted ubiquinol-cytochrome c reductase first, followed with phospholipids, the amount of inactivation after photolysis was nearly independent of the incubation time of phospho-

### TABLE II

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ubiquinol-cytochrome c reductase activity</th>
<th>% Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol c reduced/min. mg</td>
<td>%</td>
</tr>
<tr>
<td>1. dQCR + azido-Q</td>
<td>91.5</td>
<td>11.9</td>
</tr>
<tr>
<td>2. dQCR + azido-Q + PL</td>
<td>92.3</td>
<td>24.0</td>
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<tr>
<td>3. dQCR + PL + azido-Q</td>
<td>92.2</td>
<td>51.6</td>
</tr>
<tr>
<td>4. dQCR + (PL + azido-Q)</td>
<td>92.2</td>
<td>29.5</td>
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lipids in the reconstituted system (see Fig. 4B). This suggests that once the azido-Q derivative is bound to the Q-binding site, it cannot be removed or displaced by phospholipids, and, indirectly, suggests that phospholipids facilitate the Q-binding, as supported by the fact that stabilization of the ubiquinone radical is phospholipid-dependent (23).

Since two subunits in ubiquinol-cytochrome c reductase were heavily labeled with [3H]azido-Q derivative upon photolysis, it is of interest to see whether the decreased azido-Q uptake by ubiquinol-cytochrome c reductase in the presence of phospholipids results from a decrease of the azido-Q binding by a specific Q-binding subunit or by both Q-binding subunits. Fig. 5 compares the 3H-radioactivity distribution patterns, after photolysis and SDS-PAGE, among subunits of the complex from samples treated with various phospholipid incubation times. When phospholipids were preincubated with the depleted enzyme complex for 0, 1, and 5 h before the addition of [3H]azido-Q derivative, the inactivation after photolysis was 88, 50, and 10%, respectively. The 3H radioactivity incorporation at the M_r = 17,000 protein decreased as the inactivation after photolysis decreased. This result demonstrates that the M_r = 17,000 protein in ubiquinol-cytochrome c reductase is a Q-binding protein, with a Q-binding site easily masked by phospholipids in the absence of Q. By contrast, the radioactivity incorporation at the M_r = 37,000 or 30,000 protein was relatively constant with varying phospholipid incubation, indicating that this Q-binding site is less affected by phospholipids. Although the incorporation of [3H]azido-Q derivative at the M_r = 37,000 or 30,000 protein showed no correlation with the inactivation after photolysis, the constant incorporation of azido-Q into this protein makes it unlikely that this is a nonspecific uptake. It is not clear at present whether or not the covalently linked azido-Q molecule in the Q-binding site of the M_r = 37,000 protein is still functionally active, that is, whether or not displacement or rotation of the Q molecule in this Q-binding site is required during the redox reaction. Although two different Q-binding sites in ubiquinol-cytochrome c reductase have been documented through inhibitor studies (7), further clarification of the M_r = 37,000 protein as a Q-binding protein is needed. One possible approach to this problem is to identify the Q-binding site, particularly the amino acid residues involved at the Q-binding site. Investigation of this aspect is currently underway in our laboratory.

Effect of Inhibitors on the Binding of Azido-Q Derivative to Ubiquinol-Cytochrome c Reductase—The inhibitory effect of antimycin A and UHDBT on ubiquinol-cytochrome c reductase has been well established (29) both with the intact enzyme complex and the Q- and PL-depleted form. The reversal of the antimycin A (30) or UHDBT (31) inhibition by addition of Q derivatives has also been claimed or implied. It is, therefore, of interest to see whether the azido-Q derivative can reverse the inhibitory effect of antimycin A or, especially, that of UHDBT, which contains a quinone group and has been suggested to be a quinone analogue. Addition of the azido-Q derivative to the Q- and PL-depleted ubiquinol-cytochrome c reductase either before or after addition of antimycin A or UHDBT did not alter the inhibitory action of these compounds, compared to the enzyme samples to which no azido-Q derivative was added. The distribution of the 3H-
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Fig. 6. Restoration of the HQNO-inhibited activity by Q derivatives. 4.2-ml aliquots of intact succinate-cytochrome c reductase, 1.1 mg/ml (4.5 μM cytochrome b), in 50 mM phosphate buffer, pH 7.4, containing 0.4% sodium cholate and 3% ammonium sulfate were mixed with 21 μl of 95% ethanol (O,Δ,□) or 21 μl of 29.4 mM HQNO in 95% (□,●). Aliquots of 0.2 ml were withdrawn from the control and HQNO-treated samples and mixed with 5 μl of ethanolic solution containing the indicated concentrations of QnCpBr (O,●), 3-azido-2-methyl-5-methoxy-Q2, or duroquinol (□,●). After incubation in the dark for 20 min, the succinate-cytochrome c reductase activity was assayed.

Radioactivity among the subunits of the enzyme upon photolysis and SDS-PAGE was practically identical in the presence or absence of these inhibitors, indicating that the Q-binding sites in ubiquinol-cytochrome c reductase are probably not the inhibitor binding site(s) of antimycin A or UHDBT, even though UHDBT has often been referred to as a Q analogue.

The same results were obtained when the arylazido-Q derivative was the photoaffinity labeling reagent (6).

It is well established (29) that HQNO inhibits electron transfer in the cytochrome b-c1 segment of the mitochondrial electron transfer chain. It inhibits the oxidation of ubiquinol.

This inhibition can be reversed by addition of Q derivatives (30). Whether or not the binding site of HQNO is the same as the Q-binding site, however, is not established. The effect of Q on inhibition by HQNO must be studied in succinate-cytochrome c reductase, intact or reconstituted, since HQNO is not inhibitory in the ubiquinol-cytochrome c reductase assay. Fig. 6 shows the concentration-dependent reversal of the HQNO inhibition by Q derivatives. When QnCpBr, at a concentration of 3 mol/mol cytochrome b, was added to the HQNO-treated succinate-cytochrome c reductase, 87% of the original succinate-cytochrome c reductase activity was restored. When the azido-Q derivative, at 1.5 mol/mol cytochrome b, was added to the HQNO-treated sample, about 67% of the original succinate-cytochrome c reductase activity was restored. However, when duroquinol was used, no restoration of the HQNO-inhibited activity was observed. In the absence of inhibitor, a 10–20% activation of intact reductase by addition of QnCpBr was observed. This is due to a partial deficiency of Q in the enzyme preparation resulting from the isolation procedure. The amount of QnCpBr required for this activation was very low: less than 1 mol/mol cytochrome b.

No detectable increase of activity in intact reductase was observed in samples treated with azido-Q derivative, even when high concentrations of azido-Q derivative were used. This is probably due to the low electron transfer efficiency of the azido-Q derivative. When the succinate-cytochrome c reductase samples which were inhibited by HQNO and reactivated by the azido-Q derivative were subjected to photolysis, no decrease of the succinate-cytochrome c reductase activity or ubiquinol-cytochrome c reductase activity was observed. These results can be explained in several ways: 1) the HQNO is probably not bound to the Q-binding site and the reversal of the HQNO inhibition by Q analogues does not involve direct displacement of HQNO from the Q-binding site; 2) the binding of HQNO to the Q-binding site is Q-concentration-dependent and native Q binds better than does azido-Q derivative; or 3) the HQNO is bound to a Q-binding site which requires Q for activity but does not require physical movement of the Q molecule, so that even a covalently linked Q (after photolysis) is still functionally active. If the last were true, then HQNO may be bound to the Q-binding site of the M2 = 37,000 protein subunit. This is suggested by the observation that intact ubiquinol-cytochrome c reductase, when photolyzed with azido-Q, incorporated azido-Q into the M2 = 37,000 protein, but underwent no loss of activity. More experiments are needed before the mode of action of HQNO is clear.

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Additional references are found on p. 973.
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Synthesis of 2-methyl-4-(3-ethyl-5-pentyl-1,4-hexadienyl)benzoic acid (25 mg) was dissolved in 1 mL of acetic acid (95%) and the mixture was stirred until dissolved. The solution was then filtered and diluted with water, and the filtrate was evaporated to dryness. The residue was recrystallized from methanol, yielding 3.5 g of yellow needles of 2-methyl-4-(3-ethyl-5-pentyl-1,4-hexadienyl)benzoic acid (II, m.p., 152-153°C. EBOH (COOH): 1.34 (1H, d, J = 2 Hz). High-resolution mass spectrum, m/z 299.1959 (25).}

Synthesis of 2-methyl-4-(3-ethyl-5-pentyl-1,4-hexadienyl)-3-nitrobenzoic acid (VII) was carried out by the following method. The yield of this product was determined by comparing the absorbance of compound VII with that of authentic sample in a 1-cm cell. The purity and structure of compound VII were confirmed by NMR (CDCl3) and UV (EBOH) spectra.

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When the 

\[ \text{ubiquinone derivatives were illuminated with a long wavelength of light, the compounds were partially decomposed. The photo decomposition was found to follow first order kinetics. Fig. 2 shows the time course of the spectral change upon illumination of 3-azido-1-methyl-1,4-benzoquinone, 3-azido-1-methyl-5-methoxy-1,4-benzoquinone, and 2-azido-1-methyl-5-methoxy-1,4-benzoquinone. The spectra were taken at 10 min intervals. The decrease in absorption at 306 nm was accompanied with a spectral blue shift, proceeding with illumination time. A plot of logarithm of concentration versus time gives a straight line (insert of Fig. 2), proving that the photodecomposition reaction follows the first order kinetics. Analysis of the photodecomposed products of 3-azido-1-methyl-1,4-benzoquinone by thin layer chromatography showed several compounds, one of which has been identified as 3-azido-1-methyl-5-methoxy-1,4-benzoquinone. Although a similar photodecomposition phenomenon was also observed in other azido-Q derivatives, the photodecomposition rate varies greatly among the azido-Q derivatives.} 

\[ \text{Fig. 1. Absorption spectra of } 3\text{-azido-1,4-benzoquinone (--), 3-azido-1,4-benzoquinone (- -), 3-azido-1,4-benzoquinone (-- -).} \]

\[ \text{Fig. 2. Photodecomposition of 3-azido-1,4-benzoquinone.} \]

\[ \text{Fig. 3. Titration of the } \text{Q} \text{- and } \text{P} \text{-labelled succinate-cytochrome } \text{Q} \text{- reductase with 3-azido-1,4-benzoquinone.} \]
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Electron Transfer Activity of Synthesized Azido-Ubiquinone Derivatives—
The photosynthetic activity of azido-Q derivatives given in Table 1. refers to the
ability of azido-Q derivatives in restoring electron transfer activity to the Q-
and PS-depleted succinate-ubiquinone reductase, measured after also
replenishing the Q-treated enzyme with phosphoglycerate. Fig. 1 shows the effect of various concentrations of azido-Q derivatives on restoration of activity to the
depleted enzyme. Although the concentrations required for the maximal
restoration of activity were similar, the observed activity restored by azido-Q
derivatives varied significantly (Table 1). The apparent K values, as calculated from Fig. 1, for azido-Q derivatives and QOC10Br in activity restoration are quite comparable. Therefore, the low
restoration activity of azido-Q derivatives by azido-Q derivatives to the
depleted enzyme, relative to the electron transfer efficiency of azido-Q
derivatives, is not due to the incomplete binding of azido-Q derivatives to the
Q-binding site. This deduction is further confirmed by the fact that
photolysis of the azido-Q derivative-treated enzyme complex, especially those
treated with 3-azido-2-methyl-5-thoxy-02 or 3-azido-Q2 and 5-azido-02,
not only abolished the restored activity but also caused the photolyzed preparation to become
locally inactive toward activity restoration by further addition of undamaged Q
enzymes. The restoration of activity observed by azido-Q derivatives was
due probably to the effect of azido group on the ubiquinone ring rather than
strictly to the azido group itself, as in QOC10Br, did not decrease electron transfer activity relative to QOC10Br. It should be emphasized that the inactivating effect of 3-azido-2-methyl-
5-thoxy-Q2 after photolysis is not due to the photolyzed product of
3-azido-2-methyl-Q2, but to the formation of a covalent linkage
between the 0 derivative and protein. When 3-azido-2-methyl-5-thoxy-Q2 was
photolyzed in ethanol before being added to the depleted enzyme, no inhibition
was observed and the residual activity was not sensitive to photolysis. Upon
addition of QOC10Br to the sample treated with photolyzed 3-azido-2-methyl-5-
thoxy-Q2, more than 75% of maximal activity was restored. This result
indicates that photolyzed 3-azido-2-methyl-5-thoxy-Q2 forms no covalent
linkages with the protein and is easily replaced by QOC10Br.

Since the photoactivated nitrene is known to be most reactive with tyrosyl
and histidyl residues (3), it seems likely that a tyrosyl or histidyl group may
be present in the Q-binding site. The observed difference in the Q-binding
behaviour between 3-azido-2-methyl-L-mercapto-Q2 or 3-azido-Q2 and 5-azido-Q2
further suggests that the Q-binding site is very specific with respect to the
substrates on the ubiquinone ring. The higher restoration of activity by
QOC10Br to the photolyzed 3-azido-Q2-treated than to 3-azido-Q2-treated
reductase may be due to the fact that the photoactivated nitrene is some
distance away from the tyrosyl or histidyl group and thus may react non-
covalently. Direct evidence for this deduction must await identification of the
amino acids residues covalently linked to azido-Q derivatives after photolysis.

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