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Identification of Cytochrome *b* and a Molecular Weight 12K Protein as the Ubiquinone-Binding Proteins in the Cytochrome b–c_1 Complex of a Photosynthetic Bacterium *Rhodobacter sphaeroides* R-26†

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ABSTRACT: An azidoubiquinone derivative, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone, was used to study the ubiquinone–protein interaction and to identify ubiquinone-binding proteins in photosynthetic bacterial cytochrome b–c_1 complex. When isolated *Rhodobacter sphaeroides* cytochrome b–c_1 complex is incubated with a 50-fold molar excess of the azidoubiquinone derivative in the dark, no loss of activity is observed. Photolysis of this azidoubiquinone-treated sample for 5 min at 0 °C causes a 50% decrease of ubiquinol–cytochrome *c* reductase activity. When the photolyzed [^{3}H]azidoubiquinone-treated *R. sphaeroides* cytochrome b–c_1 complex is subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, after removal of non-protein-linked azidoubiquinone by organic solvent extraction, followed by analysis of the radioactivity distribution among subunits of the complex, cytochrome *b* (M_r 43K) and a M_r 12K protein are heavily labeled, suggesting that these two proteins are the ubiquinone-binding site in this complex. The amount of radioactivity in both proteins is increased when the complex is subjected to phospholipase A_2 digestion prior to photolysis with the azidoubiquinone derivative. Pretreatment of *R. sphaeroides* cytochrome b–c_1 complex with 2-heptyl-4-hydroxyquinoline *N*-oxide has little effect on the distribution of radioactivity among subunits of the cytochrome b–c_1 complex. Pretreatment of the cytochrome b–c_1 complex with 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole, myxothiazol, or antimycin increases slightly the amount of radioactivity in cytochrome *b*. These results suggest that the active site of these inhibitors is not the same as the Q-binding site.

The cytochrome b–c_1 complex (formerly known as the cytochrome b–c_2 complex) of the photosynthetic bacterium *Rhodobactor sphaeroides*, which catalyzes electron transfer from ubiquinol to cytochrome c_2, has recently been purified and characterized in several laboratories (Gabellini et al., 1982; Yu & Yu, 1982; Yu et al., 1984; Takamiya et al., 1982; Ljungdahl et al., 1986). The essential redox components of this complex are identical with those of the mitochondrial cytochrome b–c_1 complex (Cramer & Crofts, 1982; Hauska et al., 1983). They are cytochromes *b* and c_1, iron–sulfur protein, and ubiquinone. Purified *R. sphaeroides* cytochrome

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b–c_1 complex is composed of four different molecular weight subunits with unit stoichiometry among them. The molecular weights are 43K,[1] 30K, 24K, and 12K. The three larger molecular weight proteins are identified as cytochromes b and c_1 and iron–sulfur protein, respectively. The less complex protein subunit structure of *R. sphaeroides* cytochrome b–c_1 complex compared to that of the mitochondrial complex makes it useful for resolution and reconstitution studies of this segment of electron transfer.

Cytochromes b (Yu & Yu, 1984; Iba et al., 1985; Iba & Takamiya, 1986) and c_1 (Yu et al., 1986) have been isolated from *R. sphaeroides* cytochrome b–c_1 complex and purified to homogeneity. Purified cytochrome c_1 has spectral properties and electron-transfer activity (to cytochrome c_2 or c) (Yu et al., 1986a) identical with those of cytochrome c_1 in isolated cytochrome b–c_1 complex or chromatophores, indicating that isolated cytochrome c_1 is in a functionally active form. Purified cytochrome b (Yu & Yu, 1984) contains 1 mol of heme/mol of protein and has absorption characteristics identical with those of cytochrome b in isolated cytochrome b–c_1 complex. However, EPR[2] characterization of this isolated cytochrome b preparation (Salerno et al., 1986) showed only the cytochrome b-560 signal (g = 3.40). The cytochrome b-565 signal (g = 3.75), which is present in the isolated cytochrome b–c_1 complex, was not observed, suggesting that either the heme of cytochrome b-565 has been lost or the protein environment of cytochrome b-565 has been altered during the isolation process. Addition of isolated cytochrome b to a cytochrome b deficient cytochrome b–c_1 complex results in a partial reactivation of ubiquinol–cytochrome c reductase activity (Yu & Yu, 1984), suggesting that the structure of isolated cytochrome b is very close to that of the native form, and some components in the cytochrome b deficient cytochrome b–c_1 complex are able to reconstitute some of the cytochrome b-565 at the expense of cytochrome b-560. The true nature of cytochrome b-565 will not be revealed until a fully active cytochrome b preparation is obtained. It should be mentioned that, so far, none of the isolated cytochrome b preparations obtained from the cytochrome b–c_1 complex of various sources has a heme to protein ratio higher than 1 (Tsai & Palmer, 1982), even though the idea that 1 mol of cytochrome b protein contains 2 mol of heme has been stressed (Widger et al., 1984) on the basis of the amino acid sequence of cytochrome b deduced from gene sequences of many sources.

Isolated cytochrome b–c_1 complex contains 5 nmol of Q_{10}/mg of protein (Yu et al., 1984). A stable, antimycin-sensitive ubisemiquinone radical is detected in the partially reduced *R. sphaeroides* cytochrome b–c_1 complex whereas an antimycin-insensitive ubisemiquinone radical is detected in the oxidized sample. Detection of a ubisemiquinone radical in this complex and in intact chromatophores (Robertson et al., 1984) suggests the existence of specific ubiquinone-binding proteins (sites). Therefore, identification of the Q-binding proteins in this complex is essential for elucidation of the reaction mechanism of this Q-mediated electron-transfer reaction. By use of photoaffinity-labeled Q derivatives, Q-binding proteins in the cytochrome b–c_1 complex of bovine heart (Yu et al., 1985) and of yeast mitochondria (Yu et al., 1986a) and the cytochrome b_6–f complex of chloroplast (Oettmeier et al., 1982) have been identified. Two proteins, cytochrome b and a small molecular weight protein, are responsible for the Q binding in bovine heart and yeast mitochondrial cytochrome b–c_1 complexes. Since *R. sphaeroides* cytochrome b–c_1 complex is analogous to mitochondrial cytochrome b–c_1 complexes both functionally and structurally (Hauska et al., 1983), it is of interest to known whether or not the Q-binding proteins involved in this complex are similar to those in its mitochondrial counterpart.

Herein, we report experimental conditions for the identification of the Q-binding proteins in the *R. sphaeroides* cytochrome b–c_1 complex using an azido-Q derivative, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]octyl)-1,4-benzoquinone, and the interaction between this azido-Q derivative and the cytochrome b–c_1 complex under various conditions.

Materials and Methods

Materials. Horse cytochrome c, type III, bee venom phospholipase A_2, antimycin, and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were from Sigma. Myxothiazol was from Boehringer Mannhein, Mannhein, West Germany, *N*,*N*′-diallyltartardiamide (DATA) from Kodak, and Insta-gel liquid scintillation cocktail from Packard Instrument Co. 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was synthesized according to Friedman et al. (1973) with minor modifications. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (Q_0), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2) and its reduced form (Q_2H_2), 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone (azido-Q), and 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]-octyl)-1,4-benzoquinone ([^{3}H]azido-Q) were synthesized according to methods previously reported (Yu et al., 1985). The strain of *R. sphaeroides* R-26 was a gift from Drs. G. Feher and M. Okamura of the Physics Department, UCSD. The bacteria were grown and harvested according to Feher and Okamura (1978). The cytochrome b–c_1 complex of *R. sphaeroides* was prepared and assayed essentially according to Yu et al. (1984) with slight modifications. Cells were broken with a French pressure cell (20 mL/min, 1000 psi) instead of sonification, and the treatment with poly(ethylene glycol) was omitted.

Methods. All enzymatic assays were performed with a Cary spectrophotometer, Model 14 or 219, at 23 °C. The gel column used for SDS–PAGE was made of 10% acrylamide and a cleavable cross-linker, DATA. The conditions for electrophoresis and photolysis, determination of azido-Q uptake by the protein, and organic extraction of non-protein-bound azido-Q adducts from the photolyzed, azido-Q-treated cytochrome b–c_1 complex were performed as previously reported (Yu et al., 1985, 1986b). Phospholipase A_2 treated cytochrome b–c_1 complex was prepared according to Yu et al. (1984). To 0.2 mL of *R. sphaeroides* cytochrome b–c_1 complex, 1 mg/mL, in 50 mM phosphate buffer, pH 7.0, containing 10% glycerol was added 5 μL of bee venom phospholipase A_2 (2 mg/mL in 50 mM phosphate, pH 7.4, containing 10% glycerol). The solution was incubated at room temperature for 45 min before the addition of 50 μL of 0.2 M EDTA, pH 7.0, to terminate the phospholipase A_2 reaction. Radioactivity was determined with a Beckman scintillation counter, Model SL-3150T.

[1] The apparent molecular weight of cytochrome b was reported to be 48K (Yu et al., 1984) in isolated *R. sphaeroides* cytochrome b–c_1 complex but 43 K in the isolated cytochrome b (Yu & Yu, 1984). The true molecular weight of cytochrome b will not be obtained until the amino acid sequence is worked out.

[2] Abbreviations: azido-Q, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone; DATA, *N*,*N*′-diallyltartardiamide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PAGE, polyacrylamide gel electrophoresis; PL, phospholipid; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; SDS, sodium dodecyl sulfate; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

Results and Discussion

Effect of Azido-Q Concentration on Ubiquinol–Cytochrome c Reductase Activity of R. sphaeroides Cytochrome b–c_1 Complex after Photolysis. It has been shown, in bovine heart (Yu et al., 1985) and yeast (Yu et al., 1986b) mitochondrial cytochrome b–c_1 complexes, that the interaction of azido-Q derivatives with the cytochrome b–c_1 complex requires prior removal of endogenous Q and phospholipid from the complex. The reason for this is twofold; the binding affinity of azido-Q derivatives to the Q-binding proteins (sites) is weaker than that of endogenous Q_{10}, and the Q-binding site(s) in these complexes are masked by phospholipid when Q is absent from the complex. Since a method for the reversible removal of the Q and phospholipds from the *R. sphaeroides* cytochrome b–c_1 complex has not yet been developed, the cytochrome b–c_1 complex, as isolated, is used in this study. Specific interaction between added azido-Q and this isolated cytochrome b–c^1 complex is expected only when the binding affinities of Q_{10} and phospholipid in the complex are weakened, thus facilitating azido-Q competition with endogenous Q_{10} for the Q-binding site. Furthermore, the amount of azido-Q derivative required for this interaction will be higher than that needed with the Q- and PL-depleted preparation. Therefore, developing a condition that enables the cytochrome b–c_1 complex to effectively interact with this azido-Q derivative is a prerequisite for its use in identifying the Q-binding protein(s) in this complex. In the search for appropriate interaction conditions the cytochrome b–c_1 complex, at various protein concentrations, was treated with different types of detergents, such as cholate, deoxycholate, octyl glucoside, and Tween-80, singly or in combination, with or without salt, before incubation with the azido-Q derivative and photolysis. The azido-Q to protein ratio was kept constant (50 mol of azido-Q/mol of cytochrome c_1). Interaction with the azido-Q derivative was most effective (i.e., showed the greatest degree of inactivation after pyrolysis) when the cytochrome b–c_1 complex concentration was 0.3–0.5 mg/mL (3–4 μM cytochrome c_1), in the presence of 0.5% sodium cholate and 50 mM ammonium sulfate. Apparently the presence of both cholate and ammonium sulfate lowers the binding affinity of Q_{10} and phospholipid, thus facilitating the binding of the azido-Q derivative. Therefore, this condition was used throughout this study.

Figure 1 shows the effect of azido-Q concentration on the inactivation of the ubiquinol–cytochrome c reductase of *R. Sphaeroides* cytochrome b–c_1 complex after photolysis. When treated as described above with varying concentrations of azido-Q derivative, followed by photolysis, the activity in these samples decreases as the concentration of azido-Q derivative in the sample increases. Maximum inhibition (50%) is observed when 50 mol of azido-Q/mol of protein is used, on the basis of cytochrome c_1. Since ubiquinol–cytochrome c reductase activity is assayed in the presence of excess Q_2H_2 (30 μM), the extent of inactivation of this reductase in the azido-Q-treated cytochrome b–c_1 complex, after photolysis, is a measure of the degree of the Q-binding sites covalently linked to azido-Q.

When intact bovine heart mitochondrial cytochrome b–c_1 complex was photolyzed with azido-Q under conditions identical with those used with *R. sphaeroides* cytochrome b–c_1 complex, less than 3% inactivation was observed. The higher inactivation observed with *R. sphaeroides* cytochrome b–c_1 complex suggests that the binding environment of Q and PL in the bacterial complex is different from that in its mitochondrial counterpart or that the isolated bacterial cytochrome b–c_1 complex is somewhat Q deficient, or both. In fact, if we assume that the *R. sphaeroides* cytochrome b–c_1 complex behaves like its mitochondrial counterpart in which the maximal ubiquinol–cytochrome c reductase activity requires unit stoichiometry between cytochrome c_1 and Q (Yu et al., 1978), then the isolated bacterial complex is about 37% deficient in Q, because it contains, in nmol per milligram of protein, 5 and 8.3 of Q and cytochrome c_1, respectively (Yu et al., 1984). If we assume that only the Q-depleted molecules are able to bind the azido-Q and subsequently inactivate enzymatic activity upon photolysis, one would expect to see a maximum inhibition of 37%. The observation of 50% inactivation suggests that, in addition to occupying a vacant Q site, the azido-Q has also partially replaced endogenous Q_{10} in the complex.

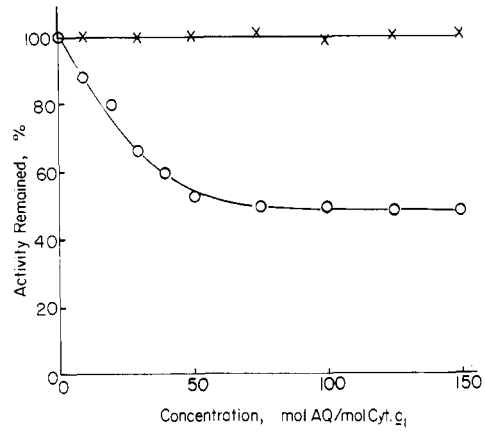

FIGURE 1: Effect of azido-Q concentration on ubiquinol–cytochrome c reductase activity after photolysis. Aliquots (0.2 mL) of *R. sphaeroides* cytochrome b–c_1 complex, 3.4 μM cytochrome c_1, in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 50 mM ammonium sulfate were mixed with 10 μL of an alcoholic solution of azido-Q derivative (concentrations indicated), in the dark. After incuation at 0 °C for 10 min, the samples were photolyzed for 5 min at 0 °C. Ubiquinol–cytochrome c reductase activity was assayed before (×) and after (○) photolysis. One hundred percent activity is the ubiquinol–cytochrome c reductase of the cytochrome b–c_1 complex without treatment with azido-Q and without photolysis.

If azido-Q can occupy 50% of the Q-binding sites in the complex, one would expect to see a decrease in the activity of the azido-Q-treated sample *before* photolysis because this derivative is reported to have a lower electron-transfer efficiency than that of other Q homologues (25% efficiency as compared to that of Q_2) (Yu et al., 1985). The failure to observe a decrease in activity prior to photolysis, even with samples having azido-Q to protein ratios as high as 200:1, is due to the fact that in the assay mixture the concentration of the substrate, Q_2H_2, is still several orders of magnitude higher than that of azido-Q. Thus Q_2H_2 can easily displace azido-Q from the binding sites, and the inferior electron-transfer activity of azido-Q is not expressed. After photolysis, the covalently linked azido-Q cannot be displaced by Q_2H_2 and inhibition of activity occurs.

Correlation between Inactivation of Ubiquinol–Cytochrome c Reductase and Azido-Q Uptake by Protein upon Photolysis. Figure 2 shows the effect of illumination time on inactivation of ubiquinol–cytochrome c reductase activity and azido-Q uptake by the cytochrome b–c_1 complex protein. When the azido-Q-treated cytochrome b–c_1 complex is photolyzed at 0 °C for various periods of time, biphasic inactivation kinetics are observed. When the illumination time is less than 5 min, activity decreases rapidly and proportionally to time. However, when the illumination time surpasses 5 min, a slower rate of inactivation is observed. The slower phase is similar to the photoinactivation of cytochrome b–c_1 complex in the absence

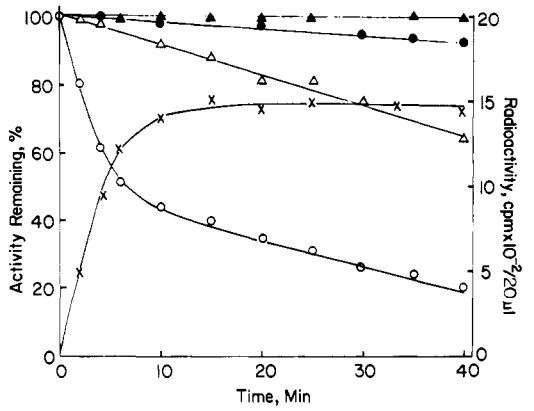

FIGURE 2: Photolysis time dependence of azido-Q uptake and inactivation of ubiquinol–cytochrome *c* reductase. Aliquots (0.4 mL) of *R. sphaeroides* cytochrome b–c_1 complex, 3.5 μM cytochrome c_1, in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 50 mM ammonium sulfate were mixed with 9 μL of [^{3}H]azido-Q derivative, 8.6 mM in 95% ethanol with a specific radioactivity of 31 000 cpm/nmol, in the dark. After incubating for 10 min at 0 °C in the dark, samples treated with [^{3}H]azido-Q (○, ●) and controls (alcohol only) (△, ▲) were divided into two equal portions: one was placed in the test tube and wrapped with the aluminum foil (●, ▲), and the others were placed in a quartz cuvette with a 0.2-cm light path (○, △). All the samples were put in the same photolysis chamber and illuminated with a long-wavelength UV light. The temperature in the photolysis chamber was maintained at 0 °C. At indicated time intervals, 10-μL aliquots were withdrawn and assayed for ubiquinol–cytochrome *c* reductase activity. For the time-course study of [^{3}H]azido-Q incorporation into protein (×), 20-μL aliquots were spotted on Whatman No. 3 paper in the dark and kept in the dark until all samples were collected. The paper was developed with a chloroform methanol (2:1) mixture to separate the non-protein-linked azido-Q adducts from the protein-linked azido-Q. Then the protein spots (original spots) were cut out, and radioactivity was determined.

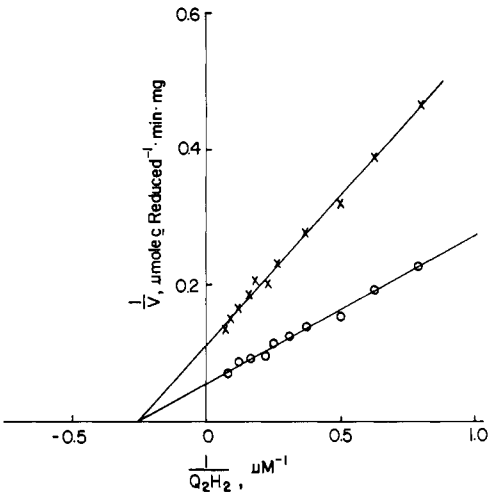

FIGURE 3: Lineweaver–Burk plot of the cytochrome b–c_1 complex photolyzed with or without azido-Q derivative. To 0.2-mL aliquots of the cytochrome b–c_1 complex, 4 μM cytochrome c_1, in 50 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 50 mM ammonium sulfate was added 10 μL of solution with (×) or without (○) azido-Q derivative (10 mM in 95% ethanol). After incubation at 0 °C for 10 min, azido-Q-treated and control samples were subjected to photolysis, side by side, for 5 min at 0 °C. Ubiquinol–cytochrome *c* reductase activity was assayed in the presence of various concentrations of Q_2H_2.

of azido-Q. It should be noted that this cytochrome b–c_1 complex has a higher photoinactivation rate than the cytochrome b–c_1 complexes from bovine heart (Yu et al., 1985) and yeast (Yu et al., 1986b); about 20% of the activity is lost after illuminating for 20 min. The higher photoinactivation rate is probably due to the lower protein concentration used; the presence or absence of cholate and ammonium sulfate has no effect. Perhaps the photolabile nature of *R. sphaeroides* cytochrome b–c_1 is an intrinsic property of this enzyme complex which has a much simpler subunit structure, devoid of core proteins, than do the mitochondrial complexes.

In order to establish that the inactivation observed results from covalent linkage of azido-Q to protein in the complex, azido-Q uptake and extent of inactivation were determined for different periods of photolysis. When the illumination time is less than 5 min, the incorporation of azido-Q into the cytochrome b–c_1 complex is correlated with activity loss. However, when illumination time is more than 5 min, no further azido-Q uptake is observed, while photoinactivation continues at a slower rate, indicating that the latter phase of inactivation results from photodenaturation of protein, not from covalent linkage of azido-Q to Q-binding protein. Maximum incorporation of azido-Q is about 1 mol per mole of cytochrome c_1. It is not clear whether all the azido-Q taken up is covalently linked to protein. At least part of the azido-Q is linked to a tightly bound to protein phospholipid molecule that is not dissociated from the protein during chromatography. Whether or not modification of such a tightly bound phospholipid, at the Q-binding site, by azido-Q also causes inactivation remains to be investigated.

Effect of Azido-Q Binding on the Kinetic Parameters of the Cytochrome b–c_1 Complex. Since, under the conditions used, the maximal inactivation of ubiquinol–cytochrome *c* reductase is about 50%, it is important to know if 50% inactivation results covalent linkage of azido-Q to 50% of the enzyme molecules of the cytochrome b–c_1 complex or results from the binding of azido-Q to part to the Q proteins in the complex, if more than one Q protein exists in the complex, thus giving partial inactivation. One way to differentiate between these two possibilities is to determine the kinetic parameters of the photolyzed, azido-Q-treated cytochrome b–c_1 complex. Figure 3 shows the Lineweaver–Burk plot of photolyzed, azido-Q-treated- and -untreated cytochrome b–c_1 complexes. The K_m for ubiquinol in the photolyzed, azido-Q-treated cytochrome b–c_1 complex is the same as that of the photolyzed cytochrome b–c_1 complex, whereas the V_{max} is only 50% of that of the control. This indicates that the 50% inactivation is a result of covalent linkage of azido-Q to all Q-binding sites in 50% of the enzyme molecules; Q-binding sites in the remaining 50% of the enzyme are not affected by the treatment with azido-Q.

Radioactivity Distribution of Azido-Q among Subunits of the Cytochrome b–c_1 Complex under Various Conditions. As described in the previous section, the 50% inactivation of photolyzed, azido-Q-treated enzyme results from total inactivation of 50% of the enzyme population not from 50% inactivation of total enzyme. Therefore, the distribution of azido-Q among the subunits of *R. sphaeroides* cytochrome b–c_1 complex can be used to identify the subunits responsible for *q* binding. Figure 4, panel A, shows the radioactivity distribution among subunits of the cytochrome b–c_1 complex. The complex was extracted with organic solvent to remove most of the non-protein-bound azido-Q, such as free azido-Q or detergent–azido-Q or lipid–azido-Q adducts, before being subjected to SDS–PAGE. Radioactivity was found predominately in proteins with molecular weights of 43K and 12K, suggesting that these two proteins are responsible for the specific Q binding in this segment of the electron-transfer chain. The M_r 43K protein is cytochrome *b* protein (Yu et al., 1984; Yu & Yu, 1984; Gabellini & Hauska, 1983); the M_r 12K protein possesses no detectable prosthetic group. The amount of radioactivity in the M_r 12K protein is about 60% of that observed in the M_r 43*K* protein.

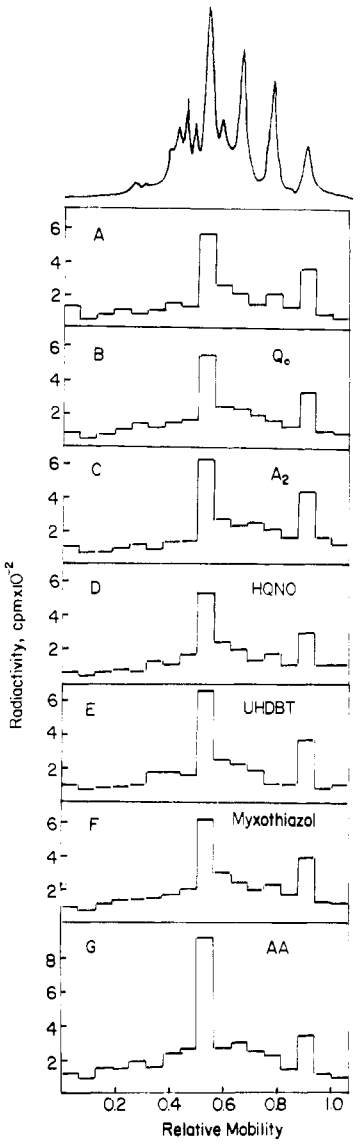

FIGURE 4: Ratioactivity distribution among subunits of the cytochrome b–c_1 complex under various conditions. To 0.2-mL aliquots of the cytochrome b–c_1 complex, 3.4 μM cytochrome c_1, in 50 mM phosphate buffer, pH 7.0, containing 0.5% sodium cholate and 50 mM ammonium sulfate was added 5 μL of 95% alcoholic solution containing (A) none, (B) Q_0 (10 mM), (C) none (the phospholipase A_2 treated sample), (D) HQNO (10 mM), (E) UHDBT (2.1 mM), (F) myxothiazol (2 mM), or (G) antimycin A (1 mM). After incubation for 10 min at 0 °C, 4 μL of [^{3}H]azido-Q (8.6 mM in 95% ethanol, 20800 cpm/nmol) was added to each sample. These were then subjected to photolysis at 0 °C for 5 min after incubation at 0 °C for 10 min. The photolyzed samples were dialyzed against water, overnight, and extracted with organic solvent to remove the non-protein-bound Q. Methanol present in the aqueous phase was removed with N_2, and the samples were lyophilized. A 120-μL aliquot of 20 mM K_2HPO_4 containing 1% SDS was added to each lyophilized sample. The protein concentration of the samples were determined and equalized before addition of β-mercaptoethanol to 1%. After incubation at 37 °C for 2 h they were subjected to SDS–PAGE with the SDS–DATA gel system. Fifty microliters of sample was loaded on each gel column. After staining and destaining processes, the gels were sliced according to the protein bands. The portions of the gel not showing protein stain were also sliced to the same size as those of protein bands. The gel slices obtained from one gel column were dissolved in 2% periodic acid at room temperature for 1 h before the radioactivity was measured.

In order to be sure that the labeling in the M_r 43K and 12K proteins is the result of a specific interaction with azido-Q derivative and not a nonspecific binding of the 1,4-benzoquinone moiety of the azido-Q molecule, the cytochrome b–c_1 complex prior to photolysis with the [^{3}H]azido-Q derivative was treated with a Q homologue, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), which contains no alkyl side chain on the 6-position of the 1,4-benzoquinone molecule and shows no electron-transfer activity in this region of the chain. If the observed radioactive labeling is a result of nonspecific interactions, one would expect to see a decrease in the radioactivity on the M_r 43K and/or 12K proteins. As shown in Figure 4B, addition of Q_0 to the system has no effect on the radioactivity distribution in the subunits, suggesting that the labeling of the M_r 43K and 12K proteins is not due to nonspecific interactions.

Table I: Effect of Q_2 on Azido-Q Inactivation of and Binding to *R. sphaeroides* Cytochrome b–c_1 Complex after Photolysis[a]

concn of Q_2 (mol of Q_2/mol of azido-Q)	inactivation after photolysis (%)[b]	[^{3}H]azido-Q uptake (mol/mol of protein)
0	55	0.98
0.5	30	0.61
1.0	10	0.28
1.5	8	0.24

[a] To 0.2-mL aliquots of cytochrome b–c_1 complex, 3 μM cytochrome c_1, in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate and 50 mM ammonium sulfate was added 4 μL of [^{3}H]azido-Q (9 mM in ethanol with a specific radioactivity of 16000 cpm/nmol) in the dark. After incubation for 10 min at 0 °C in the dark, 6-μL aliquots of ethanolic solution containing indicated concentrations of Q_2 were added. The mixture continued to incubate for 10 min in the dark before being subjected to photolysis. [b] Percent inactivation was calculated on the basis of the same sample without being subjected to photolysis. The specific activity of the cytochrome b–c_1 complex was 15 μmol of cytochrome c reduced/(min·mg), at 23 °C.

In contrast to Q_0, Q_2 is the substrate for the cytochrome b–c_1 complex, its binding to the Q-binding site is expected. Table I shows the effect of Q_2 on the azido-Q binding to the *R. sphaeroides* cytochrome b–c_1 complex. Addition of Q_2 to the azido-Q-treated complex in the dark results in a decrease in the extent of azido-Q inactivation after photolysis. The fact that an almost complete prevention of the azido-Q inactivation is observed when an equal molar ratio of Q_2 to azido-Q is added, and the fact that the extent of prevention of the azido-Q inactivation by Q_2 is independent of the addition sequence of azido-Q and Q_2, indicates that the binding affinity of Q_2 to the *R. sphaeroides* cytochrome b–c_1 complex is slightly stronger than that of azido-Q. As expected, the amount of [^{3}H]azido-Q incorporation into the complex, after photolysis, is decreased as the amount of Q_2 in the system increased (Table I). The amount of radioactivity on both the M_r 43K and M_r 12K proteins is drastically decreased in the system containing Q_2 as compared to that with no Q_2 added.

It has been shown that there is a close relationship between Q and phospholipid in the mitochondrial cytochrome b–c_1 complex (Yu & Yu, 1980). Since the *R. sphaeroides* cytochrome b–c_1 complex used contains PL, it is important to know whether or not phospholipid affects the azido-Q labeling pattern. When *R. sphaeroides* cytochrome b–c_1 complex is subjected to phospholipase A_2 treatment, about 50% of ubiquinol–cytochrome c reductase activity is abolished; activity is partially restored by addition of phospholipid prepared from the *R.* sphaeroides cytochrome b–c_1 complex or by a mixture of asolectin and phosphatidylglycerol (Yu et al., 1984). This indicates that some of the phospholipid in the complex is sensitive to phospholipase A_2 digestion. Photolysis of the phospholipase A_2 digested cytochrome b–c_1 complex with azido-Q derivative results in a decrease of the remaining activity to 32%. Figure 4C shows the radioactivity distribution among subunits of phospholipase A_2 treated cytochrome b–c_1 complex. An increase in labeling of both M_r 43K and M_r 12K is observed, compared to the sample not digested with phos-

pholipase A_2. The increase is greater for M_r 12K protein than for M_r 43K. These results suggest that the binding sites of Q on both the M_r 43K and M_r 12K proteins are somewhat masked by phospholipid and the latter is more closely related to phospholipid. Comparable results were obtained with the mitochondrial enzyme, in which incorporation of azido-Q on the smaller molecular weight (M_r 17K) Q-binding protein is phospholipid dependent and incorporation is proportional to inactivation after photolysis (Yu et al., 1985).

Effect of Electron-Transfer Inhibitors on the Azido-Q Labeling of R. sphaeroides Cytochrome b–c_1 Complex. Recently, many laboratories have employed specific inhibitors with chemical structures similar to that of Q to probe the electron-transfer mechanism of Q in the cytochrome b–c_1 region (VonJagow & Link, 1986). It is of interest to see if the action site of these inhibitors is the same as the Q-binding site in *R. sphaeroides* cytochrome b–c_1 complex. Figure 4 shows the effect of electron-transfer inhibitors on the radioactivity distribution patterns. The complex was treated with either HQNO, UHDBT, myxothiazol, or antimycin before being photolyzed with [^{3}H]azido-Q derivative. The enzymatic activity of the inhibitor-treated samples was assayed before and after the addition of azido-Q in the dark to be sure that inhibition has been properly exerted by these inhibitors. If the binding site of these inhibitors is the same as the Q-binding site, one would expect to see a decrease in the radioactivity in the M_r 43K and/or 12K proteins. HQNO shows little effect; UHDBT, myxothiazol, and antimycin, in contrast, cause an increase in radioactivity in the M_r 43K protein with little effect on the M_r 12K protein, indicating that the site of action of these inhibitors is not the same as that of Q binding. It should be mentioned that the lack of inhibitor effect on the radioactivity labeling pattern of *R. sphaeroides* cytochrome b–c_1 complex is not due to the lack of equilibrium between the azido-Q and inhibitor under experimental conditions, because results similar to those showing in Figure 4 were obtained when the incubation temperature was increased (up to 25 °C) and the incubation time was lengthened (up to 20 min).

The fact that HQNO has little effect on azido-Q incorporation into the M_r 43K and 12K proteins may be explained by assuming the action site of HQNO is the same as that of Q and that the inhibition of HQNO is reversed by added azido-Q in the dark. Since, like the mitochondrial system, the inhibitory effect of HQNO in the *R. sphaeroides* cytochrome b–c_1 complex is easily reversed by the addition of Q, inhibition cannot be monitored by assaying ubiquinol–cytochrome c reductase because Q_2H_2 is used as substrate in the assay mixture. However, inhibition of *R. sphaeroides* cytochrome b–c_1 complex by HQNO can be estimated by assaying the reconstituted succinate–cytochrome c reductase formed from HQNO-treated *R. sphaeroides* cytochrome b–c_1 complex and mitochondrial succinate–Q reductase.

The reason for the increase in radioactivity on the M_r 43K protein after pretreatment of the complex with either UHDBT, myxothiozol, or antimycin is not known. Antimycin has been shown to bind a small molecular weight protein (<8K) in mitochondrial cytochrome b–c_1 complex (Ho et al., 1985), and the binding of antimycin caused a profound change in the redox behavior of cytochrome b. The antimycin binding site in *R. sphaeroides* cytochrome b–c_1 complex has been located on the M_r 12K subunit by using photoaffinity-labeled antimycin analogue (Wilson et al., 1985). It is possible that a perturbation of the protein structure of cytochrome b caused by the binding of antimycin to its binding site increased the vulnerability of cytochrome b to be labeled by photoactivated azido-Q.

It is rather difficult to visualize the increase in the azido-Q uptake by cytochrome b in the presence of UHDBT or myxothiazol. UHDBT has been reported to affect both Rieske's iron–sulfur protein and the redox behavior of cytochrome b (Bowyer, 1981; Trumpower, 1981). Myxothiazol has been speculated to bind cytochrome b and displace the quinone from its binding site (VonJagow et al., 1984). Recently, it has been shown (Yang et al., 1986) that inhibition of ubiquinol–cytochrome c reductase by UHDBT results from "perturbation of the phospholipid environment of the enzyme complex", because when mitochondrial cytochrome b–c_1 complex was photolyzed with a [^{3}H]azido-UHDBT derivative, radioactivity was located on phospholipid molecules rather than on protein subunits. Whether this perturbation of phospholipid environment by UHDBT or myxothiazol is responsible for the slight increase of azido-Q uptake by the M_r 43K protein in the *R. sphaeroides* cytochrome b–c_1 complex remains to be elucidated.

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Membrane Reconstitution of High-Affinity α_2 Adrenergic Agonist Binding with Guanine Nucleotide Regulatory Proteins†

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ABSTRACT: Regulation of adenylate cyclase by α_2 adrenergic receptors requires the inhibitory guanine nucleotide binding protein N_i. A role for this protein has also been suggested in the high-affinity binding of agonists to the α_2 receptor. We recently reported that alkaline treatment can selectively inactivate α_2 agonist binding and N_i in human platelet plasma membranes [Kim, M. H., & Neubig, R. R. (1985) *FEBS Lett. 192*, 321-325]. Binding of the full α_2 agonists epinephrine and 5-bromo-6-[*N*-(4,5-dihydroimidazol-2-yl)amino]quinoxaline (UK 14304) to these membranes was determined by competition and direct radioligand binding, respectively. The high-affinity GTP-sensitive binding of the agonists is lost after alkaline treatment. Binding of [^{3}H]UK 14304 was reconstituted by poly(ethylene glycol)-induced fusion of alkaline-treated platelet membranes with cell membranes containing N_i but no α_2 receptor or with lipid vesicles containing purified guanine nucleotide binding proteins (N-proteins) from bovine brain. The reconstituted binding was of high affinity (K_d = 0.4 ± 0.1 nM), accounted for a substantial fraction of the total α_2 receptors (B_{max} for [^{3}H]UK 14304 was 78 ± 23% of the B_{max} for [^{3}H]yohimbine), and was abolished in the presence of guanosine 5′-(β,γ-imidotriphosphate) (GppNHp). The brain-specific protein N_o (predominant guanine nucleotide regulatory protein from bovine brain) was also effective in reconstituting high-affinity α_2 agonist binding. The results presented here show that a guanine nucleotide regulatory protein of the N_o or N_i type is necessary for high-affinity α_2 agonist binding. These methods should also prove useful for future studies of receptor N-protein interactions.

Adenylate cyclase activity is stimulated by β-adrenergic receptors and is inhibited via α_2 adrenergic receptors [see Ross and Gilman (1980) and Limbird (1981) for review]. Stimulation of enzyme activity requires the guanine nucleotide regulatory protein N_s,[1] while inhibition of adenylate cyclase requires a distinct guanine nucleotide regulatory protein, N_i (Ross & Gilman, 1980; Kurose et al., 1983). Another GTP binding protein of unknown function, termed N_o, has recently been purified from bovine brain (Sternweis & Robishaw, 1984; Neer et al., 1984). The interactions of purified β-adrenergic receptors with N_s, N_i, and the catalytic subunit of adenylate cyclase have been examined after reconstitution into phospholipid vesicles (May et al., 1985; Cerione et al., 1983). In addition, Cerione and collaborators have recently reconstituted α_2-receptor-stimulated GTPase activity using purified N-proteins and partially purified α_2 receptors (Cerione et al., 1986).

In addition to the role of guanine nucleotide regulatory proteins in producing responses, there is evidence that the affinity of agonist binding to many receptors including α_2 (Hoffman et al., 1982; Michel et al., 1980) and β-adrenergic (Maguire et al., 1976; Blume, 1978), muscarinic (Jakobs et al., 1979), and opiate receptors (Kurose et al., 1983; Katada & Ui, 1982) is regulated by these proteins. Guanine nucleotides such as the nonhydrolyzable GTP analogue GppNHp selectively reduce the affinity of α_2-adrenergic agonist binding without reducing antagonist binding (Hoffman et al., 1982; Michel et al., 1980). Modification of the N_i protein by pertussis-toxin-catalyzed ADP-ribosylation in NG108-15 cells results in a loss of receptor-mediated inhibition of adenylate

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[1] Abbreviations: buffer A, 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.0, 2 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl; GppNHp, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); N-proteins, guanine nucleotide binding proteins; N_i, inhibitory guanine nucleotide regulatory protein; N_o, predominant guanine nucleotide regulatory protein from bovine brain; N_s, stimulatory guanine nucleotide regulatory protein; NAD, nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol); POB membranes, platelet membranes pretreated with phenoxybenzamine; SDS, sodium dodecyl sulfate; TED buffer, 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol (DTT); TME buffer, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA); UK 14304, 5-bromo-6-[*N*-(4,5-dihydroimidazol-2-yl)amino]quinoxaline.

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