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Localization of the Primary Quinone Binding Site in Reaction Centers from *Rhodopseudomonas sphaeroides* R-26 by Photoaffinity Labeling†

T. D. Marinetti,‡ M. Y. Okamura,§ and G. Feher*

ABSTRACT: We have prepared a radioactive photoaffinity label, 2-azido[^{3}H]anthraquinone, to substitute for ubiquinone as the primary electron acceptor in reaction centers from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. When the label was illuminated with ultraviolet light, it photolyzed to yield an intermediate, most likely a triplet nitrene, which was observed at 80 K by optical and EPR spectroscopy. Reacton centers that had the ubiquinone replaced by the label showed photochemical activity (bleaching at 865 nm and light-induced EPR signals) at room temperature, 80 K, and 2.1 K. When reaction centers reconstituted with the label were illuminated with ultraviolet light at 80 K and subsequently warmed, some of the label became covalently attached to the protein. Similar results were obtained with infrared illumination, showing that nitrene formation can be mediated by the tetrapyrrole pigments. Analysis of photolyzed protein samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that the M subunit of the reaction center was selectively labeled, as compared to control preparations in which the primary quinone binding site was filled by ubiquinone before labeling. These results show that the primary quinone binding site is located on or very close (within ~5 Å) to the M subunit of the reaction center.

The first steps in bacterial photosynthesis, namely, the absorption of light and formation of a donor–acceptor ion pair, take place in a membrane-bound pigment–protein complex called the reaction center (RC),[1] which has been isolated in a highly pure form from various bacteria. The details of the primary photochemistry and the identities of the molecules involved in the electron transfers are fairly well understood [for recent reviews see Parson & Cogdell (1975), Loach (1977), and Feher & Okamura (1978)]. The most thoroughly

† From the Department of Physics, University of California, San Diego, La Jolla, California 92093. *Received January 4, 1979.* Supported by grants from the National Science Foundation (PCM 74-21413) and the National Institutes of Health (GM-13191).
‡ Recipient of National Institutes of Health Postdoctoral Fellowship F32-GM05750.
§ Recipient of National Institutes of Health Career Development Award 1 K04 GM00106.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; RC(s), reaction center(s); LDAO, lauryldimethylamine oxide; AzAQ, 2-azidoanthraquinone; DEAE, diethylaminoethyl; UQ, ubiquinone.

0006-2960/79/0418-3126$01.00/0

studied and characterized reaction centers are those obtained from the blue-green mutant, R-26, of *Rhodopseudomonas sphaeroides* (Clayton & Wang, 1971; Feher, 1971; Feher & Okamura, 1976, 1978). The results of many studies have shown the RC to be a complex of three nonidentical polypeptide chains denoted L, M, and H with apparent molecular weights (obtained from $NaDodSO_4$–polyacrylamide gel electrophoresis) of 21 000, 24 000, and 28 000 (Okamura et al., 1974). The complex contains four bacteriochlorophyll and two bacteriopheophytin molecules (Reed & Mayne, 1971; Straley et al., 1973), one iron atom (Feher, 1971; Feher et al., 1974), and two molecules of ubiquinone (Okamura et al., 1975). Absorption of light results in the transfer of an electron from the donor, a special bacteriochlorophyll dimer (Norris et al., 1971; Feher et al., 1973, 1975; Norris et al., 1973, 1975), to the primary acceptor. The acceptor is an ubiquinone molecule (Clayton & Straley, 1970; Loach & Hall, 1972; Slooten, 1972; Feher et al., 1972; Cogdell et al., 1974; Okamura et al., 1975) magnetically coupled to Fe^{2+} (Bolton & Cost, 1973; Feher et al., 1974; Okamura et al., 1975).

Little is known about the structural arrangement of the various components of the RC. We therefore decided to probe the structure of the RC using a chemical labeling method to determine on which subunit the primary acceptor site is located. The information available on the arrangement of the ubiquinones comes mainly from optical and EPR studies of the electron transfers. In response to a series of light flashes, either the primary or the secondary ubiquinone can be reduced preferentially (Vermeglio & Clayton, 1977; Wraight, 1977). This enables one to study the characteristics of either quinone separately. Both quinones give similar optical changes at 450 nm upon formation of the respective semiquinone, but the band shifts of the bacteriochlorophyll and bacteriopheophytin depend on whether the primary or secondary quinone is reduced (Vermeglio & Clayton, 1977; Vermeglio, 1977; Wraight, 1977). There are also differences in the line width of the EPR signal due to the quinone–iron complex, depending on whether the electron is on the primary or secondary quinone (Okamura et al., 1978; Wraight, 1978). The two ubiquinones also differ in their binding to the reaction center, allowing preparation of active reaction centers with the secondary quinone removed (Okamura et al., 1975). The above data indicate that the two quinones are close to each other and to the iron atom but differ in their orientation with respect to the pigments and in their interactions with the reaction center protein.

In the experiments described here we have employed the published procedures for removing the quinones (Okamura et al., 1975), followed by reconstitution of reaction centers with a photoaffinity label, 2-azidoanthraquinone. In the technique of photoaffinity labeling, one replaces the natural ligand with an analogue, usually radioactively tagged, containing a group that couples covalently to the protein after activation with light. These analogues are most commonly carbenes or nitrenes generated from photolyses of the corresponding diazo or azido precursor, respectively. Subsequent analysis of the photolyzed sample (e.g., by gel electrophoresis) can be used to determine which protein components have been labeled. This technique has been applied with considerable success to a wide variety of proteins [for a comprehensive review, see Bayley & Knowles (1977)], including membrane proteins such as the mitochondrial ATPase (Wagenvoord et al., 1977; Verheijen et al., 1978) and the adenine nucleotide carrier protein (Lanquin et al., 1978).

The photoaffinity label that we have synthesized and used in this work is 2-azido[^{3}H]anthraquinone. We have shown that it replaces ubiquinone to form a photochemically active reaction center and, upon photolysis, couples preferentially to one of the subunits.

Materials and Methods

Reaction Centers. RCs were prepared from *R. sphaeroides* R-26 as described by Okamura et al. (1975). Concentrations were assayed spectrophotometrically by using the extinction coefficient $\epsilon_{802} = 288\ mM^{-1}\ cm^{-1}$ (Straley et al., 1973). RCs with approximately zero and one ubiquinone were prepared as described by Okamura et al. (1975). Typically, the number of ubiquinones per reaction center was 0.1–0.15 for the depleted preparations and 0.87–0.93 for those with only the secondary quinone removed. Unless stated otherwise, the buffer used was 10 mM Tris-HCl, pH 8.0, and 0.1% LDAO.

Determination of Photochemical Activity and Quinone Content. Photochemical activity was determined by measuring the transient absorbance change, ΔA (i.e., bleaching), of the 865-nm band following a saturating flash of light. Purified native RCs as isolated show a $\Delta A/A = 0.93$ at 865 nm at room temperature. The fraction of RCs with an active primary acceptor quinone was determined from the fractional bleaching. Alternatively, the number of active acceptors was determined by monitoring the fast phase of the light-induced oxidation of cytochrome *c* in a sample containing RCs (1–2 μM) in the presence of 10–20 μM reduced horse heart cytochrome *c*. The number of donor–acceptor pairs formed in the light was obtained from the optical change at 550 nm using the differential extinction coefficient for cytochrome *c*, $\Delta\epsilon_{550}^{red-oxid} = 20\ mM^{-1}\ cm^{-1}$ (Straley et al., 1973). Control experiments showed that for RCs with one or less quinone per reaction center the activity determined by cytochrome photooxidation or by transient bleaching at 865 nm was the same. All optical measurements were performed on a CARY 14-R; for low-temperature measurements a glass dewar was inserted into the sample compartment.

Preparation of 2-Azidoanthraquinone. 2-Azidoanthraquinone was prepared in two steps from 2-aminoanthraquinone (Aldrich, technical grade) by first diazotizing the amine with isoamyl nitrite (Aldrich) by using a modification of the Knoevenagel method (Saunders, 1949) and converting the diazonium chloride to the azide with sodium azide. Suthankar et al. (1973) have synthesized AzAQ and other related azidoanthraquinones using nitrosylsulfuric acid to diazotize the amines.

First, the amine was partially purified and converted to a fine powder by dissolving it in concentrated sulfuric acid and then diluting it into a large volume of water. The slurry was filtered and dried in vacuo for 3 days. This preparation showed one major spot and one minor spot on thin-layer chromatography (Eastman Chromagram) with CH_2Cl_2 as the solvent.

The powdered amine (10.8 mg) was dissolved in 2 mL of glacial acetic acid to which 0.08 mL of concentrated HCl had been added. The mixture was heated to 90 °C in a water bath in a sealed, Teflon-capped test tube until most of the quinone had dissolved (about 10 min). It was cooled to room temperature and 0.09 mL of isoamyl nitrite was added. After 20 min at room temperature, diethyl ether was added to a final volume of 9–10 mL. The diazonium chloride precipitate was collected by centrifugation. The pellet was washed by resuspension in 6–7 mL of ether and then spun down again. The supernatant was discarded and the pellet was dissolved in 6–7 mL of 2 N HCl. During all subsequent operations the sample was shielded from light. Sodium azide (2.0 M; 0.029 mL) was added, whereupon the solution turned cloudy and effervesced, and a light tan flocculent precipitate formed. The mixture

was shaken with 2–3 mL of CH_2Cl_2, yielding a light yellow organic phase after centrifugation. The CH_2Cl_2 phase was washed several times with water, diluted to 5 mL with fresh CH_2Cl_2, and dried by addition of anhydrous $MgSO_4$. After centrifugation, the clear yellow supernatant was removed and concentrated to about 0.5 mL by evaporation using dry N_2.

The product was purified by passing it over a small column (6 × 150 mm) of silicic acid (Bio-Sil A; 200–350 mesh; Bio-Rad Laboratories) packed in ethyl acetate and washed with several column volumes of CH_2Cl_2. The product was applied and eluted with CH_2Cl_2. A dark red band formed at the top which moved very slowly, preceded by a faint yellow band moving almost with the solvent front. This latter band was collected and dried by using dry N_2. The yield of pure azide was 6.5 mg (54%). It was stored in CH_2Cl_2 solution and wrapped in aluminum foil to prevent exposure to light. This material gave a single spot on thin-layer chromatography.

2-Azido[^{3}H]anthraquinone was prepared as above with appropriate reduction in scale, by using 2-aminoanthraquinone which had been labeled by the Amersham Corp. with tritium by using the TR 1 catalytic exchange process. The specific activity of the purified azide was 2750 Ci/mol.

Reconstitution of Reaction Centers with 2-Azidoanthraquinone. Parallel samples of RCs containing approximately zero and one ubiquinone per reaction center were reconstituted with 2-azidoanthraquinone as follows. In 1.5-mL polypropylene centrifuge tubes (Eppendorf), about 150 nmol of AzAQ in CH_2Cl_2 solution was dried down with nitrogen or argon gas. LDAO (10%) (20–25 μL) was added to suspend the quinone, followed by 0.45 mL of a solution of reaction centers (A_{800}^{1cm} = 22). The mixtures were wrapped in foil and placed in a reciprocating shaking table at room temperature for 4–5 h. They were then applied to DEAE-cellulose columns (about 0.5-mL bed volume in a Pasteur pipet plugged with glass wool), washed with buffer (4–5 column volumes) to remove excess AzAQ and LDAO, and then eluted with 10 mM Tris-HCl, pH 8.0, 0.1% LDAO, and 1 M NaCl and stored in the dark at 4 °C. Samples were assayed for photochemical activity as described above, and aliquots of the assay sample were counted to measure the total radioactive quinone present. The content of AzAQ depended on the time of washing on the DEAE column; the conditions chosen were a compromise between the complete removal of unbound quinone and the concomitant stripping of the bound AzAQ. All operations were carried out in the dark.

Photolysis and Preparation of Samples for $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. About 35 μL of reconstituted RCs was taken up in 100-μL (Clay-Adams) micropipets, frozen immediately in liquid nitrogen, and kept in a covered dewar until use. Photolysis in the ultraviolet was carried out using a Xe arc lamp (Bausch & Lomb 33-86-20-01) filtered through a Pyrex tissue culture bottle filled with 0.2 M $CuSO_4$ (path length about 2.5 cm; effective band-pass 330 < λ < 600 nm). The light was focused with a quartz lens onto the plane of the sample. The sample was immersed in an unsilvered Pyrex dewar filled with liquid nitrogen and was turned throughout the photolysis by a low-speed motor (30–60 rpm) to ensure that all parts of the sample were evenly illuminated. Photolysis in the infrared was carried out similarly, except that the light source was a Leitz Prado 500 projector (500 W) filtered through a tissue culture bottle filled with water, a Corning CS-2-64 color filter (λ > 660 nm passed), and a Corion BA 9000 interference filter (λ_{max} = 888 nm). Light intensities were measured with a YSI Model 65 radiometer (Yellow Springs Instrument Co.). During infrared photolyses the room lights were turned off. After photolysis the samples were transferred to a liquid nitrogen dewar for storage until all samples within a given series were photolyzed. Control experiments showed that the length of time of storage in liquid nitrogen had no effect on the degree of incorporation of radioactivity.

The nitrenes trapped in the photolyzed samples were allowed to react by thawing the sample in a 1.5-mL centrifuge tube to which LDAO was added to bring the final concentration to 1% (v/v). After thawing, 0.9 mL of acetone–water (9:1) was added, and the samples were covered with aluminum foil to prevent exposure to light and allowed to stand 20 min at room temperature to precipitate the protein. Unbound AzAQ was removed by three cycles of resuspending the pellet in 90% acetone (0.9 mL) and then spinning for 2 min and carefully removing the supernatant liquid. The remainder of the solvent on the last wash was removed by blowing dry nitrogen gas over the pellet. The precipitated protein was dissolved in 100 μL of 2% $NaDodSO_4$ and 1% dithiothreitol in 10 mM Tris-HCl, pH 8.0, and placed on a heating block at 65 °C for 30 min. A solution (25 μL) of 50% sucrose containing bromphenol blue was added, and the samples were stored at −20 °C until the gels were run.

Sodium Dodecyl Sulfate Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by Okamura et al. (1974), except that the cross-linking agent was *N,N'*-diallyltartardiamide (Eastman) (Anker, 1970), which is cleavable with periodic acid. Gels were scanned at 280 nm in a home-built gel scanner designed to operate in the CARY 14-R. Markers were placed in the gels before scanning by stabbing the gels with a 25-gauge needle. Gels were cut by using a home-built apparatus consisting of an aluminum V-shaped holder with fine lateral cuts every 1 mm and a cutter strung with stainless steel wire (diameter 0.004 in.). Gels were frozen with powdered dry ice after aligning them in the holder by using the gel scans and the markers. When the gels had thawed to a firm but not rock-solid consistency, they were cut by slowly drawing the wires through the grooves of the holder. Slices were placed in scintillation vials and were dissolved by addition of 0.7 mL of 2% (w/w) periodic acid, followed by shaking on a shaking table for 1 h.

Parallel gels were run and fixed, stained and destained, and scanned at 560 nm and compared to gels run with untreated RCs to quantitate the amount of protein in each band, as described by Okamura et al. (1974).

Determination of Radioactivity. Radioactivity was determined by suspending samples in 10 mL of Scintisol (Isolab) and counting them in a Beckman LS-100 scintillation counter by using the wide-range ^{14}C isoset. Using [^{3}H]hexadecane as the internal standard (Amersham), we determined the counting efficiency to be approximately 30% for the dissolved gel samples and 37% for the protein solutions used for the quinone determinations.

EPR Measurements. RC samples were prepared by adding glycerol to a final concentration of 50% (v/v) and then rapidly freezing in liquid nitrogen in the dark. Low-temperature EPR was performed as described by McElroy et al. (1972, 1974).

Experimental Results

Photolysis of 2-Azidoanthraquinone at Room Temperature. Before AzAQ could be used as a label, it was necessary to demonstrate that it could be photolyzed to give reactive products. Figure 1 shows the results of optical experiments at room temperature. The spectrum of AzAQ in absolute ethanol shows two strong absorptions at 270 (ϵ_{270} = 37.3 mM^{-1}

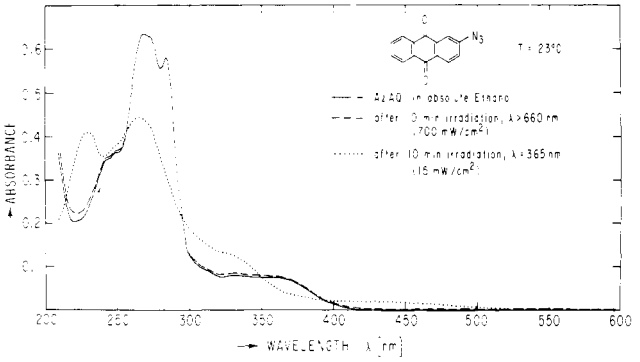

FIGURE 1: Optical spectra of 2-azidoanthraquinone and light-induced changes at room temperature. (—) Spectrum in absolute ethanol. (- - -) Same sample after illumination for 10 min with infrared light (Leitz-Prado projector (500 W) filtered through 3 cm of H_2O and a Corning CS-2-64 filter; I = 700 mW/cm^2). (···) Same sample after illumination with ultraviolet light for 10 min (light source as above, except the filter was a Corning CS-7-39; I = 15 mW/cm^2). Actinic illuminations were done in a water bath at 22.5 ± 0.5 °C.

cm^{-1}) and 284 nm, with weaker absorptions at longer wavelengths (Figure 1). Spectra of more concentrated solutions show that these bands are at 330 and 365 nm. In comparison to the parent anthraquinone, the azide compound is red-shifted by roughly 20–30 nm. Hence, the band at 330 probably arises from the azide. Reiser et al. (1966) have reported that aromatic azides give rise to n → π^* transitions at wavelengths from 300 to 350 nm, the longer wavelengths being associated with the larger aromatic skeletons. When the sample was subjected to photolysis in the infrared region for 10 min, no significant changes were seen in the spectrum (Figure 1). When the sample was photolyzed for 10 min with ultraviolet light, large changes, notably blue shift, were observed in the spectrum (Figure 1). The temperature of the samples was maintained at 22–23 °C during photolysis by immersing them in a water bath. This was done to avoid thermal decomposition of the azide due to heating of the sample during photolysis, especially with the high intensity of light used for the infrared photolysis. Thin-layer chromatography of similarly photolyzed samples showed the appearance of new spots, which were different from both the AzAQ and the precursor amine.

Photolysis of 2-Azidoanthraquinone at 80 K. To characterize the products of photolysis further, experiments were conducted at 80 K where short-lived intermediates, such as nitrenes, could be trapped and observed. AzAQ was dissolved in ethanol–isopentane–ether (2:5:5) which forms a glass at low temperature (Scott & Allison, 1962). When the sample was illuminated by broad-band irradiation (330 < λ < 600 nm; 100 mW/cm^2) while immersed in liquid nitrogen, the color changed from straw yellow to a bright orange. The upper traces of Figure 2 show optical spectra in the visible region of a sample photolyzed in the CARY 14-R spectrophotometer. In agreement with the qualitative observations above, new sharp bands appear in the optical spectrum at 510 and 550 nm. These bands disappear if the temperature of the sample is raised, by removing it from the liquid nitrogen for several seconds. Hence, the product responsible for the sharp bands is quite unstable, consistent with the properties of a nitrene.

The lower trace of Figure 2 shows the EPR spectrum at 8.8 GHz of a sample similar to the one used for the low-temperature optical studies, except that the intensity of the illumination was increased (500 mW/cm^2 for 30 min). The spectrum shows two prominent lines, one at 3178 G (g = 2), where one would expect to see signals from free radicals, and another at 6540 G. The high-field line is most likely due to

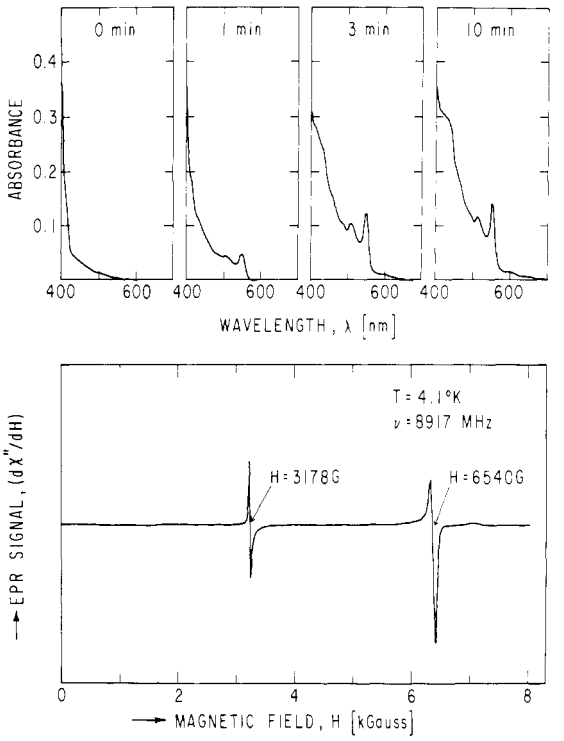

FIGURE 2: Optical and EPR spectra of the photolysis products of 2-azidoanthraquinone at 80 K. Upper traces: optical spectra of AzAQ in EPA (ethanol–isopentane–ether, 2:5:5) (Scott & Allison, 1962) after illumination for the times indicated. The actinic light source was a Leitz-Prado projector (500 W) filtered through 3 cm of H_2O and a Corning CS-O-53 filter, giving an effective band-pass of 330 < λ < 1300 nm; I = 100 mW/cm^2. Illumination and spectra were performed in an optical dewar in the CARY 14-R. Lower trace: EPR of AzAQ in EPA (1 mg/mL) photolyzed in a dewar of liquid nitrogen for 30 min. The actinic light was a Bausch & Lomb Xe arc lamp (33-86-20-01) (I = 500 mW/cm^2). The EPR spectrum was recorded at 4.1 K; microwave power was 2 × 10^{-7} W; field modulation amplitude was 20 G at 95 Hz. The low-field line corresponds to a g value of 2.0043 ± 0.0002 and is due to a free radical; the high-field line is assigned to a triplet.

a triplet state having a large zero-field splitting. It disappeared when the sample was thawed and refrozen; its behavior paralleled that of the sharp optical bands produced by the illumination. Since a nitrene is expected to have a triplet ground state, the observation of the high-field lines is further confirmation of the production of such an intermediate.

Reconstitution of Photochemically Active Reaction Centers with AzAQ. Reaction centers were depleted of ubiquinone and reconstituted with AzAQ by incubation in the presence of an excess of the solid quinone. They were tested for photochemical activity by measuring bleaching of the 865-nm band with strong actinic illumination. Figure 3a–c shows such spectra taken at 80 K in 50% (v/v) glycerol. As can be seen from Figure 3c, incubation with AzAQ resulted in the restoration of photochemical activity.

To test further the nature of the reconstituted RCs, experiments were conducted at 2.1 and 80 K. Figure 4a shows the EPR signal produced upon illumination of the sample in the EPR cavity. The signal had a g value of 2.0026 ± 0.0001, which is identical with the value seen in RCs with UQ in the primary acceptor site. At 2.1 K there was also a broad light-induced signal at g = 1.8 normally associated with the ferroquinone complex. Its width and size were similar to the corresponding line formed in native RCs.

With the magnetic field adjusted to the maximum of the narrow signal, the kinetic response of the RCs to a pulse of light was obtained at 80 K (Figure 4b). The decay time was

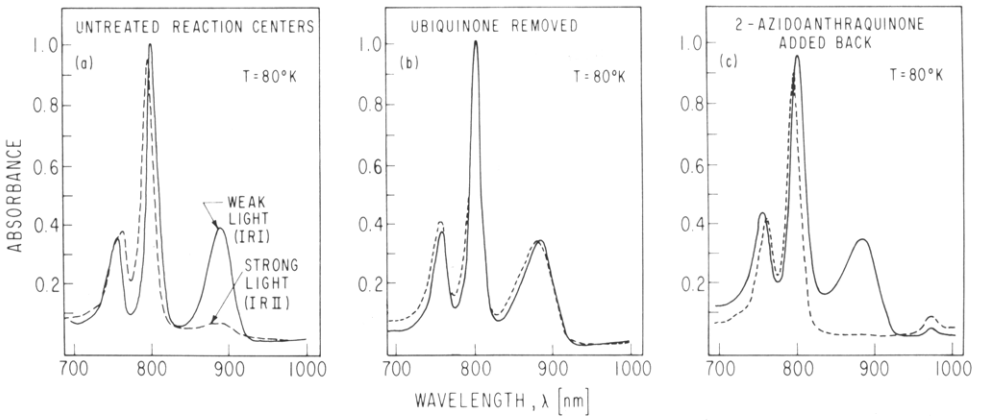

FIGURE 3: Reconstitution of reaction centers with 2-azidoanthraquinone. Reaction center samples were made 50% (v/v) with glycerol; final $A_{802}^{1cm} = 7.5$. They were loaded into optical cells (1-mm path length), mounted on phosphor bronze frames, and immediately frozen in liquid nitrogen. All operations were carried out in the dark. Spectra were recorded in an optical dewar in the CARY 14-R, with the sample mounted in a block of copper immersed in liquid nitrogen to maintain constant temperature. The actinic light was obtained by using the IR-II mode of the CARY 14-R (dashed traces). (a) Normal RCs as isolated. (b) Reaction centers after removal of ubiquinone. (c) Reaction centers as in (b) after incubation with excess 2-azidoanthraquinone.

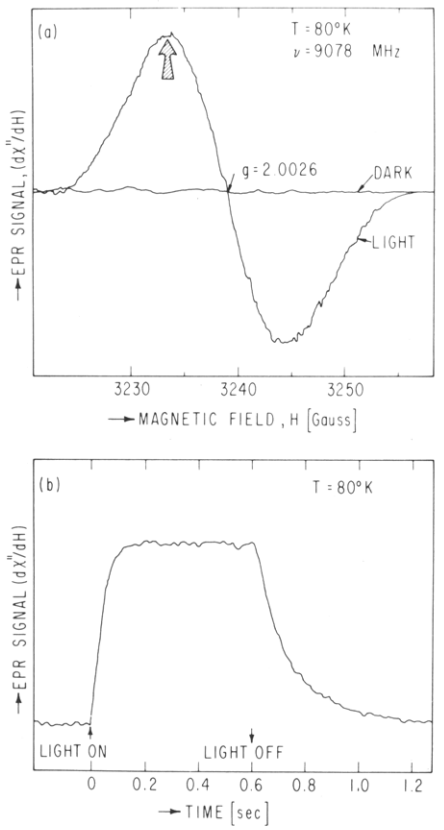

FIGURE 4: Light-induced EPR signal and kinetics at 80 K from RCs reconstituted with AzAQ. (a) Narrow signal at $g = 2.0026$. Microwave power was 2×10^{-5} W; field modulation amplitude was 10 G at 100 Hz. The dark trace was recorded immediately before the light trace. The light source was a 650-W tungsten lamp, filtered through a Corion 8500 filter; intensity at the sample was 20 mW/cm^2. (b) Kinetics of the light-induced signal at $g = 2.0026$. Actinic light was the same as described in (a). The magnetic field was adjusted to the maximum of the light-induced signal [see arrow in (a)], and the response of the system to a 0.6-s pulse of light was recorded. Microwave power was 2×10^{-5} W; field modulation was 10 G at 400 Hz. Instrumental time constant was 3 ms.

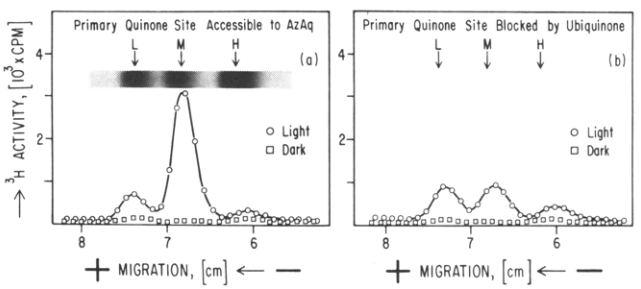

FIGURE 5: NaDodSO$_4$–polyacrylamide gel electrophoresis profiles of reaction centers reconstituted and labeled with 2-azido[^{3}H]-anthraquinone by using *ultraviolet* light during photolysis. (a) Reaction centers depleted of ubiquinone reconstituted with AzAQ, photolyzed, and run on gels (see text). (b) Reaction centers with the primary quinone site filled by ubiquinone, treated exactly as described in (a). Both samples were photolyzed at 80 K by using a Xe arc lamp. The light intensity at the sample was 180 mW/cm^2. (○) Samples exposed to light. (□) Samples handled identically except the light source was blocked by an aluminum plate. The L, M, and H markers refer to the position of the respective subunits from a scan at $\lambda = 280$ nm of the gel before it was sliced. The insert shows an identical gel stained with Coomassie Blue.

found to be 132 ± 10 ms, in agreement with the value found for RCs reconstituted with anthraquinone (Okamura et al., 1975). The light-induced signal remained almost totally reversible following a short pulse of light down to a temperature of 2.1 K (97% reversible following a 0.6-s pulse of light). The only difference between RCs reconstituted with UQ and AzAQ is that in the latter case *prolonged* strong illumination caused partially irreversible bleaching even at cryogenic temperatures. For instance, only about 50% of the absorbance at 890 nm was recovered in the sample shown in Figure 3c after leaving it in the dark for 30 min. This indicates that some chemical processes had occurred that prevented the return of the electron to the oxidized donor.

Photoaffinity Labeling with AzAQ. Reaction centers reconstituted with radioactive AzAQ were prepared, photolyzed, and run on NaDodSO$_4$–polyacrylamide gels. When photolysis was carried out at room temperature, it was found that the light caused extensive denaturation of the protein when samples were exposed for a time sufficient to photolyze the AzAQ. Furthermore, very little incorporation of radioactivity in the protein was obtained. We therefore photolyzed at 80 K, since the RCs are stable to illumination at this temperature. Covalent attachment of the nitrene occurred upon subsequent warming. Parallel samples were prepared by using RCs with the secondary quinone removed but with UQ still in the primary acceptor site. Differences in the labeling of the two samples should, therefore, be associated with the AzAQ molecules occupying the primary quinone site. Figure 5a,b

Table I: Covalent Labeling of Reaction Centers with 2-Azido[^{3}H]anthraquinone[a]

wavelength used during photolysis (nm)	(A) primary quinone site accessible to AzAQ (mol %)			(B) primary quinone site blocked by UQ (mol %)			(C) difference (A − B) (mol %)		
	L	M	H	L	M	H	L	M	H
330–600	1.0	7.7	0.3	2.0	2.5	0.5	−1.0 ± 0.4	5.2 ± 1.6	−0.2 ± 0.1
900	0.5	2.1	0.2	0.2	0.4	0.3	0.3 ± 0.1	1.7 ± 0.4	−0.1 ± 0.1
480	0.5	8.6	0.5	0.3	0.9	0.3	0.2 ± 0.1	7.7 ± 1.7	0.2 ± 0.1
dark	0.14	0.05	0.07	0.09	0.06	0.08	0.05 ± 0.03	−0.01 ± 0.03	−0.01 ± 0.03

[a] Data from Figures 5 and 6 and other gels not shown. The numbers given in the table are the number of AzAQ molecules bound per mole of subunit expressed as a percent. The estimated statistical errors in the above are ±20% of the values listed, except for the values in column C.

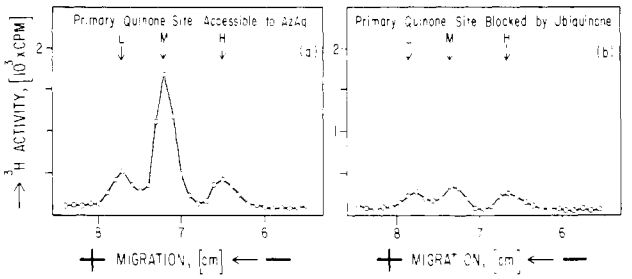

FIGURE 6: NaDodSO$_4$–polyacrylamide gel electrophoresis profiles of reaction centers reconstituted and labeled with 2-azido[^{3}H]-anthraquinone, by using *infrared* light during photolysis. The experiment was performed as described in Figure 5, except the light source was a Leitz-Prado projector (500 W) filtered through 2.5 cm of H_2O, a Corning CS-2-64 color filter, and a Corion BA 9000 interference filter. Light intensity at the sample was 65 mW/cm^2. L, M, and H denote the positions of the respective subunits from a scan at λ = 280 nm of the gel before slicing.

shows the gel profiles for two such samples. The insert in Figure 5a shows a gel run in parallel that was stained with Coomassie Blue. The UQ-depleted sample contained 0.1 UQ per RC before incubation, 0.6 AzAQ per RC after incubation, and 0.54 active[2] quinones per RC as determined from the photochemical activity. The corresponding numbers for the sample of Figure 5b are 0.93 UQ per RC, 0.33 AzAQ per RC, and 1.06 active quinones per RC. The samples were illuminated with broad-band irradiation and treated as described under Materials and Methods. The gel profiles clearly show selective labeling of the M subunit in the sample from which UQ was removed. Gel profiles of identical samples which were not exposed to the irradiation are also included in the figures and show virtually no incorporation of radioactivity.

Figure 6 shows a set of samples similar to those used for Figure 5 which were photolyzed in the infrared. The UQ-depleted sample contained 0.1 UQ per RC and 0.63 AzAQ per RC, with 0.47 total quinones per RC photochemically active as acceptors. The corresponding numbers for the undepleted sample were 0.93 UQ per RC, 0.32 AzAQ per RC, and 1.05 active quinones per RC. The samples were photolyzed for 30 min at 80 K by using an interference filter (λ = 888 nm; $\Delta\lambda$ = 100 nm; 66 mW/cm^2) to irradiate the RC band at 890 nm (P865 shifts to 890 nm at 80 K). The gel profiles again show preferential labeling of the M subunit. The total incorporation of radioactivity is less, but the distribution of label is the same as that shown in Figure 5.

The results on the fractional labeling of the subunits for different wavelengths used during photolysis for RCs that had the primary quinone site either blocked by UQ or accessible to AzAQ are summarized in Table I. To correct for differences in the amount of protein in different gel samples, the results are given as moles of AzAQ per mole of subunit. One gel of a duplicate pair was sliced and counted to determine the amount of AzAQ; the other was stained and scanned to estimate the protein in each band. The area of each band of the stained gel was compared with that of a parallel sample of unmodified RCs. When the primary quinone site was accessible to AzAQ, preferential labeling of the M subunit was observed (column A, Table I). When the primary quinone site was blocked, no preferential labeling was obtained (column B). The residual labeling in this case is presumably due to attachments to nonspecific sites. The differences in labeling between RCs whose sites are accessible to AzAQ and those whose sites are blocked are tabulated in column C and show unequivocally the specificity of the labeling to the M subunit.

Summary and Discussion

The success of a photoaffinity labeling experiment depends on meeting three necessary criteria: (a) the label should photolyze to produce an active intermediate that reacts with any neighboring group on the protein; (b) the label should bind specifically to the binding site of the macromolecule with minimal structural perturbations; (c) it should couple covalently to the protein with a high enough efficiency to permit subsequent detection above background labeling due to nonspecific attachments. As discussed by Bayley & Knowles (1977), organic nitrene labels combined several experimental advantages, such as high reactivity, stability of the parent azide, and relative ease of synthesis. Most successful applications of azide photolabels have employed aromatic azides because they are longer lived and less prone to internal rearrangement than alkyl azides [see Bayley & Knowles (1977) and references cited therein]. Hence, the label of choice would be both an aromatic and a quinone. Since Okamura et al. (1975) had already shown that anthraquinone could replace UQ in RCs, an azidoanthraquinone was chosen. It had the additional advantage that in being aromatic it could be easily radiolabeled by catalytic exchange with tritium. It should be noted that analogues with the azide moiety on the quinone ring are unsuitable since they behave like vinyl azides and decompose by internal rearrangement without generating nitrenes (Weyler et al., 1973; Germeraad et al., 1974).

The first step after synthesis was to show that AzAQ satisfies criterion a. Figure 1 shows that qualitatively the compound is sensitive to ultraviolet light and insensitive to high intensities of infrared light, as expected from its optical spectrum. To show that photolysis of AzAQ produced the desired nitrene, we trapped it by photolysis at 80 K. Reiser et al. (1966) reported optical spectra at 77 K on a series of aromatic azides and nitrenes produced upon photolysis in an organic solvent glass. These intermediates give characteristic sharp optical bands shifted to the red compared to the parent azide. We have found a similar behavior as shown in the upper traces of Figure 2. Furthermore, Reiser et al. (1966) observed

[2] The activity of this sample as compared to the one shown in Figure 3 is due to the *excess* AzAQ used in the sample of Figure 3.

that the sharp optical bands associated with the nitrene disappeared above 90 K. The same behavior was observed with AzAQ.

The ground state of the nitrene is a triplet. EPR spectra for several aromatic nitrenes have been reported by Smolinsky et al. (1962). At 9 GHz they observed a high-field line between 6.7 and 7.8 kG and with some compounds a low-field line at 1.6 kG. The EPR spectrum of the lower trace of Figure 2 shows a high-field transition at 6.54 kG which is consistent with the spectra reported by Smolinsky et al. (1962). The line at $g = 2$ corresponds to a spin $^1/_2$ free radical produced by the ultraviolet light and is not directly related to the line at high field. Both the EPR and the optical data therefore indicate that photolysis of 2-azidoanthraquinone yields a nitrene. Suthankar et al. (1973) have reported that azidoanthraquinones attach to nylon fibers when activated by heat or light, presumably by a nitrene intermediate.

The next step was to determine if AzAQ could replace UQ in the primary acceptor site (criterion b). The data of Figure 3 show clearly that this is the case, since at 80 K, AzAQ was able to restore the photochemical activity of RCs whose UQ had been removed. While it is possible that restoration of activity at room temperature could be due to AzAQ acting as an exogenous (diffusible) acceptor, this is unlikely at 80 K. This conclusion is further strengthened by the observation of reversible photochemical activity at 2.1 K, a temperature at which no process involving molecular diffusion is possible. Hence, we conclude that AzAQ can replace UQ in the primary quinone binding site of the RC.

The photolysis experiments with RCs reconstituted with AzAQ presented one difficulty due to the fact that RCs are denatured at room temperature by the high light intensities needed for photolysis. We therefore carried out the photolysis at 80 K where the RCs are stable to illumination even with high light intensities. This allowed us to use higher light intensities than would be possible at room temperature, resulting in a higher degree of labeling (criterion c). An additional advantage of performing the photolysis at 80 K is that AzAQ molecules that are not bound to the RC are immobilized in the solvent matrix and cannot label the protein by a nonspecific collision after the azide has been photolyzed; the nitrenes start decomposing at about 100 K, a temperature at which the nitrene cannot diffuse. The results of the labeling experiments performed at different wavelengths of illumination are shown in Figures 5 and 6 and summarized in Table I, which gives the amounts of AzAQ bound per subunit normalized for the amount of protein in each band of the gel. While all bands show some incorporation of radioactivity, only the M subunit shows a large differential labeling (Table I, column C) depending on whether or not the primary quinone site is occupied by ubiquinone. These results show that the primary quinone binding site is on or very close (within $\sim$5 Å) to the M subunit.

The labeling of the L subunit, while lower, is still significant when compared to the labeling of the H subunit. In samples which contained an excess of AzAQ over RCs, the total labeling was increased with both the L and M subunits about equally labeled while the H subunit labeling remained low. These results could be due to the secondary quinone binding site being on or close to the L subunit. However, as we were unable to show that AzAQ acts as a secondary electron acceptor, nonspecific binding to the L subunit cannot be ruled out at present.

The usual hazards of nonspecific labeling inherent in these types of experiments can be ruled out with respect to the labeling of the M subunit by the following observations and arguments. (1) AzAQ was found to be active as a primary acceptor. Furthermore, the low-temperature kinetics and EPR spectra showed it to be identical with anthraquinone. We do, of course, have to assume in this argument that the covalently bound fraction is representative of the total AzAQ. (2) The labeling of the M subunit was reduced when the primary site had been filled by UQ. (3) The photolysis performed at 900 nm shows that the AzAQ must be on or very near the primary binding site to be activated by the primary photoprocess.

The assumption that the covalently bound fraction is representative of the total AzAQ is related to the absolute efficiency of labeling which, therefore, warrants some discussion. Few of the large number of reported labeling experiments (Bayley & Knowles, 1977) quote the efficiency. Knowles (1972) reported that 65% of the antibody sites of an antibody directed against a hapten containing a nitrophenyl azide were labeled. Hixson & Hixson (1973) reported 7% incorporation of an azido-NAD analogue in yeast alcohol dehyrogenase, of which 3% was specific. More recently, the mitochondrial ATPase from bovine heart (Wagenvoord et al., 1977) and *Escherichia coli* (Verheijen et al., 1978) has been labeled with 8-azido-ATP. From the data presented in those papers one can estimate the incorporation of label to be almost 100% for the β subunit of the former and about 45% for the α subunit of the latter, assuming one ATP binding site per subunit of the respective enzymes. These data show a difference in coupling efficiency of more than 1 order of magnitude, depending on the particular system. The 7–8% labeling of the M subunit was obtained under conditions where the occupancy of the binding site was about 60%, corresponding to a labeling efficiency of 12–13%. This efficiency is toward the low end of the range of reported values, presumably because the internal rearrangement of the nitrene competes favorably with the covalent attachment to the RC binding site.

The localization of the primary quinone binding site on the M subunit is consistent with the fact that isolated LM particles (RCs with the H subunit selectivity removed) show photochemical activity (Okamura et al., 1974; Feher & Okamura, 1978). Therefore, the primary donor and primary quinone must be associated with LM particles. Two other components of the RC are likely to be close to the primary quinone: the transient intermediate which transfers the electron from the donor to the quinone (Kaufman et al., 1975; Rockley et al., 1975; Fajer et al., 1975) and the iron atom which is magnetically coupled to the quinone (Feher, 1971; Feher et al., 1974). Thus, it is reasonable to expect that the transient intermediate and the iron atom are also on or close to the M subunit.

An interesting observation is that labeling occurred even when samples of RCs reconstituted with AzAQ were irradiated at 900 nm, where AzAQ does not absorb but where bacteriochlorophyll has an absorption peak (see Figures 1 and 6). Moreover, sustained illumination of the RCs resulted in permanent bleaching even at 2.1 K. Evidently, during continuous illumination, when a steady-state charge separation exists, a process occurs which results in activation of the azide. The mechanism for this process is not known but one can rule out a direct energy transfer from bacteriochlorophyll to form an excited state of AzAQ. By assuming a one-photon process, the energy of a 900-nm photon is only half of the 22 000 cm^{-1} which separates the ground state and first excited triplet state of anthraquinone (Kusuhara & Hardwick, 1964; Tolkachev, 1975). A possible explanation of the infrared activation process is that an electron transfer from the donor to the quinone either

directly or indirectly results in formation of the nitrene, which then labels the protein. If formation of the semiquinone promotes activation of the azide, then AzAQ would be a particularly useful probe to label other quinone–protein complexes specifically in electron transfer chains, since only those sites involving reduction and oxidation of quinones would be labeled. This functional discrimination would eliminate spurious labeling of hydrophobic regions in membrane systems.

Acknowledgments

We are grateful to E. Abresch, who grew the bacteria and prepared the reaction centers, R. A. Isaacson, who recorded the EPR data, and L. C. Ackerson, A. Darszon, M. Montal, and D. Rosen for helpful discussions.

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