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UBIQUINONE IN *RHODOPSEUDOMONAS SPHAEROIDES*

SOME THERMODYNAMIC PROPERTIES

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Summary

In *Rhodopseudomonas sphaeroides* chromatophores there are 25 ± 3 ubiquinone (Q) molecules/reaction center protein. They comprise several thermodynamically and functionally different ubiquinone complements. There are approx. 19 ubiquinones (E_{m7} = 90 mV) in the main ubiquinone complement which, within experimental resolution, appears thermodynamically homogenous and follows the redox reaction $Q + 2e + 2H^+ \rightleftharpoons QH_2$ from pH 5—9. A method which takes advantage of the $2H^+$ bound/molecule of Q reduced is described for measuring the time course of light-activated reaction center-driven reduction and oxidation of the 19 Q complement. No stable semiquinones were detected in the constituents of the 19 Q complement. There are approx. 6 ubiquinones of lower E_m which are currently unaccounted for, although one or possibly two of these can be assigned to the quinones of the reaction center protein. The remainder may be associated with the NADH-ubiquinone oxidoreductase.

Introduction

It has long been known that quinone is vital to the overall process of photophosphorylation [1—3]. In photosynthetic bacteria the chemical forms of the quinone occupying the different species are well documented [4—6], as is the fact that quinone is present in the membrane in considerable excess over the

Abbreviations: Q, ubiquinone; Q·H and $Q^{\cdot-}$, ubisemiquinone protonated and unprotonated, respectively; QH_2, ubiquinol; Z and ZH_2, hypothetical electron donor to cytochrome c_2 in oxidized and reduced form, respectively; MOPS, morpholinopropane sulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $(BChl)_2$ and $(BChl)_2^+$, reaction center bacteriochlorophyll dimer in reduced and oxidized form, respectively; BChl, bacteriochlorophyll.

redox centers involved in electron transfer [7].

In *Rhodopseudomonas sphaeroides* the quinone (Q) complement is made up solely of quinone-10 [4,5]. One molecule of Q is located in the photochemical reaction center protein where, in association with an iron atom [8—10], it receives an electron from the light-generated reduced bacteriopheophytin radical to form Q^-(Fe) in approx. 150 ps half-time [11]. The functional E_m for the Q^-(Fe)/Q(Fe) couple is —180 mV [12]. At equilibrium the reduced Q(Fe) takes the form of a stable semiquinone and in the physiological pH range it slowly binds a proton; the pK on the reduced form (i.e., Q·H(Fe)/Q^-(Fe)) is at pH 9.8. Other bacterial species are similar in having a pK on the primary semiquinone but the values are lower [12]. A second Q is associated with the reaction center and although this is bound less tightly [9] it also appears to interact with the iron atom [13,14]. There is less quantitative information about the redox properties of this secondary Q; in contrast to the primary Q, it appears not to have a stable semiquinone form at equilibrium although during light-driven electron transfer the semiquinone is very probably a functional species [15,16]. The primary Q reduces the secondary Q, with an approximately 100 μs half-time. Proton binding to the chromatophore from the external aqueous phase is seen concomitant with the reduction of this secondary Q, and the product is considered to be Q·H; the secondary Q·H/Q^- displays a pK at 8.5 [18].

The photoactivated reaction center, in addition to delivering one reducing equivalent to Q located near enough to the outside the chromatophore membrane to be readily protonated [18], also serves to oxidize one of the two cytochrome c_2 hemes located on the inner membrane/aqueous interface within 1 ms [19]. The reducing equivalent on the Q·H is cycled in a series of steps back to the oxidized cytochrome c_2 via a ubiquinone-cytochrome b/c_2 oxidoreductase. Several of these steps have been described [18,20—23] but a complete and unambiguous scheme of electron and proton translocation remains exclusive Most working models [22—26] for the function of the Q-b/c_2 oxidoreductase and analogous systems of other organelles invoke the involvement of quinones or quinone-like redox agents. However, in contrast to the specialized quinones associated with the reaction center, the number and character of the other Q molecules is still uncertain.

In this report we have investigated some basic thermodynamic properties of the Q complements in *Rps. sphaeroides* using redox potentiometry together with in vivo redox analysis by electron paramagnetic resonance spectroscopy (EPR) and spectrophotometry after solvent extraction, a method used in several biological systems [27—30]. We have examined the number of Q molecules in the membrane per reaction center protein and have assessed their physical-chemical homogeneity. We have investigated the redox state of the Q complement under various metabolic conditions as well as the effect of ubiquinone extraction and recnstitution on flash-activated electron and proton movements in the Q-b/c_2 oxidoreductase of the chromatophores.

Materials and Methods

Rps. sphaeroides strain Ga was grown and chromatophores were prepared as previously described [31]. The total bacteriochlorophyll concentration of the

chromatophore suspension was measured in vivo as described by Clayton [52] using the extinction coefficient at 850 nm. The concentration of reaction center protein in the chromatophore suspension was determined using the reduced minus oxidized extinction coefficient at 605 nm of the reaction center $(BChl)_2$ [31].

Determination of total content of ubiquinone

An outline of the method has already been described [28]. 5.0 ml of acetone/methanol mixture (1 : 1, v/v) was added to 0.5 ml of the chromatophore suspension, and ubiquinone was extracted with occasional shaking for 15 min at room temperature. 0.02 ml of 0.2 M $FeCl_3$ solution was added to the acetone/methanol extract to oxidize all the extracted ubiquinone and, after 2–3 min, 5 ml of light petroleum (35–60°C, b.p.) was added to the extract which was shaken by a Vortex mixer for 10 s. The upper, petroleum layer was transferred to a separation funnel, and the light petroleum extract was washed four times with 5.0 ml of 95% methanol in water (v/v) and then evaporated in vacuo. The dried extract was dissolved in a 10.0 ml of ethanol, and the absorbance at 275 nm of the ethanol solution was measured before and after the addition of a few crystals of sodium borohydride. To obtain the concentration of Q in the ethanol we used the value of 14 $mM^{-1} \cdot cm^{-1}$ for the oxidized minus reduced extinction coefficient [32]. Control experiments done in triplicate using pure ubiquinone showed that the Q recovery was close to 94%. In this report the numbers quoted are not corrected for this small loss as it is less than the standard deviation of the experimental determinations.

Determination of ubiquinone redox level under various experimental conditions.

Chromatophores were suspended to about 0.5 mM bacteriochlorophyll in a 20 mM morpholinopropane sulfonate (MOPS) buffer (pH 7.0) containing 100 mM KCl and 1 mM $MgCl_2$. The chromatophores were poised under anaerobic conditions at a chosen redox potential in an anaerobic redox vessel described elsewhere [33]. Usually, 100–200 μM potassium ferrocyanide, 20 μM each of 2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazonium methosulfate, *N*-ethylphenazonium ethosulfate and pyocyanine were used as redox mediators. In some cases, 800 μM hydroquinone was used instead of ferrocyanide, or 20 mM succinate was used as substrate instead of all of these redox mediating dyes. An aliquot of 0.5 ml was taken anaerobically from the redox vessel with a syringe and it was immediately transferred to a test tube or a flat bottomed cylindrical glass vessel(2.3 cm diameter) in which air had been replaced by argon gas. Then 5.0 ml of acetone/methanol mixture (1 : 1, v/v) cooled to −72°C with an ethanol/solid CO_2 mixture was rapidly injected to the test tube or the cylindrical vessel through the rubber septum. This injection promptly disrupted the chromatophores. The extraction mixture was warmed to room temperature and allowed to stand for 15 min with occasional shaking to extract the ubiquinone. The method for determination of the concentration of oxidized ubiquinone in the extract was the same as that used to determine the total ubiquinone except the ferric chloride addition was omitted. Control experiments using pure, reduced ubiquinone in the presence of the same con-

centration of dyes and chromatophores showed that re-oxidation of reduced ubiquinone during the extraction procedure was less than 2%.

Reduced ubiquinone was made as follows: A few crystals of sodium borohydride were added to an ethanolic solution of pure commerically purchased ubiquinone-10 (Sigma) to reduce the ubiquinone. A drop of conc. HCl was added to the ethanolic solution to neutralize it and then a similar volume of light petroleum was added and the mixture was shaken. Distilled water was added in order to separate the light petroleum layer, which was transferred to a test tube and washed several times with distilled water to remove traces of sodium borohydride. Usually, this yielded a preparation in which there existed about 10% of oxidized ubiquinone; however, the reduced ubiquinone was not auto-oxidized further after standing either in a solid state or in petroleum ether.

Isooctane extraction of ubiquinone and reconstitution of extracted chromatophores

Chromatophores washed with and resuspended in 1 mM MOPS, pH 7.3, containing 1 mM KCl and 1 mM $MgCl_2$, were lyophilized and extracted with isooctane as described previously [34].

Reconstitution was carried out by addition of an appropriate amount of pure ubiquinone in isooctane to lyophilized, extracted chromatophores. After the isooctane was completely evaporated in vacuo, the chromatophores were resuspending in the same buffer containing 100 mM KCl and 1 mM $MgCl_2$.

Light-induced electron transfer measurements

The time course of cytochrome c_2 oxidation and reduction was measured under controlled ambient redox potentials with a rapidly responding dual wavelength spectrophotometer equipped with a signal averager as described before [31].

Light-induced proton binding measurements

Light-induced proton binding was measured spectrophotometrically using 25 μM chlorophenol red as the pH indicator dye under controlled ambient redox potentials as described previously [18] except that 200 μM ferrocyanide and a catalytic amount of 2,3,5,6-tetramethylphenylenediamine (0.5 μM) were added as redox mediators and also to promote electron donation to the oxidized reaction center bacteriochlorophyll dimer. Antimycin (5 μM) was added to inhibit electron flow through the Q-b/c_2 system, and carbonyl cyanide *p*-trifluoromethoxyphenolhydrazone (FCCP; 10 μM) was added to collapse in ms any membrane potential generated during illumination, and to promote the re-appearance outside the chromatophore membrane of any protons released internally; protons retained by the chromatophore under these conditions were considered to be bound in the reduced redox component(s) of the Q-b/c_2 oxidoreductase.

Electron paramagnetic resonance

EPR measurements were done using a Varian E109 spectrometer under controlled temperatures from 193 to 1 K. Samples were taken from the same vessel

as used in the direct extraction described above under controlled redox potentials as described before [35].

Results

Determination of total amount of ubiquinone

In chromatophores from *Rps. sphaeroides* there are 25.3 ± 2.6 (S.D.) ubiquinone molecules/reaction center protein. This value was determined using five chromatophore preparations made from different cell batches grown for 20—30 h (started with a 10% inculum from a similar culture). This value is comparable to those of 20 and 25 quinones/reaction center in chromatophores of *Chromatium vinosum* and *Rps. capsulata*, respectively (Takamiya, K., unpublished data). In contrast it has recently been reported [36] that *Rhodospirillum rubrum* chromatophores contain as few as 5 Q molecules/reaction center protein. However, other determinations of the Q content of *R. rubrum* [7] have yielded protein values that are closer to those given above for *Rps. sphaeroides*, *Rps. capsulata*, and *C. vinosum*. The reason for the discrepancy is not apparent, although a possibility is that the determinations that yielded low Q levels [36] were made on material obtained from young cells grown from a low inoculum (approx. 0.1%); we have found that *Rps. sphaeroides* chromatophores prepared from cells grown for longer than 3 days generally contained relatively higher amounts of Q/reaction center, yielding values of 30 or more.

Redox titrations of ubiquinone in chromatophores

Fig. 1A is a typical redox titration done at pH 7.0. The titration curve reveals that about 75% of the total Q can be fitted with a Nernst curve with E_m of 92 mV and $n = 2$; the remaining approx. 25% seems to represent a separate thermodynamic population. Thus the E_m = 92 mV portion of Q accounts for approx. 19 of a total 25 Q molecules. The approx. 6 ubiquinones not reduced at the low potential end point of the titration (this was usually 100 mV lower than the E_m for the 19 Q molecules) must have lower E_m values. However, detailed redox potentiometric investigations of the low potential population have not yet been pursued.

Titrations done at various pH values (Fig. 1B) indicate that each titration accomodates a Nernst curve with $n = 2$ and demonstrates an E_m/pH relationship of —60 mV/pH unit from pH 5 to 9 (Fig. 1C). This shows the ubiquinone redox reaction at equilibrium in this pH range to be $Q + 2e^- + 2H^+ = QH_2$.

The results shown in this paper compare with the E_{m7} of 65 mV ($n = 2$, —60 mV/pH) of the main Q complement of mitochondria [37] but differs somewhat from the E_{m7} of 2 mV reported in *R. rubrum* [38].

The oxidized minus reduced difference spectra of Q in ethanol, extracted at various pH values and various redox potentials (not shown here), indicated that ubisemiquinone was not present in the extracts. From what we know of quinone in water in this pH range (see [39]) this is not unexpected. This fact introduces us to a weakness in the solvent extraction technique: Although the method will still provide the in vivo state of reduction of the Q complement at a specified redox potential and yield the appropriate n-value of the Nernst curve for a stable ($n = 1$) or unstable semiquinone ($n = 2$) population in vivo,

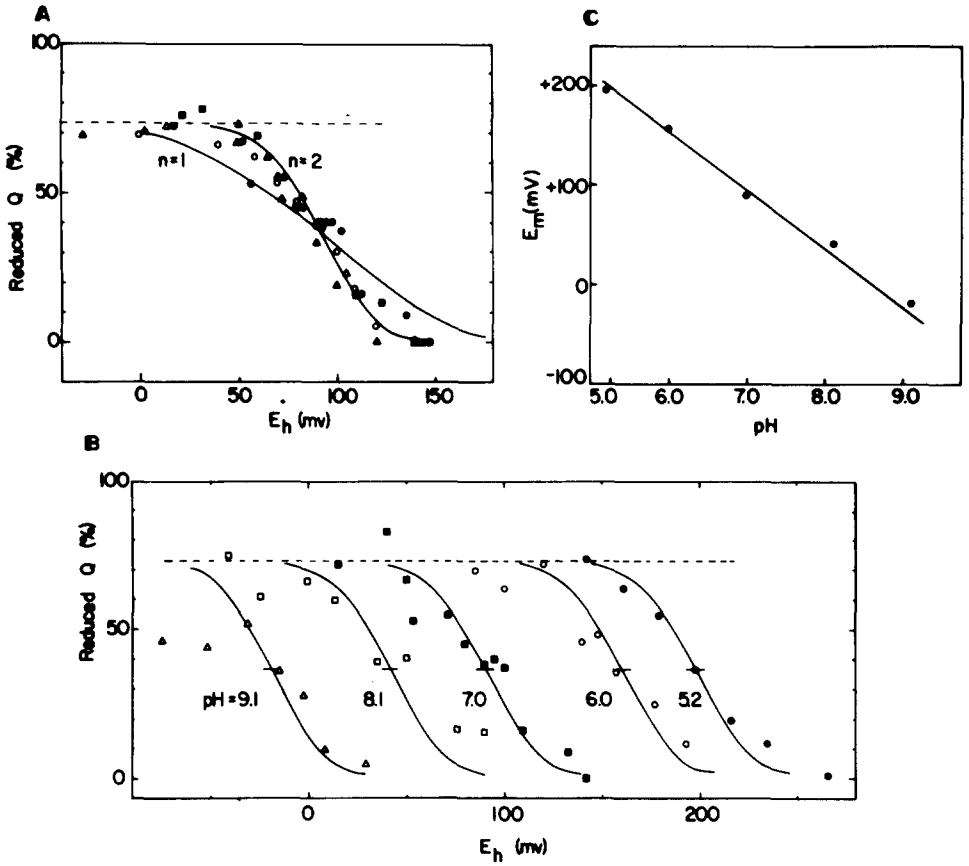

Fig. 1. (A) Redox titrations of ubiquinone at pH 7.0. Chromatophores (approx. 0.5 mM BChl) were suspended in 100 mM KCl, 1 mM $MgCl_2$ and 20 mM MOPS buffer at pH 7.0. Redox mediator dyes used were 100 μM ferrocyanide, 10 μM each of 2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazonium methosulfate, *N*-ethylphenazonium ethosulfate and pyocyanine. The total amount of ubiquinone was determined in every measurements by adding $FeCl_3$ to the acetone/methanol extract. The different symbols represent titrations done with different preparations. The curves drawn through the points are Nernst curves for an E_m value of 92 mV with *n*-values of 1 and 2. The dashed line is the maximal reduction level of ubiquinone obtained in these titrations. (B) Redox titrations of ubiquinone at various pH values. Experimental conditions were the same as in (A) except that at pH 8.1 and 9.1 the buffer used was 50 mM glycylglycine. (C) The pH dependency of the E_m of ubiquinone. E_m values obtained in (B) were plotted against pH. The line through the points has a slope of —60 mV/pH unit.

the presence of the ubisemiquinone itself will not be revealed directly. Thus, a semiquinone state that exists stably in vivo, once extracted into organic solvents, will disproportionate in vitro (i.e., $2Q{\cdot}H = Q + QH_2$). It is conceivable therefore that the existence of some of the approx. 6 apparently low potential Q complement result from the presence in vivo at higher potentials of twice as many stable ubisemiquinones which disproportionate following dissolution in ethanol. Although the Nernst curve of the 19 Q complement described in Fig. 1A can be best fitted by a single $n = 2$ curve suggesting that the above point may not be applicable here, the scatter of the data points in some titrations can also accomodate $n = 1$ curves. This made it necessary to substantiate

the results by examining in vivo for stable ubisemiquinone using EPR spectrometry.

Electron paramagnetic resonance investigation for semiquinones

EPR spectra of chromatophores were surveyed under a wide variety of redox conditions imposed potentiometrically. Fig. 2 is a typical set of low temperature spectra fo chromatophores, poised in the dark before freezing at redox potentials spanning the redox centers of the Q-b/c_2 oxidoreductase. The signal that is observed in the $g = 2$ region is centered between the $g = 2.0045$ expected for a semiquinone and a $g = 2.0026$ signal typified by radicals of the kind displayed by chlorophylls as shown int he bottom trace (Fig. 2) by reaction center $(BChl)_2^+$; the signal observed therefore could contain semiquinone. However, the signal is rather insensitive to the state of reduction of the Q-b/c_2

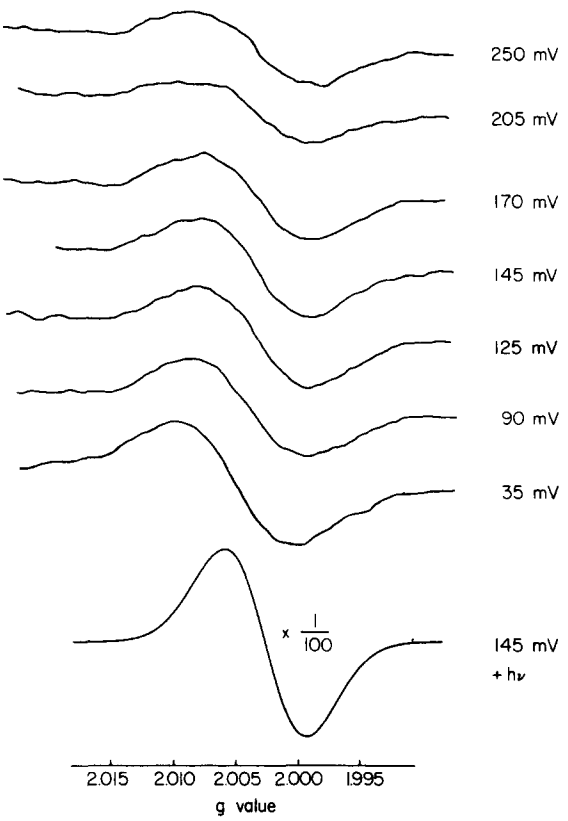

Fig. 2. EPR spectra at various ambient redox potentials at pH 6.0. The redox mediating dyes used in the redox titration were all $n = 2$ redox agents chosen for their lack of semiquinone formation. They were 20 μM each of 1,4-naphthoquinone, duroquinone, 1,4-naphthoquinone-2-sulfonate and 200 μM ferrocyanide. Dimethylsulfoxide was used as the stock solvent for the mediators; this is because ethanol has been reported to affect the EPR properties of certain iron-sulfur proteins and semiquinones [51]. The temperature was 30 K and microwave power was 0.5 mW. The microwave power was not saturating. In the bottom trace, the sample in the cavity was illuminated with 150 W tungsten-iodine lamp through 3 cm water layer. The reducing factor of 1/100 was calculated from both the gain of the EPR spectrometer and actinic light saturation of the illuminated sample.

oxidoreductase redox centers including the 19 Q complement (E_{m6} = 150 mV). Signals in samples redox poised near the E_m value of the 19 Q complement (where semiquinones concentrations are maximized in an n = 2 redox system) are no different from background. There is a steady increase in the radical signal amplitude as the redox potential is lowered but it seems unlikely that this will be of any significance; the signals are very small. Even assuming that the entire dark signal represents semiquinone it represents only 0.5% of the signal identified with the light-induced reaction center $(BChl)_2^+$ radical (Fig. 2, bottom) which represents only 1 spin/reaction center. The comparison provides us with a stability constant for the main Q complement of 10^{-7}; however, because of the assumption, this is regarded as an upper limit. Essentially the same results were obtained at pH 7.5 and 9.0. In each case it was established that the dark- and light-induced signals were similarly microwave non-saturated.

Further searches for semiquinone radicals were made with chromatophore samples under the following conditions: Samples at the same redox potentials as shown in Fig. 2 were illuminated in the presence or absence of 10 μM FCCP and 5 μM antimycin for about 30 s followed by prompt or a 10 s wait in the dark before freezing in liquid nitrogen in the dark. The same experiments were repeated except that the redox mediator dyes and potentiometric control were replaced by 1 mM ascorbate or 20 mM succinate under anaerobic conditions. The above experiments were also done at pH 7.5 and 9.

In all cases we failed to detect signals that would represent stable quinone radicals. A search over a wide range of magnetic field strength for EPR signals that might represent semiquinone radicals which are significantly modified was also negative, and we could not resolve the characteristic split signal seen in mitochondria which is apparently caused by the spin interaction between two stable semiquinone radicals [40,41] that are close to the iron-sulfur protein center S3 of the succinate dehydrogenase. In this respect it is pertinent to point out that the succinate dehydrogenase of *Rps. sphaeroides* is similar in redox centers to the mitochondrial enzyme [42,43].

Thus the EPR analysis supports the n-value of 2 obtained in the redox titrations of the main 19 Q complement, and efforts so far have not revealed stable semiquinone species identiable with the 19 Q complement, succinate dehydrogenase, or the Q-b/c_2 oxidoreductase.

Measurements of the redox state of ubiquinone using solvent extraction

Incubation of chromatophores with succinate in the dark for 5—10 min led to the reduction of up to 16 Q molecules, probably members of the 19 Q complement (Table I). From the E_{m7} of 25 mV and 92 mV for the succinate/fumarate couple and the 19 Q complement respectively, a high level of reduction of the Q by an excess of succinate is expected if these two can approach redox equilibrium.

Table II shows that there was little effect on the redox level of the Q complement during continuous illumination of coupled chromatophores poised at various redox potentials. The same was true in the presence of uncoupler or antimycin although with a notable exception at higher potentials where, at 300 mV, about 6 of the total 25 ubiquinones became reduced in the light. In order to study this effect more fully, an alternate and prompt way of measur-

TABLE I

REDUCTION OF UBIQUINONE BY SUCCINATE IN THE DARK

Chromatophores were suspended (400 μM BChl) in 20 mM MOPS, 100 mM KCl, 1 mM $MgCl_2$, pH 7.0 in test tubes. In aerobic experiments the test tube containing 0.5 ml of the suspension was shaken vigorously in the air for 30 s and immediately a cooled acetone/methanol solution was added. In the anaerobic experiments the air was replaced by argon gas and anaerobiosis was maintained for about 20 min before the cooled acetone/methanol was added. The incubation time with 20 mM succinate was for about 10 min. The total amount of ubiquinone was determined in each determination separately by adding $FeCl_3$ to the acetone/methanol extract.

Aerobic or anaeriobic	Succinate (20 mM)	Antimycin (10 μM)	% reduced
Aerobic	—	—	0
Aerobic	+	+	65
Anaerobic	+	—	52
Anaerobic	+	+	65

ing the redox level of the Q complement was developed; this was based on the protons bound concomitantly with Q reduction.

Measurement of the redox state of ubiquinone complement by proton binding

We have confirmed here that two protons are required at equilibrium per two electron reduction of each Q in the 19 Q complement. It has previously been established in kinetic/stoichiometry experiments [18,23] that two protons/reaction center are taken up by the chromatophore following a single turnover flash. One of the protons bound is antimycin-sensitive (H^+_{II}) while the other (H^+_I) is not. Although the details concerning H^+_{II} are still unclear, the agent responsible for the antimycin-insensitive H^+_I binding under these non-equilibrium conditions is considered to be Q^- to form Q·H ($Q{\cdot}H/Q^-$, $pK = 8.5$; see ref. 18). In the presence of antimycin, at pH values well below this pK and also below the $pK = 7.5$ on the reduced form of cytochrome b_{50} [21], cyto-

TABLE II

REDOX STATE OF UBIQUINONE AT VARIOUS E_h VALUES WITH OR WITHOUT ILLUMINATION AND ANTIMYCIN AT pH 7.0

Chromatophores were suspended (approx. 500 μM BChl) and redox poised in 20 mM MOPS, 100 mM KCl, 1 mM $MgCl_2$, pH 7.0 in the presence of 100 μM ferrocyanide, 10 μM each of 2,3,5,6-tetramethylphenylenediamine, *N*-methylphenazonium methosulfate, *N*-ethylphenazonium ethosulfate and pyocyanine. When the samples were illuminated the period in the light was 30 s before addition of the cooled acetone/methanol. The total content of ubiquinone was determined in each determination. The values in the third and fourth column are average percent reduction and standard deviation; the number in parenthesis is the number of experiments done with different chromatophore preparations.

E_h (mV)	Light or dark	— Antimycin	+ Antimycin (10 μM)
25	D	74 (1)	76 (1)
25	L	70 (1)	77 (1)
90	D	53 ± 7 (3)	60 ± 5 (5)
90	L	51 ± 7 (3)	55 ± 8 (5)
150	D	8 ± 6 (3)	8 ± 5 (4)
150	L	17 ± 6 (3)	32 ± 10 (4)
300	D	0 ± 0 (2)	0 ± 0 (5)
300	L	3 ± 1 (2)	22 ± 4 (5)

chrome b_{50} is reduced by Q·H in $t_{1/2} \sim 2$ ms to form ferrocytochrome b_{50} H [21]. Thus the Q-b/c_2 oxidoreductase chemically retains H_I^+, and under these conditions the rate of re-appearance of the H_I^+ in the external aqueous phase of the chromatophores is not promoted by uncoupler; the appearance is governed by the time (s) that cytochrome b_{50} H takes in the antimycin-inhibited state to become re-oxidized and release the H^+ (for more details seen the accompanying manuscript [48] and ref. 21). Thus we may surmize that if the 19 Q complement is in contact with the reaction center Q-cytochrome b_{50}, we would expect H_I^+, retained in the presence of uncoupler, to reflect the degree of reduction of the Q complement (minus one H^+ for cytochrome b_{50}). However, since the number of reducing equivalents available for delivery to the cytochrome b_{50} and quinones via the reaction center, in the presence of antimycin, is restricted to the $(BChl)_2$ and the two cytochrome c_2 hemes, an external electron donor is required that is capable of reducing the light-oxidized $(BChl)_2$ or cytochrome (see also [25]). Fig. 3 shows such an experiment done at pH 6.0. Using ferricyanide (plus a catalytic amount of 2,3,5,6-tetramethylphenylenediamine, to aid electron transfer through the membrane and increase the reliability of the

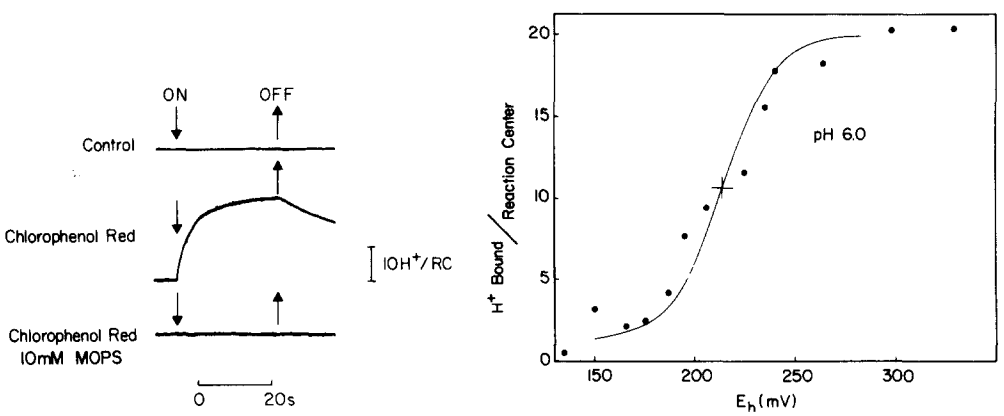

Fig. 3. Time course of proton binding to chromatophore membranes. Chromatophores (reaction center protein concentration 150 nM) were suspended in 100 mM KCl, 1 mM $MgCl_2$ in the presence of 200 μM ferrocyanide, 0.5 μM 2,3,5,6-tetramethylphenylenediamine, 10 μM FCCP, 5 μM antimycin. pH and E_h were adjusted to 6.0 and 300 mV, respectively. Absorbance changes were monitored at 586 nm. The top trace is a control taken without addition of chlorophenol red as pH indicator. In the middle trace 25 μM chlorophenol red was present. In the bottom trace 25 μM chlorophenol red and 10 mM MOPS (pH 6.0) were present. Because of the very slow course of proton efflux, a new 'dark-adapted' sample was employed for each determination. In all cases, to avoid pre-illumination effects the monitoring beam was turned on by manual shutter several seconds before the continuous illumination was turned on. Continuous illumination was provided through a 2 cm water layer and Wratten 88A filter. Calibration of the absorbancy change of the pH indicating dye was done by measuring the absorption change at 586 nm upon addition of standard HCl solution.

Fig. 4. Relationship between H^+ binding and ambient redox potential. Chromatophores (reaction center concentration 162 nM) were suspended in 100 mM KCl, 1 mM $MgCl_2$ in the presence of 200 μM ferrocyanide, 1 μM 2,3,5,6-tetramethylphenylenediamine, 10 μM FCCP, 5 μM antimycin, and 25 μM chlorophenol red. Each point represents the proton binding of a fresh sample raken from the same stock chromatophore suspension and adjusted to pH 7 and to the specified ambient potential. In this experiment a train of 192 flashes spacing 356 ms was used instead of continuous illumination; the results were similar using either source of illumination. Calibration of the absorbance change at 586 nm was done with standard HCl for each determination.

redox potentiometry assay) as the artificial electron donor with this system, a maximum value of 19 protons (i.e., equivalent to 9—10 QH_2) were bound/reaction center (Fig. 3); this is in agreement with 8—9 QH_2 obtained with the organic solvent extraction method measured in the same preparation under similar conditions. Consistently good agreement was obtained between the two methods. Obviously the H^+ binding method suffers from being indirect and its deployment is quite restricted. However, when conditions permit, it provides a ready method of measuring the time course of Q redox changes, and, compared to the solvent extraction method, requires 25 times less material.

The E_h dependency of the light-induced Q reduction in the presence of antimycin

The extent of Q reduction measured in the presence of antimycin and uncoupler, using ferrocyanide as the electron donor, and activating with saturating, single-turnover flashes delivered every 356 ms, was found to be dependent on the ambient redox potential (Fig. 4). At pH 6.0 and E_h = 150 mV, there was little net proton binding on a seconds timescale. At E_h values below 150 mV, where the 19 Q complement becomes predominantly reduced at equilibrium at pH 6, there was no evidence of light-induced net oxidation of the QH_2. The 150 mV (pH 6.0) value is equivalent to the 90 mV (pH 7.0) value of Table II where, in agreement, there was no light-induced net

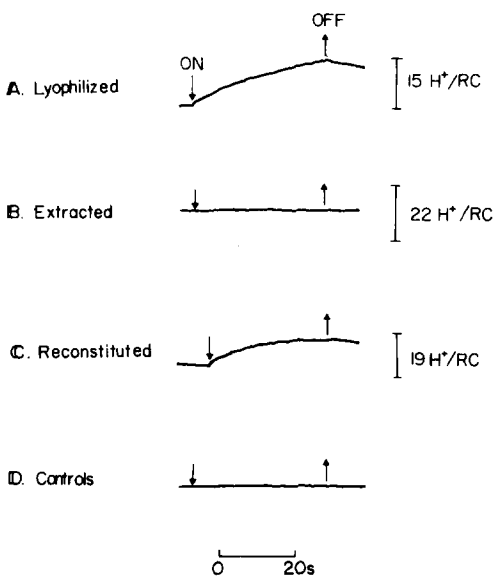

Fig. 5. Effect of extraction of ubiquinone and reconstitution with ubiquinone on proton binding to chromatophores in the presence of antimycin and uncoupler. Chromatophores were suspended in 100 mM KCl, 1 mM $MgCl_2$ in the presence of 200 μM ferrocyanide, 0.5 μM 2,3,5,6-tetramethylphenylenediamine, 10 μM FCCP, 2 μM valinomycin, 5 μM antimycin, and 25 μM chlorophenol red as pH indicator. pH and E_h of suspensions were adjusted to 6.0 and 300 mV, respectively. Proton binding was monitored by the absorbance increase at 586 nm which was calibrated by addition of standard HCl. The reaction center $(BChl)_2$ concentration was 180 nM in A, 110 nM in B, 130 nM in C. In D the 'controls' trace is typical of the absence of absorbance changes displayed by all chromatophore preparations (lyophilized, extracted or reconstituted) either in the absence of chlorophenol red or after addition of 10 mM MOPS of pH 6.0 in the presence of chlorophenol red.

reduction or oxidation. Above 150 mV the extent of proton binding as a function of redox potential showed a Nernst curve with $n = 2$ and E_{m6} of about 210 mV. The kinetics of Q reduction and dark re-oxidation were similar throughout the range. The curve coincides the Nernst curve of the ubiquinone-cytochrome b/c_2 oxidoreductase redox center ZH_2/Z [44] which is considered to be an immediate electron donor to cytochrome c_2.

Effect of isooctane extraction and reconstitution on light-induced quinone reduction

Fig. 5 shows that proton binding was greatly diminished by isooctane extraction and was restored by addition of ubiquinone to the extracted chromatophores. The extraction was a partial extraction since at least the primary Q of the reaction center must still remain after extraction because of an unaltered light-activated electron transfer in the reaction center and between the $(BChl)_2^+$ and cytochrome c_2. In each step of the experiment it was established that photochemistry and cytochrome c_2 oxidation was normal both in amplitude and kinetics.

Discussion

The redox data obtained in dark titrations indicate the presence of at least two thermodynamically different ubiquinone complements in the chromatophores. Accuracy of the direct solvent extraction/redox titration method did not itself yield indications of further heterogeneity of the Q complement of *Rps. sphaeroides*, but with information from other sources some assignments may be discussed. The reaction center primary Q ($E_{m7} = -15$ mV; $n = 1$) would in the titration presented here be identified with the low potential group. Lack of data on the secondary Q currently does not permit any assignment; it may be a special low potential QH_2/Q couple although it is also possible that it has the same properties as the 19 Q complement. Thus the low potential group is for the most part unaccounted for and more work is required. It is pertinent to note however, that a similar group exists in mitohcondria which has been identified with the NADH-ubiquinone oxidoreductase [29].

Two pieces of evidence, neither of which are conclusive, suggest that the immediate electron donor to cytochrome c_2 could be a special Q: the donor, desginated Z [44], has an E_{m7} of 155 mV and at equilibrium from pH 5 to 11 the redox reaction is $Z + 2e^- + ZH^+ = ZH_2$, a reaction similar to quinone reduction and oxidation [22]. Furthermore, it has recently been demonstrated in *Rps. capsulata* [3] that partial Q extraction dramatically affected light-oxidized cytochrome c_2 re-reduction and light-induced cytochrome b reduction and re-oxidation. A similar study with *Rps. sphaeroides* using single-turnover flash activation has yielded a similar result [45,46]; in the partially extracted chromatophores, redox poised at 100 mV (pH 7.0), flash oxidized cytochrome c_2 was reduced with a half-time of up to 100 ms instead of less than 5 ms, whereas re-addition of Q reconstituted the <5 ms reaction. If Z is indeed a special Q with an E_{m7} of 155 mV it should be observed as an $n = 2$ step at the high potential end of the redox titration curves of the 19 Q complement of Fig. 1. However, in *Rps. sphaeroides* evidence suggests that there is only one

Z per Q-b/c_2 oxidoreductase [47] (similar work has been done on *Rps. capsulata* [25]), and currently the analysis is not of sufficient accuracy to resolve one Q with an E_m only 60 mV higher than the others of 19 Q complement.

Z has proven to be a component central to the operation of the reaction center Q-b/c_2 oxidoreductase cycle [22]. An aspect of the control exerted by Z on the system may be evident in the antimycin-induced, uncoupler-insensitive Q reduction seen in Table II and Figs. 3 and 4. The role of Z in this process can be partially explained as a steady state phenomenon as follows: Depending on the state of reduction of Z at the time of flash activation, the rates of electron transfer through the Q-b/c_2 part of the system are markedly altered under coupled or uncoupled conditions even in the presence of antimycin [22,48]. For instance if Z is reduced before light activation, the entire reaction center Q-b/c_2 oxidoreductase can turnover with a half-time of a few ms; addition of antimycin extends electron transfer through the Q-b/c_2 oxidoreductase into the 200—300 ms half-time range. With Z oxidized, the flash-actiavated electron transfer through the Q-b/c_2 oxidoreductase takes in the region of 50—100 ms half-time, while in the presence of antimycin this half-time becomes about 10 s [48]. As such the extent of light-induced reduction of the 19 Q complement could be governed by the prevailing rate of electron transfer through the Q-b/c_2 oxidoreductase, compared to the various rates of redox interaction by the added redox mediator agents with various points in the system. It can readily be appreciated that when the rate of electron transfer through the Q-b/c_2 oxidoreductase is slow, it is more likely that added redox agents, natural and artificial, will act as net electron donors or acceptors to or from the oxidoreductase. Thus, when chromatophores are poised with Z oxidized in the presence of antimycin, the seconds half-time for the re-oxidation of light-generated ferrocytochrome *b* [48] and re-reduction of ferricytochrome c_2 permits ferrocyanide to be competitive as an electron donor for the long-lived light-generated $(BChl)_2^+$ directly or via ferricytochrome c_2. In the experiment of Fig. 4 ferrocyanide was chosen because it is an electron-only redox agent (obligatory for the H^+ assay method) and because its concentration does not change greatly over the range of study (i.e., the E_h range is well away from its E_m) which this minimizes unwanted contributions to the extent of Q reduction furnishes by a variation in electron donor rates; concerning this possibility it was also established that 2,3,5,6-tetramethylphenylenediamine, added to provide a more reliable E_h reading, had no detectable effects on the results in the 0.5—5 μM range. Thus with 200 μM ferrocyanide with 0.5 μM 2,3,5,6-tetramethylphenylenediamine present, $(BChl)_2^+$ became half reduced in 440 ms and 275 ms at 300 mV and 200 mV, respectively, after each flash delivered every 356 ms. The flash interval chosen delivered to the reaction center quinones approx. 0.4 and 0.6 electron equivalents/reaction center per flash at 300 and 200 mV, respectively. The overall effect therefore is that light drives the electrons from ferrocyanide via the reaction center to the Q-b/c_2 oxidoreductase, and when Z is oxidized and the system is slow, electrons accumulate in the 19 Q complement. With Z reduced before activation, the approx. 250 ms half-time for cytochrome b_{50} re-oxidation [48] and cytochrome c_2-reduction is fast enought to compete with the ferrocyanide and greatly diminish the extent of accumulation of reduced Q. These factors may be expected to yield

an E_h dependency of QH_2 reduction that approaches the Nernst curve of the ZH_2/Z couple ($E_{m6} = 215$; $n = 2$) as was shown to be the case in Fig. 4.

If the above explanation is correct, and the fractional reduction of the 19 Q complement is a steady state manifestation, then use of a faster electron donor to $(BChl)_2^+$ and ferricytochrome c_2 will yield a higher level of Q reduction with Z oxidized and possibly some when Z is reduced. The water-soluble hydroquinone is a much faster donor than ferrocyanide and interacts not only with the $(BChl)_2^+$ but also the ferricytochrome c_2, and experiments with it support the steady state explanation. Thus with Z oxidized before continuous illumination of chromatophores in the presence of antimycin and uncoupler hydroquinone (800 μM) served as the electron source to drive up to 80% (i.e. 15 of the 19 Q complement) of the total Q complement reduced. This was analyzed by solvent extraction. Controls done with ferrocyanide on the same chromatophores and analyzed similarly yielded no more than 35% of the total in agreement with results already presented. Further support for the steady state explanation comes from the observation that 800 μM hydroquinone is fast enough to promote Q reduction even with Z predominantly reduced before illumination.

In general it is easier to design experiments to promote net reduction of the Q complement rather than, starting with the Q complement reduced, experiments to promote net oxidation of QH_2. This is because the control conferred on the system by Z favors high potential experiments and also because, in general, redox agents are more likely to interact more proficiently with $(BChl)_2^+$ or ferricytochrome c_2 than they do with cytochrome b_{50} (see [49]). It is also pertinent to add that in the absence of external interference from substrate level concentration of succinate/fumarate, $NADH/NAD^+$ or artificial redox agents, a cyclic system of the kind formed by the reaction center Q-b/c_2 oxidoreductase, which possess a relatively few redox centers, has the capacity to effect only minor changes in the steady state level of the 19 Q complement. More dramatic redox changes in the 19 Q complement and perhaps the low potential complement may be encountered in studies on reverse electron flow from succinate to NAD^+, driven by energy provided by the light-driven reaction center Q-b/c_2 oxidoreductase.

The following additional tentative conclusions can be drawn from this work. (a) The QH_2 generated in the light as described above clearly reacts directly with the ferro/ferricyanide (with catalytic amounts of 2,3,5,6-tetramethylphenylenediamine) only on a very slow time scale. Similarly it is clear that in the presence of antimycin the QH_2 reacts extraordinary slowly with the predominantly oxidized Q-b/c_2 oxidoreductase. For example, after illuminating at an ambient redox potential of 330 mV which holds cytochrome c_2 in a predominantly oxidized state in the dark, the QH_2 takes over 10 min to approach completion in complete darkness. (b) Two pieces of information are pertinent to the recognition of Z as a molecule separate from the 19 Q complement. (i) The first point is that if Z represented any one Q of the 19 Q complement (i.e., assuming the 19 Q complement is integral to the Q-b/c_2 oxidoreductase (see [50]) then the E_h dependency of the reduction of one Q out of 19 would have a half-point at some 45 mV higher than the true E_m of the 19 Q complement. At pH 7.0 this would be 135 and 195 mV at pH 6.0, which is 20 mV

lower than the ZH_2/Z Nernst curve; also the Nernst curve would be stronly asymetric (see [22,26]), a possibility which is not observed. (ii) The second point is less reliant of experimental accuracy. If any of the 19 quinones reduced is ZH_2, then at high potentials (i.e., starting with Z already oxidized before illumination), once one Q molecule is reduced in the light then this would be equivalent to starting with Z reduced and thus after the reduction of one Q, further Q reduction would not be expected, a sharp contrast to the observed results.

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References

1 Rudney, H. (1961) J. Biol. Chem. 236, PC39
2 Okayama, S., Yamamoto, N., Nishikawa, K. and Horio, T. (1968) J. Biol. Chem. 243, 2995–2999
3 Melandri, A.B. and Melandri, B.A. (1977) FEBS Lett. 80, 459–464
4 Carr, N.G. and Exell, G. (1965) Biochem. J. 96, 688–692
5 Takamiya, K., Nishimura, M. and Takamiya, A. (1967) Plant Cell Physiol. 8, 79–86
6 Maroc, J., DeKlerk, H. and Kamen, M.D. (1968) Biochim. Biophys. Acta 162, 621–623
7 Kakuno, T., Bartsch, R.G., Nishikawa, K. and Horio, T. (1971) J. Biochem. 70, 79–94
8 Cogdell, R.J., Brune, D.C. and Clayton, R.K. (1974) FEBS Lett. 45, 344–347
9 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3491–3495
10 Tiede, D.M., Leigh, J.S. and Dutton, P.L. (1978) Biochim. Biophys. Acta, in press
11 Dutton, P.L., Prince, R.C., Tiede, D.M., Petty, K.M., Kaufmann, K.J., Netzel, T.L. and Rentzepis, P.M. (1976) in Brookhaven Symposia in Biology: No. 28 (Olson, J.M. and Hind, G., eds.), Brookhaven National Laboratory, Upton
12 Prince, R.C. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 329–334
13 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1978) Biophys. J. 21, 8a
14 Wraight, C.A. (1978) Biophys. J. 21, 8a
15 Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516–524
16 Wraight, C.A. (1977) Biochim. Biophys. Acta 459, 525–531
17 Parson, W.W. (1969) Biochim. Biophys. Acta 189, 384–396
18 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 335–345
19 Prince, R.C., Baccarini-Melandri, A., Hauska, G.A., Melandri, B.A. and Crofts, A.R. (1975) Biochim. Biophys. Acta 387, 212–227
20 Prince, R.C. and Dutton, P.L. (1975) Biochim. Biophys. Acta 387, 609–613
21 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 346–353
22 Prince, R.C. and Dutton, P.L. (1977) Biochim. Biophys. Acta 462, 731–747
23 Petty, K.M., Jackson, J.B. and Dutton, P.L. (1977) FEBS Lett. 84, 299–303
24 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
25 Crofts, A.R. and Bowyer, J. (1977) in The Proton and Calcium Pumps (Azzone, G.D., Varon, M., Metcalfe, J.C., Quagliariello, E. and Siliprandi, N., eds.), pp. 55–64, Elsevier, North Holland
26 Dutton, P.L., Bashford, C.L., van den Berg, W.H., Bonner, H.S., Chance, B., Jackson, J.B., Petty, K.M., Prince, R.C., Sorge, J.R. and Takamiya, K. (1977) in Photosynthesis '77, Proc. 4th Int. Congr. Photosynthesis (Hall, D.O., Coombs, J. and Goodwin, T.W., eds.), Biochem. Soc. London
27 Redfearn, E.R. (1967) Biochim. Biophys. Acta 131, 218–220
28 Takamiya, K. and Takamiya, A. (1969) Plant Cell Physiol. 10, 363–373
29 Kröger, A. and Klingenberg, M. (1973) Eur. J. Biochem. 39, 313–323
30 Erecinska, M. and Wilson, D.F. (1976) Arch. Biochem. Biophys. 174, 143–157
31 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556

32 Barr, R. and Crane, F.L. (1971) Methods Enzymol. 23, Part A, 372—408
33 Dutton, P.L. (1978) Methods Enzymol., in press
34 Takamiya, K. and Nishimura, M. (1975) Plant Cell Physiol. 16, 1061—1072
35 Dutton, P.L. and Leigh, J.S. (1973) Biochim. Biophys. Acta 314, 178—190
36 Morrison, L., Runzquist, J. and Loach, P. (1977) Photochem. Photobiol. 25, 73—84
37 Urban, P.F. and Klingenberg, M. (1969) Eur. J. Biochem. 9, 519—525
38 Kakuno, T., Hosoi, K., Higuti, T. and Horio, T. (1973) J. Biochem. 74, 1193—1203
39 Michaelis, L., Schubert, M.P., Reber, R.K., Kuck, J.A. and Granick, S. (1938) J. Am. Chem. Soc. 60, 1678—1683
40 Ruzicka, F.J., Beinert, H., Schefler, K.L., Dunham, W.R. and Sands, R.H. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2886—2890
41 Ohnishi, T., Salerno, J.C., Blum, H., Leigh, J.S. and Ingledew, W.J. (1977) in Bioenergetics of Membranes (Packer, L., Papgeorgiou, G.C. and Trebst, A., eds.), Elsevier, Holland
42 Ingledew, W.L. and Prince, R.C. (1977) Arch. Biochem. Biophys. 178, 303—307
43 Ohnishi, T. (1973) Biochim. Biophys. Acta 301, 105—128
44 Cogdell, R.J., Jackson, J.B. and Crofts, A.R. (1972) Bioenergetics 4, 413—429
45 Takamiya, K. and Dutton, P.L. (1978) Abstr. 6th Int. Biophys. Congr., Kyoto
46 Bashford, C.L., Prince, R.C., Takamiya, K. and Dutton, P.L. (1979) Biochim. Biophys. Acta 545, 223—235
47 Prince, R.C., Bashford, C.L., Takamiya, K., van den Berg, W.H. and Dutton, P.L. (1978) J. Biol. Chem., in press
48 Petty, K., Jackson, J.B. and Dutton, P.L. (1979) Biochim. Biophys. Acta 546, 17—42
49 Dutton, P.L. and Prince, R.C. (1978) FEBS Lett., in press
50 Crofts, A.R., Crowther, D. and Tierney, G.V. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.) pp. 233—241, Elsevier, North Holland
51 Salerno, J.C. and Ohnishi, T. (1976) Arch. Biochem. Biophys. 176, 757—765
52 Clayton, R.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L.P., eds.), p. 498, Antioch Press, Yellow Springs