Determination of the amount and the type of quinones present in single crystals from reaction center protein from the photosynthetic bacterium *Rhodopseudomonas viridis*

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The identity and amount of quinone in single crystals from reaction center protein from *Rhodopseudomonas viridis* have been determined using HPLC, optical spectroscopy and mass spectrometry. The ratio menaquinone-9/ubiquinone-9 for solutions of reaction center protein of *Rps. viridis* was 0.93 ± 0.05. After crystallization of the reaction center solution, the quinone ratio was 1.6 ± 0.1. These observations show that about half of the ubiquinone-9 can be lost during crystallization.

Photosynthesis Rhodopseudomonas viridis *Crystal* *Menaquinone* *Ubiquinone* *HPLC*

1. INTRODUCTION

In the reaction center protein from *Rhodopseudomonas sphaeroides* R-26 the structure and function of the two quinone acceptors have been studied extensively. Previous workers reported that this protein contained two Q-10 molecules [1–4]. From optical [5,6] and ESR experiments [7] it was concluded that these two quinones act in series as primary and secondary electron acceptors. In other organisms a similar function for the quinones was found [8,9].

Very recent results, however, may complicate the notion that these quinones act only in series. X-ray and EPR analyses of crystals from *Rps. viridis* [10,11] suggest that the reaction center protein may provide two different but symmetrical pathways for charge separation. The possibility of a dual pathway raises questions about the function of the quinones in the reaction center protein. If each pathway includes one quinone then the quinones may also act in parallel; if one pathway contains both quinones, then symmetry is broken and the other potential pathway would appear to be without a clear function. X-ray data have given no definitive answer to this problem, but do possibly suggest a twin chlorophyll-pheophytin pathway in which a single quinone exists only in one half of the electron donor acceptor assembly with approximate C2 symmetry [10].

The identity and an estimation of the amount of quinones present in crystals from reaction centers of *Rps. viridis* are relevant to the question of symmetry as well as to the X-ray data. The quinone content is crucial to the interpretation of both charge separation and structure. However, up to now, consistent information about the identity and the amount of quinone acceptors in the reaction center has not been available. Conflicting reports indicate that the reaction center protein of *Rps. viridis* contains either MQ [12], or one MQ-9 and one Q [13] or one MQ-7 and one Q-10 [14]. The initial X-ray data are consistent with either MQ-6 or Q-6 [10]. Even if the exact quinone content were

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Abbreviations: LDAO, lauryldimethylamine oxide; HPLC, high-performance liquid chromatography; Q, ubiquinone; MQ, menaquinone

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known for the reaction center solution, such data would only set an upper limit to the amount present in a crystal since quinone may be lost during the crystallization.

In this study, using the combined strength of HPLC, optical spectroscopy and mass spectrometry, we have determined not only the identity but also the amount of quinone present in crystals and in reaction center solution from *Rps. viridis* prior to crystallization.

2. MATERIALS AND METHODS

Reaction center protein from *Rps. viridis* was isolated as in [15]. Crystals from reaction center protein from *Rps. viridis* were grown using a modified procedure based on [16,17]. The LDAO concentration of the starting solution was increased to 0.3%, and the starting concentration of reaction centers was increased to about 40 μM. From this solution, crystals grew in 3 weeks to a maximum length of 3 mm with a yield of about 50 crystals per ml starting solution. About 100 crystals were used for the quinone extraction. These crystals were washed 5 times in 15 ml of 2.5 M ammonium sulphate, 1% heptanetriol, 0.1% LDAO and 50 mM phosphate buffer, pH 6, to remove uncrystallized protein. The crystals were then dissolved in 10 ml of 0.1% LDAO, 1 mM EDTA, 10 mM Tris, pH 8, followed by overnight dialysis against the same buffer. The quinones in this solution were extracted by a slightly modified method from [18]. Briefly, 1 ml of the reaction center solution was treated with 200 μl of 0.1 M $FeCl_3$ and then with 10 ml acetone/methanol (50:50). After stirring for 5 min the quinones were extracted with three 30-ml portions of hexane. The combined hexane extracts were washed twice with 10 ml methanol/water (90:10) and twice with water, and concentrated by evaporation at room temperature. A similar procedure was applied for the isolation of quinones from a single crystal and yielded results essentially identical to using many crystals.

Stainless-steel HPLC columns (25 × 1.00 cm) packed with 5 μm Ultrasphere-ODS (Altex, Berkeley, CA) were used for quinone separation. The mobile phase consisted of a mixture of tetrahydrofuran (30%) and acetonitrile (70%). The column was operated at ambient temperature at a flow rate of 3 ml/min. The liquid chromatograph used was a modification of a Beckman Model 334 gradient liquid chromatograph (Beckman, Berkeley, CA). This system is equipped with dual constant flow pumps (model 110A), a microprocessor-controller (model 421), a sample injector (model 210) fitted with a 20 μl loop and a mixing chamber. The detector was a variable wavelength ultraviolet-visible (UV-vis) detector (model 155-10; Beckman), set at 270 nm. Spectra were recorded on an HP 3392A integrator. Separation of quinones on a preparative scale was also performed on a Zorbax ODS column (25 × 2.12 cm) (DuPont Instruments) with IR detector (Altex).

The mass spectra were obtained with a ^{252}Cf plasma desorption time-of-flight mass spectrometer (PDMS) constructed at Argonne National Laboratory [19]. The ^{252}Cf-PDMS is one of several mass spectrometric techniques developed which can be used to analyze compounds which cannot be readily volatilized thermally without decomposition. Ions with a mass to charge ratio equal to, or within a few mass units of, the original molecule are generally observed, in addition to some fragmentation products of the molecule.

The amount of quinones was determined optically using extinction coefficients of ϵ^{248} = 18900 $M^{-1} \cdot cm^{-1}$ for MQ [20] and of ϵ^{275} = 14700 $M^{-1} \cdot cm^{-1}$ for Q [21]. The molar absorptivity of reaction centers was ϵ^{960} = 12300 $M^{-1} \cdot cm^{-1}$ [22]. Reduction of the Q was done as in [23], and of MQ as in [24]. MQ standards with different isoprenoid chain lengths (MQ-6, -7, -8, -9, -10) were a generous gift from Hoffman LaRoche, Basel, Switzerland. Q standards (Q-6, -7, -9, -10) were obtained from Sigma, St. Louis. All organic solvents were HPLC grade.

3. RESULTS

Fig.1A shows the HPLC chromatogram when 5 μl of the hexane extract from reaction center solution was injected; fig.1B shows the same for the crystal extract. The identities of the species with retention times of 17 and 27 min were determined in 3 ways. First, MQ and Q standards with different isoprenoid chain lengths (MQ-6, -7, -8, -9, -10; Q-6, -7, -9, -10) were analyzed separately by HPLC. We found that the retention time dif-

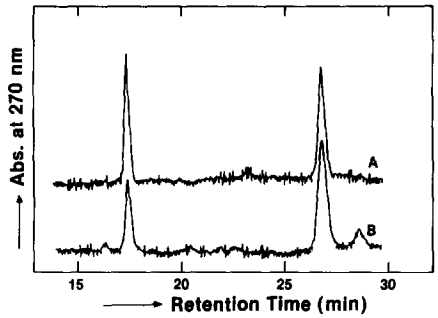

Fig.1. Chromatogram of quinones from reaction center solution (A) and from reaction center crystals (B) of *Rps. viridis*.

ference between the MQs and Qs with different chain lengths was more than 3 min. The MQ and Q standards were co-chromatographed with the hexane extract of reaction centers. The Q-9 peak coincided with the peak at 17 min and the MQ-9 peak with the 27-min peak. Secondly, the absorption spectrum of the two species with retention time of 17 and 27 min was recorded from 230 to 360 nm. A preparative HPLC column was used to obtain a sufficient amount of material for measuring the absorption spectra. The purity and the identity of the collected fraction from the preparative column were confirmed by analyzing aliquots on the analytical column. The fraction with a retention time of 17 min exhibited the characteristic absorption spectrum of Q (maximum at 270 nm [22,23]). The fraction with a retention time of 27 min showed the characteristic absorption spectrum of MQ (maxima at 248, 262 and 265 nm [21,24]). Absorption difference spectra of reduced species minus oxidized species confirmed these results. Finally, the molecular mass of the two species under study was determined by mass spectrometry. The species with retention times of 17 and 27 min corresponded to compounds with molecular masses of 795.6 ± 0.3 and 784.9 ± 0.3 Da, respectively. This is in very close agreement with the molecular masses of 794.6 and 784.6 Da expected for monoisotopic ubi- and menaquinone. The lower mass region showed a complex set of fragments reflecting a quinone profile.

The ratio of MQ to Q in the reaction center in solution and in crystals is readily observed in fig.1B. This ratio is larger in the reaction center crystals. By comparing the areas of the peaks and correcting for the slightly different extinction coefficients of MQ and Q at 270 nm [21,24], we found that the ratio MQ/Q for solution reaction centers was 0.93 ± 5%; for crystal reaction centers it was 1.6 ± 5%.

To calculate the number of MQ-9 and Q-9 molecules per reaction center, the HPLC was calibrated using mena- and ubiquinone-9 standards of different concentrations. We also tested the effectiveness of the quinone extraction method. When aliquots of the 3 hexane extractions and the methanol/water solutions were analyzed on HPLC, it was found that more than 99% of the quinones were extracted after the second hexane extraction and that less than 1% of the quinones was present in the methanol/water solution.

Using a known amount of reaction center crystals we found that the number of quinones was 1.0 ± 30% for MQ-9 and 0.6 ± 30% for Q-9. For solution reaction centers we found 1.0 ± 30% for MQ-9 and 1.0 ± 30% for Q-9 per reaction center. The largest contribution to the error in these numbers arises from the uncertainty in the extinction coefficient for the reaction centers at 960 nm [22]. To establish the homogeneous nature of the quinone content in crystals we have measured the MQ/Q ratio of one single crystal using HPLC. The single crystal results are identical to those mentioned above.

4. DISCUSSION

We find only MQ-9 and Q-9. Table 1 summarizes our results by listing the appropriate quinone content per reaction center protein and the ratio of MQ to Q in each reaction center protein.

We have shown that some Q-9 is lost during crystallization. We suggest that the loss of quinone during crystallization may account for finding only

Table 1

	MQ-9/RC	Q-9/RC	MQ/Q
RCs prior to crystallization	1 ± 30%	1 ± 30%	0.9 ± 5%
Crystallized RCs	1 ± 30%	0.6 ± 30%	1.6 ± 5%

a single quinone acceptor by the initial X-ray analysis. In addition, the X-ray data could be refined by analyzing the structure in terms of MQ-9 or Q-9 units. We further suggest that the position of the Q-9 in the reaction center protein of *Rps. viridis* could possibly be determined by growing the crystals in a solution enriched in Q-9. The location and the number of quinones in the reaction center is relevant to the question of a single or dual pathway for electron transfer.

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