Anal. Chem. **2006,** *78,* 5046−5051

Scanning Electrochemical Microscopy of the Photosynthetic Reaction Center of *Rhodobacter sphaeroides* in Different Environmental Systems

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The present work uses a scanning electrochemical microscopy technique to study systems containing the membrane-bound reaction center protein (RC) from the purple photosynthetic bacteria *Rhodobacter spheroides* to chromatophores (spherical reorganization of cell membrane following its mechanical rupture) and liposomes (reconstituted membrane systems at lower degree of complexity). Scanning electrochemical microscopy is a useful tool to investigate redox processes involving a RC, because the effective heterogeneous rate constants for the redox reaction with different mediators can be measured. The technique is also able to provide information on the role of the outer cell membrane permeation on the kinetics of the electron-transfer processes and to obtain more insight into the nature of the species involved.

The secret to the molecular logic of photosynthetic processes resides in the specific organization of the components of the photosynthetic apparatus into protein membrane complexes, built up by several molecular classes, cooperating in complementary roles: architectonic support, light absorption, energy and electron transfer, and product separation. A series of electron-transfer reactions between donor and acceptor substances, immobilized in membranes or dissolved in the aqueous phase, are implicated in the conversion of solar energy in photosynthetic systems.[1−5]

The redox properties of the photosynthetic apparatus components are well suited for electrochemical studies that characterize species and monitor the involved processes.[6] When biological systems are composed of several classes of molecules, such as membrane proteins, difficulties arise because of the large number of species involved in the electron transport and of the dependence of the redox potential of these species on the micro- and macroenvironment in which they are imbedded. Further problems are related to the different conformations of some membrane proteins under dark and light conditions, which affect the evaluation of the physicochemical properties of the species under examination.

Various attempts to directly follow electrochemically the redox path of species involved in photosynthesis are reported in the literature.[7−12] Most of them have been carried out in the presence of redox couples that either mediate the electron transfer to an electrode or undergo selective electron transfer with one of the components of the biological system.

A recent scanning electrochemical microscopy (SECM) study by Mirkin et al.[13] used the feedback mode to probe the redox activity of individual cells of *Rhodobacter sphaeroides,* a purple bacterium that contains a membrane-bound reaction center protein (RC) with a number of bound redox cofactors.[14,15]

In the RC, photon absorption promotes the primary electron donor, a bacteriochlorophyll dimer (P), to the first excited singlet

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(1) Collins, M. L. P.; Remsen, C. C. In *Structure of Phototrophic Prokaryotes*; Stolz, F. J., Ed.; CRC Press: Boca Raton, FL, 1991; pp 49−77.

(2) Oelze, J.; Drews, G. *Biochim. Biophys. Acta* **1972**, *265*, 209−239.

(3) Verméglio, A.; Joliot, P.; Joliot, A. In *Anoxygenic Photosynthetic Bacteria*; Blankenship, R. E., Madigan, M. T., Bauer, C. E., Eds.; Kluwer Academic Publishers: Dordrecht, 1995; pp 279−295.

(4) Zuber, H.; Cogdell, R. J. In *Anoxygenic Photosynthetic Bacteria*; Blankenship, R. E., Madigan, M. T., Bauer, C. E., Eds.; Kluwer Academic Publishers: Dordrecht, 1995; pp 315−348.

(5) Kuehlbrandt, W.; Wang, D. N. *Nature* **1991**, *350*, 130−134.

(6) Crofts, A. R.; Wraight, C. A. *Biochim. Biophys. Acta* **1983**, *726*, 149−185.

(7) Munge, B.; Pendon, Z.; Frank, H. A.; Rusling, J. F. *Bioelectrochemistry* **2001**, *54*, 145−150.

(8) Kong, J.; Lu, Z.; Lvov, Y. M.; Desamero, R. Z. B.; Frank, H. A.; Rusling, J. F. *J. Am. Chem. Soc.* **1998**, *120*, 7371−7372.

(9) Zou, Y.; Zhao, J.; Chen, Z.; Kong, J.; Zeng X.; Xu, C. *Series C: Life Sci.* **2001**, *44*, 524−533.

(10) Samuel Ko B.; Babcock B.; Jennings G. K.; Tilden S. G.; Peterson, R. R.; Cliffel, D. E. *Langmuir* **2004**, *20*, 4033−4038.

(11) Munge, B.; Das, S. K.; Ilagan, R.; Pendon, Z.; Yang, J.; Frank, H. A.; Rusling, J. F. *J. Am. Chem. Soc.* **2003**, *125*, 12457−12463.

(12) Ciobanu, M. Kincaid, H. A.; Jennings G. K.; Cliffel, D. E. *Langmuir* **2005**, *21*, 692−698.

(13) Cai, C.; Liu, B.; Mirkin, M. V.; Frank, H. A.; Rusling, J. F. *Anal. Chem.* **2002**, *74*, 114−119.

10.1021/ac060228q CCC: $33.50
Published on Web 05/28/2006

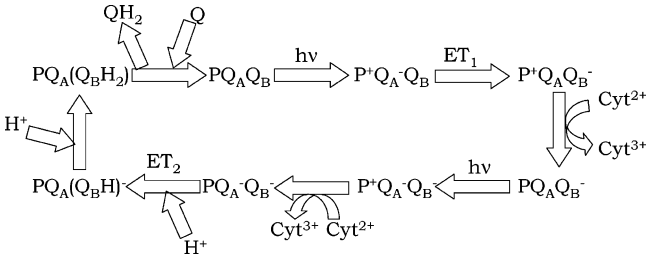

Figure 1. Quinone reduction cycle in RC. Q_B is reduced in two one-electron reactions and binds two protons. The reduced QH_2 leaves the RC and is replaced by an exogenous quinone. ET_1, first interquinone electron transfer; ET_2, second interquinone electron transfer.

state (P*). An electron is consequently transferred (via bacteriopheophytin) from P* to a first ubiquinone-10 (UQ_{10}) electron acceptor (Q_A) and generates the primary charge-separated state $P^+Q_A^-Q_B$ (Figure 1). The electron present on Q_A is then delivered to a secondary ubiquinone-10 electron acceptor, Q_B. The water-soluble cytochrome c^{2+} rapidly rereduces the photooxidized primary donor, which transfers, upon photon absorption, a second electron to the secondary quinone acceptor that leaves the RC in its fully reduced and protonated state, $UQ_{10}H_2$.[16] In vivo, the function of the quinone complex acceptor is to deliver reducing equivalents to a pool of ubiquinone molecules present in the native membrane in stoichiometric excess over the RC.[6] This occurs through the free exchange of UQH_2, at the Q_B site of the RC, with oxidized quinone from the pool.

In the reported work by Mirkin et al.,[13] two groups of redox mediators (i.e., hydrophilic and hydrophobic redox species) were used to shuttle electrons in the dark between the SECM tip electrode in solution and the RCs inside the cell. Hydrophilic mediators can only permeate the outer membrane while the hydrophobic ones can penetrate into the cytoplasm by crossing the inner membrane. Information on the permeability of the outer cell membrane to different ionic species and on the intracellular redox properties was obtained by analysis of the dependence of the measured heterogeneous rate constant of the redox reaction between mediator and cell on the formal potential of the mediators in solution. The authors attributed the different measured values of intracellular formal potential to the different abilities of the mediators to cross the outer and inner membranes or to their interaction with different redox centers inside the cell. The cellular organism investigated by Mirkin et al. has several classes of membrane proteins containing different cofactors[17−27] that can interact with the mediator at the external applied potentials. The present work therefore applies SECM to biological systems that contain the photosynthetic RC and are of a lower degree of complexity than that previously studied. The study will specifically focus on chromatophores (spherical reorganization of cell membrane following its mechanical rupture) and liposomes (reconstituted membrane systems).

The chromatophore is a specialized pigment-bearing structure obtained from the mechanical rupture of the *Rb. sphaeroides* bacterium cell wall that still contains multiprotein complexes, among which is the RC, and presents an inside-out orientation. In the liposomes, however, the RC is the only protein present and is randomly orientated as a result of liposome formation. Studies of these systems are expected to clarify the role played by the external membrane in determining the rate of the measured processes and the influence that other complexes present in the bacterial cell can exert on the electron transfer to the cofactors of the RC.

EXPERIMENTAL SECTION

Chemicals. Except where otherwise stated, all chemicals were purchased from Sigma, of the highest purity, and used without further purification. All aqueous solutions were prepared using MQ-treated water (Milli-Q, Millipore).

Electrodes. A three-electrode setup was used for SECM experiments with a 5-μm-radius Pt tip as a working electrode, a homemade Ag/AgCl electrode as a reference electrode, and a Pt wire as a counter electrode. The tips were prepared as described previously[28] and polished with 0.05-μm alumina before each experiment.

Biological Sample Preparation. The carotenoid-less *Rb. sphaeroides* strain R-26 cells were grown anaerobically in Hutner's medium (~13 L) under a tungsten light and were harvested in log phase after 2−3 days of growth at 30 °C by centrifugation at 3500*g* for 15 min. The cells were then resuspended in 20 mM Tris-HCl buffer, pH 7.5.

Chromatophores were prepared by passing (two times) the bacterial suspension previously incubated with small amounts of DNAse and lysozyme in a French pressure cell. After removal of cellular debris by a 38000*g* centrifugation, chromatophores were pelleted at 175000*g* for 2 h and resuspended in 20 mM Tris-HCl buffer, pH 7.5.

The reaction center protein was isolated from the membrane, following the Gray procedure[29] with minor changes, resulting in a preparation containing ~1.7 quinones/RC. The secondary quinone acceptor (Q_B) was removed following the procedure described by Okamura.[30]

The RC-containing vesicles (proteoliposomes) were prepared as follows. A 4-mg sample of egg yolk phosphatidylglycerol (PG) and the proper amount of ubiquinone-10 were placed in a 1.5-mL Eppendorf tube, dissolved in chloroform, dried under N_2, and solubilized in 0.5 mL of a 50 mM phosphate/100 mM KCl/4%

(14) Deisenhofer, J.; Michel, H. *Science* **1989**, *245*, 1463−1473.
(15) Feher, G.; Allen, J. P.; Okamura, M. Y.; Rees, D. C. *Nature* **1989**, *339*, 111−116.
(16) Wraight, C. A. *Isr. J. Chem.* **1981**, *21*, 348−354.
(17) Arata, H.; Parson, W. W. *Biochim. Biophys. Acta* **1981**, *636*, 70−81.
(18) Prince, R. C.; Dutton, P. L. In *The Photosynthetic Bacteria*; Clayton, R. K., Sistrom, W. R., Eds.; Plenum Press: New York, 1978; pp 439−453.
(19) Reed, D. W.; Zankel, K. L.; Clayton, R. K. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 42−46.
(20) Dutton, P. L.; Leigh, J. S.; Wraight, C. A. *FEBS Lett.* **1973**, *36*, 169−173.
(21) Arata, H.; Nishimura, M. *Biochim. Biophys. Acta* **1983**, *726*, 394−401.
(22) Prince, R. C.; Dutton, P. L. *Arch. Biochem. Biophys.* **1976**, *172*, 329−334.
(23) Maroti, P.; Wraight, C. W. *Biochim. Biophys. Acta* **1988**, *934*, 329−347.
(24) Turzo, K.; Laczko, G.; Filus, Z.; Maroti, P. *Biophys. J.* **2000**, *79*, 14−25.
(25) Mancino, L. J.; Dean, D. P.; Blankenship, R. E. *Biochim. Biophys. Acta* **1984**, *764*, 46−54.
(26) Kleinfeld, D.; Okamura, M. Y.; Feher, G. *Biochim. Biophys. Acta* **1984**, *766*, 126−140.
(27) Tandori, J.; Sebban, P.; Michel, H.; Baciou, L. *Biochemistry* **1999**, *38*, 13179−13187.
(28) Bard, A. J.; Fan, F.-R. F.; Kwak, J.; Lev, O. *Anal. Chem.* **1989**, *61*, 132.
(29) Gray, K. A.; Farchaus, J. W.; Wachtveitl, J.; Breton, J.; Oesterhelt, D. *EMBO J.* **1990**, *9*, 2061−2070.
(30) Okamura, M. Y., Isaacson, R. A., Feher, G., *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3491−3495.

sodium cholate (pH 7) buffer by sonication on ice for 10−15 s with a titanium tip. A 60-μL aliquot of 80 μM RCs was added to this solution. Detergent was removed by applying this suspension on a Sephadex G-50 (Pharmacia) column equilibrated with 50 mM phosphate/100 mM KCl (pH 7) buffer. Detergent removal resulted in RC-containing small unilamellar vesicles[31] eluting in a fraction of ~1 mL. A PG/RC mole ratio of 1000 was obtained, and the RC concentration was ~3.8 μM as evaluated by spectrophotometric assay. Three kinds of proteoliposome samples were prepared, containing different amounts of ubiquinone-50: type I contained ~1 quinone/RC (i.e., Q_A only), as Q_B-depleted RCs were used and no quinone was added externally; type II were made by using as prepared RCs and adding ubiquinone-50 in a molar ratio 3:1 with the RCs, resulting in a fully Q_B-active sample but with a negligible external quinone pool; type III were made similarly to type II, but a ratio ubiquinone-50/RC 10:1 was used, to obtain a larger quinone pool.

Instrumentation and Procedure. Voltammetry was performed in the same phosphate buffer solution (PBS) as used in the SECM experiments.

Steady-state voltammograms were obtained in a three-electrode setup using a CHI900 electrochemical workstation (CH Instruments, Austin, TX). The half-wave potentials of redox mediators were measured with respect to a Ag/AgCl reference electrode, which was also used in SECM experiments.

All SECM measurements were performed at room temperature in a plastic culture (Petri) dish, mounted on the horizontal stage of the SECM. The SECM apparatus and procedures have been described previously.[32,33] The tip current (i_T) was recorded as a function of the tip position (d) and the i_T versus d approach curves were obtained by positioning the tip above the biological sample (chromatophores or liposomes) and slowly moving it vertically down to the sample surface (scan rate of 1 μm/s).

Before each experiment, 200 μL of the biological sample was deposited on the bottom of a Petri dish using a micropipet. The solvent was removed by evaporation. If the biological sample contains liposomes a lipid bilayer is formed,[34] whereas for chromatophore samples a simple film is formed without changes to the structure.

After removal of the solvent by evaporation, optical spectra of the biological samples on the Petri dish were recorded on a Varian Cary3 UV−visible spectrophotometer, to confirm the deposition of the sample on the bottom of the culture dish. In the case of chromatophores, the spectrum was characterized by a large peak at 867 nm due to the absorption of the LH-I chlorophylls and a peak at 550 nm due to the membrane-bound *c*-type cytochromes (Figure 2a). For PG liposomes, the presence of RC was confirmed by the three characteristic peaks at 760, 800, and 860 nm (Figure 2b).[35]

Finally, 5 mL of the redox mediator solution in PBS buffer was added. The mediator solution covering the biological sample was carefully degassed. The dish was covered with Parafilm and purged with a slight positive pressure of inert gas during the entire experiment to remove oxygen interferences.

All experiments were performed under ambient light conditions that are considered as "dark" conditions.

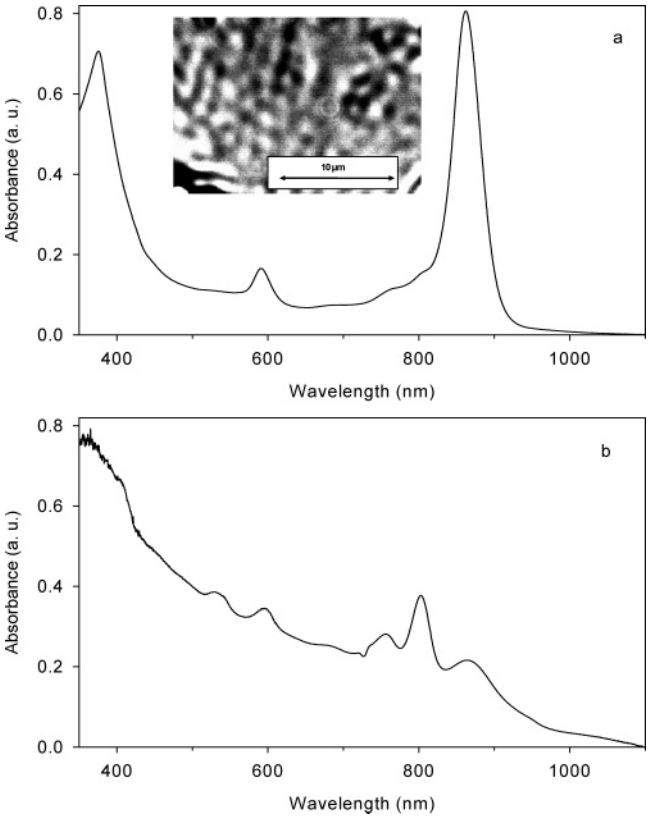

Figure 2. UV−visible spectra of (a) chromatophores and (b) PG liposomes deposited on Petri dish. Inset a: optical micrograph of chromatophores deposited on Petri dish.

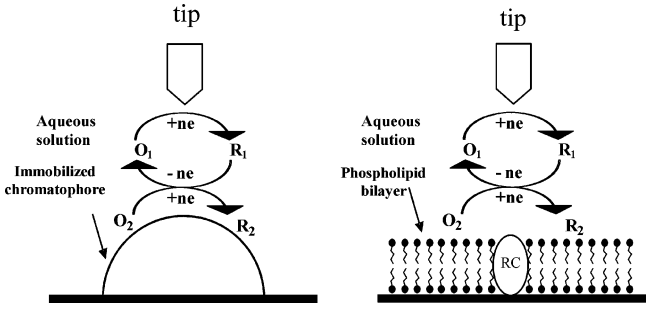

Figure 3. Schematic diagrams of the SECM feedback mode experiments with chromatophores or proteoliposomes from *Rb. Sphaeroides*. The type III liposomes have also a larger quinone pool.

RESULTS AND DISCUSSION

SECM experiments were carried out in feedback mode, with the tip ultramicroelectrode (UME) placed in the solution containing the oxidized form of a redox mediator, O_1. In this mode, the reduced species generated at the UME can diffuse into the biological substrate, eventually be reoxidized by the appropriate cofactor (Figure 3), and diffuse back to the nearby UME where it is detected. This leads to an enhancement in the current flowing at the UME (positive feedback), which depends on the normalized tip/substrate distance (d/a, where d is the separation distance and a is the tip radius). If the mediator regeneration by the

(31) Venturoli, G.; Mallardi, A.; Mathis, P. *Biochemistry* **1993**, *32*, 13245−13253.
(32) Liu, B.; Cheng, W.; Rotenberg, S. A.; Mirkin, M. V. *J. Electroanal. Chem.* **2001**, *500*, 590.
(33) Liu, B.; Mirkin, M. V. *J. Am. Chem. Soc.* **1999**, *121*, 8352.
(34) Graneli, A.; Rydsrom, J.; Kasemo, B.; Hook, F. *Langmuir* **2003**, *19*, 842−850.
(35) Frank, H. A.; Taremi, S. S.; Knox, J. R. *J. Mol. Biol.* **1987**, *198*, 139−141.

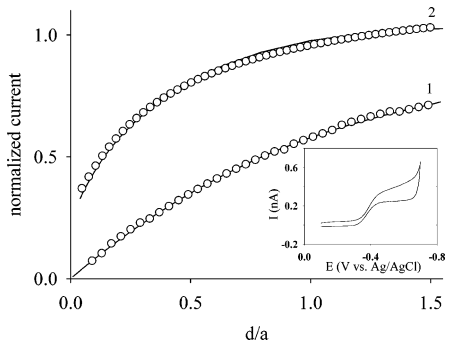

Figure 4. Approach curves of a 10-μm Pt UME to a clean Petri dish (curve 1) and to deposited chromatophores (curve 2) for a 20 μM menadione solution. The circles represent the experimental approach curve while the solid line is the SECM microdisk theory. The tip current is normalized by the $i_{t,\infty}$ value measured in the bulk solution; the tip−substrate separation distance is normalized by tip radius, *a*. Inset: voltammogram of 0.1 mM menadione at 10 μm Pt tip at 50 mV/s scan rate.

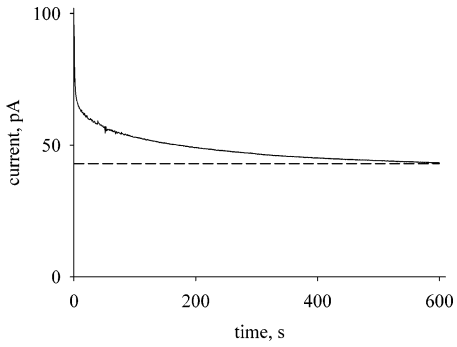

Figure 5. Current versus time dependence obtained at a 5-μm-radius Pt tip positioned at a constant distance (~5 μm) above a chromathophore. Solution contained 20 μM menadione. The dashed line represents the SECM theoretical current for negative feedback at the tip/substrate distance of 5 μm.

biological substrate is a slow process, a decrease of the tip current with a decrease of the distance, *d,* will be observed (negative feedback), caused by the hindrance of the diffusion processes to the tip. The overall reactions can be summarized as follows:

$$O_1 + ne^- \rightarrow R_1 \quad \text{(at the tip)} \qquad (1)$$

$$O_2 + R_1 \rightarrow R_2 + O_1 \quad \text{(at the biological sample)} \qquad (2)$$

The plot of the normalized tip current, i_T (ratio of the tip current by the tip current measured far from the sample, $i_{T,\infty}$), versus normalized distance, *d/a* (ratio of the tip to substrate distance by the ultramicroelectrode radius), is called an approach curve. The tip potential is kept at sufficiently negative values, to maintain diffusion-controlled conditions for reduction of the mediator, O_1, in reaction 1. Comparison of approach curves to appropriate theoretical curves can be used to determine the value of the heterogeneous rate constant (*k)* in (2). In the present study, the species susceptible of regenerating O_1, i.e., O_2, can be the oxidized RC itself, the quinone pool associated with the RC, or other oxidants.

1. SECM Measurements on Chromatophores. In Figure 4, two SECM current−distance curves are shown, both obtained with a 5-μm-radius Pt tip at an approach speed of 1 μm/s using a 20 μM solution of menadione (a well-known hydrophobic mediator frequently used in similar experiments on different systems),[13] in PBS (pH 7.5). The tip potential, according to the voltammogram of the mediator reported as the inset in Figure 4, was held at −0.55 V versus Ag/AgCl. Curve 1 represents the approach curve to the plastic surface of the culture dish (circles) and fits the theory for a diffusion-controlled process with an insulating substrate very well (solid line). Curve 2 is recorded for a chromatophore film deposited on the Petri dish (see inset Figure 2a). The menadione normalized current (measured over the biological sample) is significantly higher than that measured over the insulating substrate. This implies that menadione (RO_2) is reduced to menadiol ($R(OH)_2$) at the UME and successively reoxidized by the biological sample. This regeneration occurs at a measurable rate. By fitting the current−distance curve to the SECM kinetic theory, the value of the effective heterogeneous rate constant ($k = (4.8 \pm 0.1) \times 10^{-3}$ cm/s) expressing the overall rate of the mediator regeneration by the substrate (reaction 2)[36,37] can be extracted, although the complexity of the biological system under study does not allow at this stage a clear identification of the nature of the redox species involved in the regeneration reaction.

It is well known from the theory[36,38] that the heterogeneous rate constant is not influenced by the mediator concentration, if the concentration of redox centers in the biological samples (c_{ctr}) exceeds by a factor of almost 15 the mediator concentration (c°). The concentration of redox centers present in the studied biological samples was checked by evaluating the dependence of the heterogeneous rate constant on the mediator concentration. The rate constant *k* was essentially independent from the mediator concentration over the entire analyzed range (20 to 100 μM).

Alternatively, the concentration of redox centers in the biological samples was also determined by chronocoulometry. In a chronocoulometric experiment, the concentration of redox mediator in solution is kept sufficiently high to make the flux of mediator produced by the tip significantly higher than the rate of production of redox centers in the biological sample. Under such conditions, the concentration of biological redox centers, which regenerates the mediator, should decrease with time until all biological material is reduced (or oxidized). At this point, the biological sample is no longer capable of regenerating the mediator, and a SECM negative feedback is recorded. Figure 5 reports the tip current versus time curve obtained with a 5-μm-radius Pt tip positioned at 5-μm distance from the surface of the chromatophore sample. The mediator was always menadione (20 μM) in PBS, and as soon as the negative potential was applied to the tip, the reduction of menadione to menadiol occured. The subsequent observed slow decrease of i_T, points to the depletion of the biological oxidant responsible for regeneration of reduced menadione. In agreement with SECM theory for negative feedback at a tip/substrate distance of ~5 μm, a constant value (43 pA) of the tip current was reached after 600 s.

This indicates that the biological redox centers responsible for regeneration of the mediator have been completely reduced

(36) Liu, B.; Rotenberg, S. A.; Mirkin, M. V. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9855.
(37) Wei, C.; Bard, A. J.; Mirkin, M. V. *J. Phys. Chem.* **1995**, *99*, 16033.
(38) Barker, A. L.; Macpherson, J. V.; Slevin, C. J.; Unwin, P. R. *J. Phys. Chem. B* **1998**, *102*, 1586.

by the menadiol produced at the tip. The integration of the tip current versus time curve (i.e., the area between the curve and the dashed line in Figure 5) allows the evaluation of the total amounts of the redox centers initially present in the chromatophore. A value of $(3.4 \pm 0.2) \times 10^{-14}$ mol/chromatophore of biological redox centers was obtained.

The overall results indicate that, under the present experimental conditions, the amount (and, hence, concentration) of redox centers in the biological samples is much higher than the mediator concentration in solution, and therefore, the heterogeneous rate constant is not influenced by the mediator concentration. Furthermore, a comparison between the k values of the mediator/chromatophores and mediator/whole cells redox reaction is possible. By comparing the kinetic constant ($k = (2.6 \pm 0.2) \times 10^{-3}$ cm/s) reported for menadione/whole cell by Mirkin et al.[13] and the present value ($k = (4.8 \pm 0.1) \times 10^{-3}$ cm/s) extracted for the menadione/chromatophores sample, a significant difference is observed and can be attributed to the lack of the outer cell wall.

In fact, aside from the concentration effects, one must consider that the reaction between the mediator and the biological substrate is quite complicated and includes additional steps as diffusion of the mediator across the sample membrane and ET between the mediator species and the biological redox centers. As discussed previously,[37,39] the effective heterogeneous rate constant, k, of the multistep reaction under steady-state conditions can be written as a reciprocal sum of terms, which are characteristic for the above steps

$$1/k = c^{\circ}/k_{gen} + 2/P + 1/k_{ET}c_{ctr} \qquad (3)$$

where c° is the concentration of redox mediator in solution, k_{gen}, in our experimental conditions, represents a constant parameter determined by the cellular chemistry, independent by c°, P is the permeability coefficient, k_{ET} is the rate constant of the electron transfer (ET) between mediator species and biological substrate, and c_{ctr} is the concentration of biological redox centers. In the present case, the absence of the cell wall in the chromatophores will affect the permeability coefficient and influence the kinetics of the electron transfer between the mediator and the target redox species of the *Rb. sphaeroides*.

2. SECM measurements on Liposome Substrates. Because the experiments performed with the chromatophores do not allow a clear attribution of the nature of the *Rb. sphaeroides* redox centers (quinone pool or redox cofactors, Q_A and Q_B) reacting with the mediator, SECM experiments were carried out on a different system consisting of liposomes (types I, II and III) containing isolated RC protein.

Compared with the chromatophores, RC liposomes type I and II do not possess a membrane quinone pool, but only the RC protein quinones, Q_A (type I) and $Q_A + Q_B$ (type II). Finally, type III liposomes have also the exogenous UQ-50 in excess that is accumulated in membrane pools very similarly to that present in chromatophores and cells.

In Figure 6 are reported the approach curves relative to the bilayer formed by deposited PG liposomes of the three different types previously described obtained with a 5-μm-radius Pt tip at an approach speed of 1 μm/s with a 20 μM solution of menadione in PBS. The measured k values for type I and II liposomes are similar. In fact, k is $(1.4 \pm 0.2) \times 10^{-3}$ and $(1.3 \pm 0.2) \times 10^{-3}$ cm/s for types I and II, respectively. However, the calculated k value for type III liposomes ($(4.3 \pm 0.2) \times 10^{-3}$ cm/s) is significantly higher than that obtained from types I and II. Furthermore, this k value is close to that obtained with chromatophores. The slight difference between chromatophores and type III liposomes could be reasonably attributed to the different kind of protein environment (natural or artificial membrane).

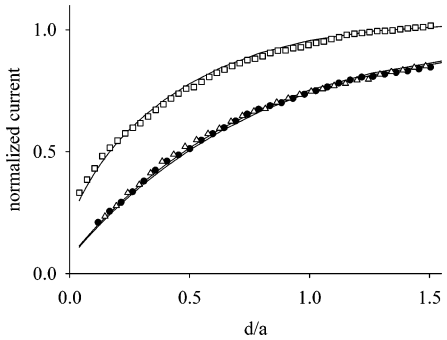

Figure 6. Approach of a 5-μm-radius Pt tip to liposomes type I (△), type II (●), and type III (□) in phosphate buffer solution containing 20 μM menadione. Solid lines are the theoretical curves.

A possible explanation of these results can be related to the presence or absence of the quinone pool. When the artificial (inside liposome type III) or natural (inside chromatophore) pool is present, the mediator regeneration reaction by the biological substrate takes place almost at the same rate. Conversely, although the mediator regeneration by liposome type I or II occurs, in the absence of the pool, the heterogeneous rate constant is much lower, due to the decreased quinone concentration in the substrate. This fact is further supported by the experimental evidence that if we repeat the scan on the same sample of chromatophores or of type III liposomes, we do not observe any change in the value of the rate constant; on the contrary, the same experiments on type I and II liposomes results in the progressive decrease of the rate constant, until it reaches a value typical of an insulating substance. This result is consistent with a decreasing concentration of the quinones to almost zero after several scans.

These observations suggest that inside of the studied biological samples (chromatophore and liposomes) the reactive species are the quinones, coming mainly from the membrane in chromatophores and liposome-containing quinone pool and directly from the RC in liposomes containing only the quinones linked to the specific protein binding sites.

This result well agrees with that report by Mirkin et al.,[13] who suggested that the oxidant species inside a *Rb. sphaeroides* cell is the quinone that resides in the cytoplasmic membrane pool.

CONCLUSIONS

Scanning electrochemical microscopy is a useful tool for the investigation of the redox processes involving whole cells of photosynthetic bacteria and has been for the first time applied to membrane fragments (chromatophores) and liposomes containing the isolated bacterial reaction center, allowing the calculation of the effective heterogeneous rate constants for the involved redox reaction.

(39) Andrieux, C. P.; Savéant, J.-M. In *Molecular Design of Surfaces*; Murray, R. W., Ed.; John Wiley & Sons: New York, 1992; p 207.

In the first system, the technique was able to provide information about the role of the outer cell membrane permeation on the kinetics of the electron transfer between a mediator and the protein. In particular, the measured kinetic constant for the menadione/chromatophores system suggests that the absence of the cell wall influences the electron-transfer kinetics between the mediator and the photosynthetic redox species of the *Rb. sphaeroides*.

The liposome system provided further insight on the nature of the involved species. The compared analysis between the rate constant values obtained for the chromatophore and the liposome, evidenced as the different chemical environment in which the RC is embedded, can affect the physical−chemical properties of the mediator−protein systems, as extensively already reported in the literature.[40] The rate constant depends on the biological substrate concentration, and the value calculated in the presence of a quinone pool in the liposome bilayer is quite similar to that relative to the chromatophores. If we repeat the scan on the same sample, the the concentration of quinones in liposomes (without pool) significantly lowers, quickly decreasing to almost zero after several scans.

(40) Trotta, M.; Milano, F.; Nagy, L.; Agostiano, A. *Mater. Sci. Eng., C* **2002**, *22(2)*, 263−267.

Future studies on the proteoliposomes, changing the lipid nature and the level of occupancy of the quinone binding sites in the protein, should yield further information on the effect of the double layer permeation to the mediator. The preparation of the proteoliposomes in dark/light conditions can additionally provide information on the accessibility of the cofactors in the charge separated state to the redox species in solution.

ACKNOWLEDGMENT

This work was supported by the National Science Foundation (CHE 0109587) and by INSTM (Prisma project). The authors gratefully thank Prof. Flavio Maran for permission to use the SECM instrument in his research laboratory and Dr. Sabrina Antonello and Dr. Emanuele Istuli for their help. We also thank Mr. Sergio Nuzzo and Mr. Giovanni Lasorella for their technical support.

Received for review February 3, 2006. Accepted April 28, 2006.

AC060228Q