Interaction of Cytochrome *c* with Reaction Centers of *Rhodopseudomonas sphaeroides* R-26: Determination of Number of Binding Sites and Dissociation Constants by Equilibrium Dialysis†

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

ABSTRACT: The number of binding sites and dissociation constants of cytochrome *c* (horse heart) and cytochrome c_2 (*Rhodopseudomonas sphaeroides*) to reaction centers of *Rhodopseudomonas sphaeroides* R-26 was determined by equilibrium dialysis. One binding site was found for both cytochromes. The dissociation constants (10 mM Tris-HCl, pH 8.0) were ~0.4 μM and ~1.0 μM for cytochrome *c* and cytochrome c_2, respectively. Oxidized and reduced forms of both cytochromes bound to reaction centers with approximately equal affinity.

The primary energy conversion process in photosynthesis involves the conversion of light energy into chemical energy by means of a series of electron-transfer reactions. In the primary photochemical event, an electron is transferred from a donor (D) to an acceptor (A) in a bacteriochlorophyll–protein complex called the reaction center (RC).[1] The photogenerated electron–hole pair associated with the donor–acceptor pair D^+A^- of the RC recombines at physiological temperatures with a characteristic time of ~0.1 s. For high quantum yields, it is necessary to prevent this recombination, i.e., the electron or hole has to be transferred to secondary reactants in a time much less than 0.1 s. On the donor side, ferrocytochrome c_2 accomplishes this task [Parson, 1968; for a review, see Dutton & Prince (1978)]. Thus, the binding and the electron-transfer rate between the cytochrome and the RC are important parameters of the photosynthetic process. In this work, we present data on the number of binding sites and the dissociation constant of cytochrome *c* to RCs. In forthcoming papers, the kinetics of binding and electron transfer (T. D. Marinetti, M. Y. Okamura, D. Rosen, and G. Feher, unpublished experiments), as well as evidence for the positions of the binding site (D. Rosen, M. Y. Okamura, and G. Feher, unpublished experiments) will be presented.

The stoichiometry of binding of cyt *c* to RCs has been investigated both in vivo and in vitro by several authors (Ke et al., 1970; Dutton et al., 1975; Bowyer & Crofts, 1979; Pachence et al., 1979). Differing results were reported by the various groups. These conflicting results may have been due to differences in the systems studied and the indirect methods that were used to study them. In this work, we used the method of equilibrium dialysis to study the binding of cyt *c* to isolated, well-characterized RCs (Feher & Okamura, 1978) from *R. sphaeroides* R-26. This required a semipermeable membrane that would pass cyt *c* but not RCs. Using a membrane with these properties, we determined the dissociation constant and number of binding sites on the RC for oxidized and reduced cyt *c* (horse heart) and cyt c_2 (*R. sphaeroides*). A preliminary account of this work has been presented earlier (Rosen et al., 1979; Rosen, 1979).

Materials and Methods

Equilibrium Dialysis. A five-cell rotating equilibrium dialysis assembly (Spectrum Medical Instruments, Los Angeles, CA) was used; the volume of each compartment was 1 mL. The membranes were of the regenerated cellulose series (Schleicher & Scheull, Keene, NH, RC-52 and RC-53). They were washed for several hours with five changes in 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100 (TT buffer) and 1 mM EDTA and then rinsed with TT buffer before use. All buffers were filtered through 0.22-μM Millipore filters to remove particles that might clog the membrane. Dialyses were performed at 4 °C in the dark for 48 h unless otherwise specified.

Reaction Centers. RCs were prepared according to published procedures (Feher & Okamura, 1978). The solubilizing detergent, lauryldimethylamine oxide (LDAO), was found to change the oxidation state of cyt; it was therefore replaced with 0.1% Triton X-100 by absorbing RCs on a DEAE column, washing with TT buffer, and eluting with 1 M NaCl in TT buffer followed by dialysis against TT buffer. Triton X-100 was treated with $NaBH_4$ (10 mM) to reduce peroxides and dialyzed overnight against water. The concentration of Triton was determined by its optical absorbance at 275 mm (ϵ_{275} = 20 cm^{-1} $\%^{-1}$).

Cytochrome c. Horse heart cytochrome *c*, obtained from Sigma (type III), was used without further purification. Cytochrome c_2 was prepared by the method of Bartsch (1978) or was kindly supplied by Drs. R. G. Bartsch and T. E. Meyer.

Reduced cytochrome *c* was prepared by adding a 3–5-fold excess of sodium dithionite to the cytochrome solution, followed by chromatography on Sephadex G-25 equilibrated in 10 mM Tris-HCl, pH 8.0. The reduced cytochrome had a concentration of ~1 mM and was stored under nitrogen at −20 °C.

Oxidized cytochrome *c* was prepared by passing a 3–4 mM cyt solution in 10 mM Tris, pH 8, over a column of Bio-Rad AG1-X8 which was 50% saturated with potassium ferricyanide. Cytochrome *c* (horse) prepared this way was ~98% oxidized. Cytochrome c_2 required two passes over the column and was 96% oxidized. The oxidized solutions were diluted in 10 mM Tris-HCl, pH 8.0, to a concentration of 1–2 mM

† From the Department of Physics, University of California—San Diego, La Jolla, California 92093. *Received April 10, 1980.* This work was supported by grants from the National Science Foundation (PCM 78-13699) and the National Institutes of Health (GM 13191). It was performed in partial fulfillment for the Ph.D. degree of D.R. (supported by NIH Training Grant PHS GM 07313). This is the first of a series of papers; the others will deal with the kinetics of electron transfer and the position of the binding site.

‡ Present address: Bell Telephone Laboratories, Holmdel, NJ 07733.

§ Recipient of National Institutes of Health Career Development Award 1 K04 GM00106.

[1] Abbreviations used: cyt, cytochrome; *R. sphaeroides*, *Rhodopseudomonas sphaeroides*; RC, reaction center; LDAO, lauryldimethylamine oxide; TT buffer, 10 mM Tris-HCl, pH 8.0, and 0.1% Triton X-100; TL buffer, 10 mM Tris-HCl, pH 8.0, and 0.025% LDAO.

0006-2960/80/0419-5687$01.00/0

and stored at −20 °C. Before each dialysis experiment, the cytochrome solutions were diluted with TT buffer.

Optical Measurements. The concentration of cytochrome was determined by measuring the optical absorbance by using the extinction coefficients $\epsilon_{550}^{red} = 27.6\ mM^{-1}\ cm^{-1}$ for cyt *c* (Margoliash & Frohwirt, 1959) and $\epsilon_{550}^{red} = 30.8\ mM^{-1}\ cm^{-1}$ for cyt c_2 (Bartsch, 1978). The RC concentration was determined by using the extinction coefficient $\epsilon_{802} = 288\ mM^{-1}\ cm^{-1}$ (Straley et al., 1973). The extinction coefficient of RCs at 550 nm was determined to be $\epsilon_{550} = 31.7\ mM^{-1}\ cm^{-1}$. Spectra were taken with a Cary 14R spectrophotometer. Base lines were taken before and after each spectrum to check for possible drifts. Before spectra were taken, the cytochrome was reduced by the successive addition of small aliquots of sodium ascorbate (solid) until no absorbance changes at 550 nm were seen. At the lowest cyt concentrations, the difference between the absorbances at 550 and 560 nm was used to correct for slight scattering of the sample. Dilutions were performed by weighing all solutions; this kept the dilution errors to less than 0.2%.

Analysis of Data. (1) Plotting of Results. Let the concentrations of total, bound, and free cytochromes be denoted by $[cyt]_{total} = C_T$, $[cyt]_{free} = C_F$, and $[cyt]_{bound} = C_B$, and similarly for the concentrations of reaction centers $[RC]_{total} = R_T$, $[RC]_{free} = R_F$, and $[RC]_{bound} = R_B$. The concentrations in the compartment that do not have RCs will be primed (i.e., $R' = 0$, $C_F' = C_F$, etc.). The concentration of cyt *c* added to one compartment at the beginning of the experiment will be denoted by C_0. With the assumption that *n* represents independent, homogeneous binding sites, the fraction of RCs having a cytochrome bound, $\bar{\nu}$ ($\bar{\nu} = C_B/R_T$), is given by the relation (Van Holde, 1971)

$$\bar{\nu} = n - (\bar{\nu}/C_F)K_D \tag{1}$$

where K_D is the equilibrium dissociation defined by

$$K_D = [RC][cyt]/[RC{\cdot}cyt] = R_F C_F/C_B \tag{2}$$

If one plots $\bar{\nu}$ against $\bar{\nu}/C_F$, *n* and K_D can be obtained from the intercept and slope of the straight line given by eq 1.

(2) Correction Due to the Donnan Effect. In the presence of charged, nondiffusible macromolecules, the equilibrium concentrations of the diffusible ions will not be the same in both compartments. Specifically, the concentration of the diffusible ion that has a charge opposite to that of the macromolecule will be larger in the compartment having the macromolecules. A negative charge on the RC of $z = -15$ was estimated at pH 8.0 from the amino acid analysis (Steiner et al., 1974) by assuming a molecular weight of 95 000 and a ratio of Glu/Glx = Asp/Asx $\simeq$ 0.6 (Dayhoff et al., 1976). The charges, *x*, on cytochrome *c* (including the free carboxyl end group and the two propionic acids on the heme) at pH 8 are $x = +6$ and $+7$ for the reduced and oxidized species (Dayhoff et al., 1972). For cytochrome c_2 at pH 8, the charges are $x = -3$ and -2, respectively (Dayhoff & Barker, 1976).

If the concentration of univalent ions, in our case Tris-HCl, is much larger than xC_F and zR, the Donnan equilibrium will be governed by the buffer and is given approximately by (Tanford, 1961)

$$\frac{[Tris^+]}{[Tris^+]'} = \frac{[Cl^-]'}{[Cl^-]} \simeq 1 - \frac{zR_F + (z+x)R_B}{2[Tris^+]} = 1 - \frac{zR_T + xC_B}{2[Tris^+]} = 1 - \epsilon \tag{3}$$

where the two terms in the numerator are due to the charges of the RCs that are free and the RCs that have a bound cyt. The expansion in terms of ϵ, defined by eq 3, is justified since $\epsilon \ll 1$ for all our measurements. The ratio of the concentrations of the polyvalent diffusible ions (cytochrome) having a charge *x* is given by (Tanford, 1961)

$$\frac{(C_F)}{(C_F)'} = \left(\frac{[Tris]}{[Tris]'}\right)^x \simeq 1 - x\epsilon \tag{4}$$

The Donnan corrections of $\bar{\nu}$ and $\bar{\nu}/R$ were obtained by using eq 3 and 4, i.e.:

$$\bar{\nu} = C_B/R_T = (C_T - C_F)/R_T = [C_T - C_F'(1 - x\epsilon)]/R_T \tag{5}$$

$$\bar{\nu}/C_F = \bar{\nu}/[C_F'(1 - x\epsilon)/R_T] \tag{6}$$

(3) Error Analysis. The errors in $\bar{\nu}$ and $\bar{\nu}/C_F$ (and hence *n* and K_D) arise mainly from uncertainties in the determination of the cytochrome concentrations; the error in the determination of the starting RC concentration is small and was neglected. Different assay procedures were used for low ($\bar{\nu} < 0.5$–0.6) and high ($\bar{\nu} > 0.5$–0.6) cytochrome concentrations. As a result, the error determination differed for the two cases. Only the two quantities C_F' and C_T need to be measured at equilibrium since $C_B = C_T - C_F'$.

At *low cytochrome concentrations*, the absorbance of RCs at 550 nm introduces a large error in C_T when one tries to determine it from the optical absorbance of the mixture of RCs and cytochrome. Consequently, we measured instead the total amount of cytochrome, C_0, introduced into the cell. This together with a measurement of C_F', gives C_T. The expressions for the errors in $\bar{\nu}$ and $\bar{\nu}/C_F$ were obtained by standard procedures and are given by

$$\sigma_{\bar{\nu}}^2 = [\sigma_{C_T+C_F'}/R_T]^2 + [2\sigma_{C_F'}/R_T]^2 \tag{7}$$

$$\sigma_{\bar{\nu}/C_F}^2 = [\sigma_{C_T+C_F'}/R_T C_F]^2 + [\sigma_{C_F}(C_T + C_F')/R_T C_F'^2]^2 \tag{8}$$

where σ represents the standard deviation of the mean of the quantity denoted by the subscript. The individual σ values were estimated in the following way: In a separate set of experiments, we determined that for both cytochromes $C_T + C_F' = (0.96 + 0.03)C_0$, i.e., about 4% of the cytochrome was absorbed to the membrane or cell wall. The percentage error in $C_T + C_F'$ was estimated to be 4%, i.e., $\sigma_{C_T+C_F'} = 0.04(C_T + C_F')$ and 2% of C_F' or 0.03 μM, whichever was larger.

At *high cytochrome concentrations*, the fractional difference between C_T and C_F' becomes small, resulting in an increased error in $C_B = C_T - C_F'$. Consequently, we found it advantageous to measure the absorbance at 550 nm of the solution containing RCs and cytochrome and to correct for the now relatively small absorbance of the RCs at that wavelength. For $R_T = 2.5\ \mu M$, this correction corresponds to 2.9 μM cyt *c* and 2.6 μM cyt c_2. With the assumption of a 5% error in this correction, it contributes an error to C_T of $\sigma_{C_T} = (2.9)(0.05) = 0.14\ \mu M$. With a 2% error in the determination of the optical absorbance of the two compartments, the numerical values for $\sigma_{\bar{\nu}}^2$ and $\sigma_{\bar{\nu}/C_F}^2$ for $R_T = 2.5\ \mu M$ are given by

$$\sigma_{\bar{\nu}}^2 = [(C_T + 2.9)(0.02)/R_T]^2 + [(C_F')(0.02)/R_T]^2 + [0.14/R_T]^2 \tag{9}$$

$$\sigma_{\bar{\nu}/C_F}^2 = [(C_T + 2.9)(0.02)/(R_T C_F)]^2 + [(C_T)(0.02)/(R_T C_F)]^2 + [0.14/(R_T C_F)]^2 \tag{10}$$

For cyt c_2, the values of 2.9 and 0.14 should be replaced by 2.6 and 0.13, respectively.

The above equations were applied to each point, and a least-squares computer fit was obtained to a straight line given by eq 1. Each value was assigned a weighting factor inversely proportional to $(\sigma_{\bar{\nu}}^2 + \sigma_{\bar{\nu}/C_F}^2)$. Equations 5 and 6 were used

Table I: Number of Binding Sites and Dissociation Constants for Reduced and Oxidized Cytochromes *c* and c_2[a]

cytochrome species	uncorrected		corrected[c]	
	n	K_D (μM)	n	K_D (μM)
cyt *c* (horse heart)				
reduced	1.00 ± 0.04[b]	0.37 ± 0.06	0.97 ± 0.03	0.35 ± 0.05
oxidized	1.08 ± 0.02	0.41 ± 0.03	1.05 ± 0.02	0.39 ± 0.02
cyt c_2 (*R. sphaeroides*)				
reduced	0.86 ± 0.02	0.87 ± 0.05	0.93 ± 0.02	1.00 ± 0.05
oxidized	1.04 ± 0.05	1.41 ± 0.15	1.08 ± 0.05	1.47 ± 0.15

[a] Dialysis was performed at $T = 4$ °C. Buffer: 10 mM Tris-HCl, pH 8, and 0.1% Triton X-100. [b] Corrected for Donnan effect (see eq 5 and y). [c] Statistical errors (standard deviations of the mean) are quoted. The uncertainties in the extinction coefficients of RCs and cyt contribute an additional fractional error of approximately 7% and 5% for n and K_D, respectively.

to correct each point for the Donnan effect. Since the Donnan corrections were found to be small, we assumed the same σ values for the corrected points. It follows from eq 9 that the higher the RC concentration, R_T, the smaller the error in $\bar{\nu}$ and $\bar{\nu}/C_F$. However, an increase in R_T produces a larger Donnan correction and also necessitates an increase in cytochrome concentration. These considerations led to the choice of $R_T = 2.5$ μM.

Besides the statistical errors discussed above, systematic errors in n and K_D may arise due to uncertainties in the values of the extinction coefficients ϵ_{cyt}^{550} and ϵ_{RC}^{802}. It is easily shown that a fractional error, δ, in ϵ_{cyt}^{550} causes a fractional error, δ, in both n and K_D (if ϵ_{cyt}^{550} used is too *high*, the measured values of n and K_D will be too *low*). Similarly, a fractional error, δ, in ϵ_{RC}^{802} causes a fractional error in n (if ϵ_{RC}^{802} used is too *high*, the measured value of n will be too high). However, in this case, no error in K_D is made. Since the extinction coefficients have been determined with a fractional error of approximately 5%, we need to increase the statistical error in n by adding to σ_n^2 a systematic component, $(0.07n)^2$. Similarly, the error in K_D is increased by adding an amount, $(0.05K_D)^2$, to $\sigma_{K_D}^2$.

Experimental Results

Selection and Characterization of the Dialysis Membrane. The first problem was to find a semipermeable membrane that passed cytochrome *c* but not RCs. About 20 different membranes were tested for these properties. Those that had a half-time for cytochrome equilibration of more than 2 days or that bound a significant fraction (>10%) of the cytochrome were discarded.

The Schleicher & Scheull RC-series membranes had the desired characteristics. Figure 1 shows the time course for the passage of reduced cytochrome *c* across the RC-52 and RC-53 membranes. The half-times for equilibration were found to be 23 and 2 h for the two membranes, respectively. For cytochrome c_2, the half-time for the RC-53 membrane was 4 h. Both of these membranes were impermeable to RCs. When RCs were put into one compartment, no detectable RCs (<0.02%) were found in the second compartment after 7 days.

Binding of Cytochrome c to RCs. Figure 2 shows the binding of reduced cyt *c* (horse heart) to RCs as a function of the cyt *c* concentration added to the compartment opposite the one containing RCs. The binding clearly saturates at about one cytochrome per RC.

In order to obtain more quantitative values for the number of binding sites, n, and the dissociation constant, K_D, the data of Figure 2 were replotted according to eq 1 (see open circles of Figure 3A). The experimental errors were determined as described under Materials and Methods. The filled circles correspond to the experimental values corrected for the Donnan effect (eq 5 and 6). The errors were assumed to be the same for both the corrected and uncorrected points. The straight line represents a least-squares computer fit to the corrected

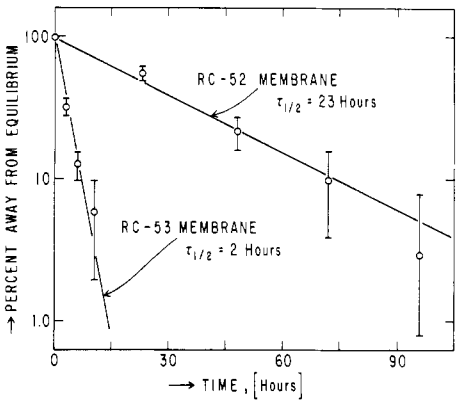

FIGURE 1: Dialysis of cyt *c* (horse heart) across RC-52 and RC-53 membranes at $T = 4$ °C. At zero time, 20 μM reduced cyt *c* in TT buffer was placed in one compartment of each of five equilibrium dialysis cells; the other compartment contained TT buffer. At different times, one cell was drained, and the cytochrome concentrations, $[cyt]_1$ and $[cyt]_2$, were determined spectrophotometrically in compartments 1 and 2. Percent away from equilibrium was defined as $([cyt]_1 - [cyt]_2)/([cyt]_1 + [cyt]_2)100\%$.

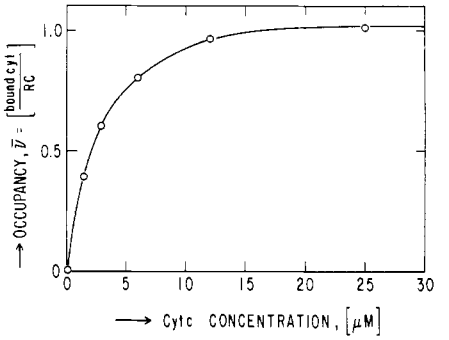

FIGURE 2: Binding of horse heart cyt *c* to RCs by equilibrium dialysis. RCs (2.5 μM) in TT buffer were introduced into one compartment of each dialysis cell. Different amounts (1.5–25 μM) of cyt *c* were introduced into the second compartment. The two compartments were separated by an RC-53 membrane. Samples were dialyzed for 48 h at $T = 4$ °C in the dark. The cells were then drained, and the samples were analyzed spectrophotometrically after reduction with sodium ascorbate. Cytochrome concentrations in each compartment were determined as described in Materials and Methods.

values. From the intercept and slope, we obtained the values n and K_D (see Table I).

Although the cyt *c* was added in the fully reduced form, at the end of the dialysis (48 h), approximately 20% of the cyt *c* was oxidized.[2] In order to determine the effect of this partial oxidation, we checked the binding of fully oxidized cyt *c* to RCs (see Figure 3B). The values for n and K_D (see Table I), obtained from Figure 3B, are sufficiently close to those ob-

[2] No deleterious effects on the cytochrome spectra due to the presence of Triton X-100 could be detected.

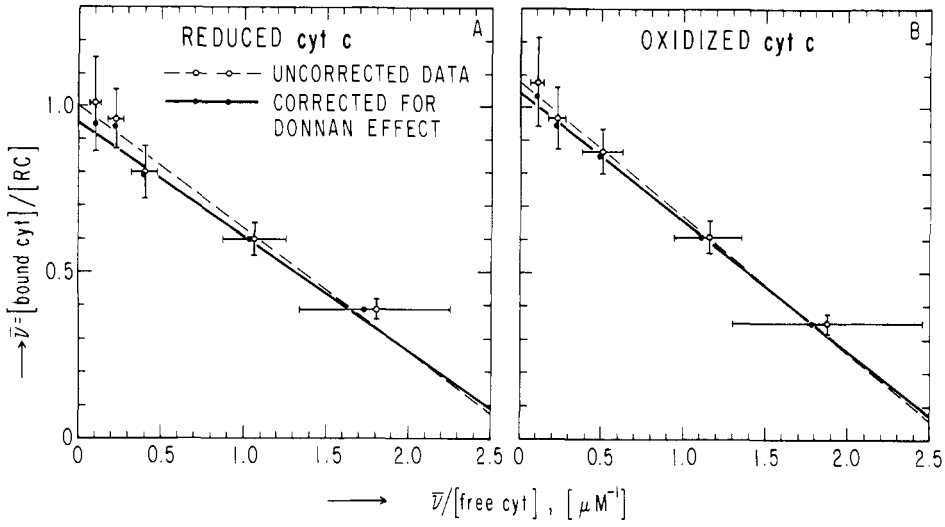

FIGURE 3: Equilibrium dialysis results of reduced (A) and oxidized (B) cyt *c* (horse heart) and RCs. From the intercept and slope of the line, the number of binding sites, *n*, and the dissociation constant, K_D, were determined (see eq 1). Open circles represent experimental result; filled circles correspond to experimental values corrected for the Donnan effect. Other conditions same as in Figure 2.

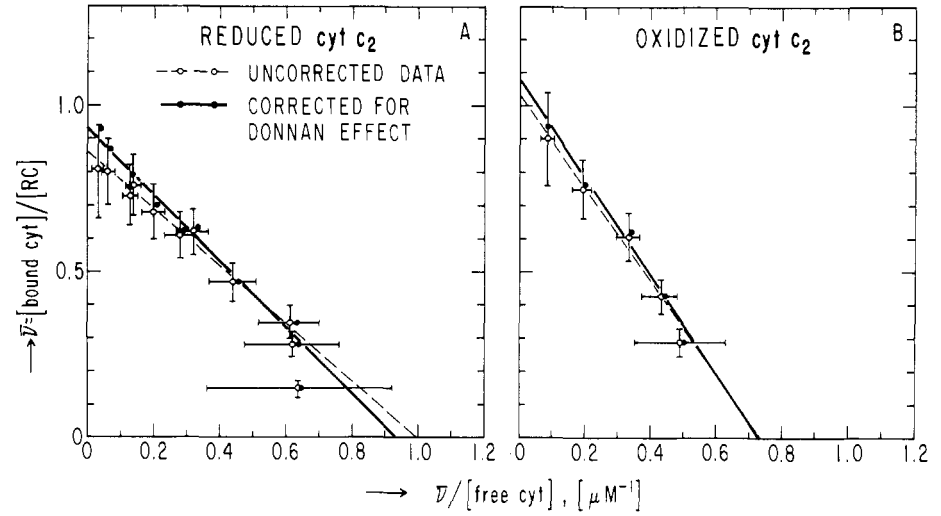

FIGURE 4: Equilibrium dialysis results of reduced (A) and oxidized (B) cytochrome c_2 (*R. sphaeroides*) and RCs. Conditions same as in Figure 3.

tained from Figure 3A to ensure that the fraction of oxidized cyt *c* has a negligible effect on the binding of reduced cyt *c*.

In order to check whether a true equilibrium had been attained after 48 h in the presence of RCs, a control experiment was performed in which cyt *c* was added to the compartment containing RCs. After a 48-h dialysis, the equilibrium concentrations were found to be the same as those obtained when the cyt was added to the compartment opposite the RCs.

Binding of Cytochrome c_2 to RCs. The equilibrium dialysis results for the binding of reduced and oxidized cytochrome c_2 from *R. sphaeroides* to RCs are shown in Figure 4A,B. The data were treated similarly to those of Figures 3A,B. The results are summarized in Table I. Less than 10% of the reduced cyt c_2 was oxidized during the course of the dialysis. Hence, in spite of the relatively larger difference in K_D between the oxidized and reduced species, the effect on the binding results was again negligible.

Competition between Binding of Cyt c and Binding of Cyt c_2 to RCs. In order to investigate whether the two cytochromes bind to the same site, the following competition experiment was performed: To the compartment of the dialysis cell that did not contain RCs reduced cyt *c* was added together with reduced cyt c_2, at concentrations of 2.5 and 10 μM, respectively. These concentrations correspond to fractional bindings (see K_D values in Table I) of $\bar{\nu} = 0.59$ and $\bar{\nu}_{c_2} = 0.80$. Thus, if the binding sites for cyt *c* and cyt c_2 were independent, a combined binding of $\bar{\nu}_{c+c_2}$ of 0.59 + 0.80 = 1.39 would be expected. Instead, we found after 48-h dialysis at 4 °C a combined binding of $\bar{\nu}_{c+c_2}$ of 0.81 ± 0.10 (uncorrected for Donnan effect). If the two cytochromes compete for the same site, one can show from simple binding theory[3] that $\bar{\nu}_{C+C_2}$ for our case should be 0.87, in agreement with the observed value.

Discussion

We have shown by equilibrium dialysis that one cyt *c* (or cyt c_2) binds to isolated RCs from *R. sphaeroides* R-26 (see Table I). Competition experiments between mammalian cyt *c* and bacterial cyt c_2 showed that only one of the cytochromes can be bound to the RC at one time. The simplest interpre-

[3] Analogous to eq 2, one can write

$$K_D^{\alpha} = (C_0^{\alpha} - C_B^{\alpha})(R_T - C_B^{\alpha} - C_B^{\beta})/2C_B^{\alpha}$$

$$K_D^{\beta} = (C_0^{\beta} - C_B^{\beta})(R_T - C_B^{\beta} - C_B^{\alpha})/2C_B^{\beta}$$

where the superscripts α and β refer to cyt *c* and cyt c_2, respectively. These two quadratic equations were solved for C_B^{α} and C_B^{β} to obtain the combined binding $\nu_{c+c_2} = (C_B + C_B)/R_T$. Corrections due to the Donnan effect were neglected.

tation of this result is that the same site can bind either cyt *c* or cyt c_2. In view of the finite accuracy and finite cytochrome concentrations used in our experiments, we cannot exclude the possiblility of an additional, weaker binding site. From the data of Figures 3 and 4, we estimate that if such a site did exist its dissociation constant K_D must be at least ten times larger than the one given for cyt *c* and cyt c_2 in Table I. The dissociation constant has been found to depend strongly on the ionic strength and pH (Ke et al., 1970; Rosen, 1979; Rosen et al., 1979). A detailed discussion of this dependence will be presented elsewhere (T. D. Marinetti, M. Y. Okamura, D. Rosen, and G. Feher, unpublished experiments).

The above results of one binding site are in disagreement with several published reports. Ke et al. (1970) estimated 24 cytochrome binding sites on isolated RCs from *R. sphaeroides* R-26. However, in this early work, the schemes for obtaining highly purified RCs had not yet been developed. Consequently, these RCs were known to contain several cytochromes, excess ubiquinones, and phospholipids (Reed & Clayton, 1968). It is likely, therefore, that nonspecific binding was observed, in particular to the negatively charged phospholipids (Kimelberg et al., 1970; Steinemann & Laüger, 1971) that are known to be constituents of the chromatophore membrane (Gorchein, 1968).

Dutton et al. (1975) reported that chromatophores of *R. sphaeroides* contained two cyt c_2 per RC. They concluded from the photooxidation of cyt in the presence of the electron-transfer inhibitor antimycin A that each RC is associated with two cytochromes. Bowyer & Crofts (1979) cast some doubt on the interpretation of these results by demonstrating that antimycin A did not completely block electron transfer. They reported a cyt *c*/RC stoichiometry of 1:1.

Pachence et al. (1979) studied the binding of cyt *c* (horse heart) to RCs incorporated into lipid vesicles. They found 2–3 binding sites per RC, depending on the lipid-to-protein ratio. The discrepancy between their measurements and those reported in this work is not fully understood; nonspecific binding could account for their results.

Another interesting question is whether the redox potential of cytochrome changes on binding to the RCs. The difference in the midpoint oxidation–reduction potential of bound (E_M^b) and free (E_M^f) cytochrome is related to the dissociation constants of the oxidized (K_D^{ox}) and reduced (K_D^{red}) species by

$$E_M^b - E_M^f = RT/(n\mathcal{F}) \ln (K_D^{ox}/K_D^{red}) \qquad (11)$$

where R is the gas constant, T is the absolute temperature, n is the number of equivalents, and $\mathcal{F}$ is the charge per equivalent. For $n = 1$ at room temperature, eq 11 reduced to 60 log (K_D^{ox}/K_D^{red}) (in millivolts). From the measured values of K_D (see Table I), eq 11 predicts for cyt c_2 a 10-mV increase in E_M on binding; for cyt *c*, no change is expected. Contrary to these results, Dutton et al. (1975) reported for chromatophores of *R. sphaeroides* Ga a *decrease* in E_M of 50 mV from the value obtained for free cyt c_2. Equation 11 predicts that this should result in a ratio of $K_D^{red}/K_D^{ox} = 7$. A possible way of reconciling these results with ours is to postulate that the phospholipid environment of the RCs in chromatophores favors the binding of oxidized cyt. The work of Kimelberg & Lee (1970) that showed that the E_M of cyt *c* decreased from 273 to 225 mV in the presence of phospholipid vesicles makes this postulate plausible.

The concentration of cyt c_2 inside chromatophores can be estimated from the work of Packham et al. (1979) to be ~0.5 mM (~10 cyt c_2 per chromatophore having a mean diameter of ~400 Å). This is close to three orders of magnitude larger than the dissociation constants (K_D) for cyt c_2 and isolated RCs reported in this work. Consequently, each RC in the chromatophore is likely to have a cyt c_2 associated with it.

It is interesting to compare the RC–cyt system with the mitochondrial electron-transport chain, the RC in the chromatophore being analogous to the mitochondrial cytochrome oxidase. Ferguson-Miller et al. (1976), using a binding assay involving gel filtration, found two binding sites on cytochrome oxidase having dissociation contants of 10^{-6} and 3×10^{-8} M. Similarly, Errede & Kamen (1978) concluded from kinetic measurements that two cyt *c* bind to the oxidase. In view of the discrepancies in the published values of the number of cytochrome binding sites on RCs, it may be of interest to repeat the cytochrome oxidase binding experiments by equilibrium dialysis.

Acknowledgments

We thank Ed Abresch for technical assistance in the preparation of the RCs, Drs. T. D. Marinetti and B. Zimm for helpful discussions, and Drs. T. E. Meyer and R. G. Bartsch for the gift of cyt c_2 and advice on bacterial cytochromes.

References

Bartsch, R. G. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) Chapter 13, pp 249–279, Plenum Press, New York.

Bowyer, J. R., & Crofts, A. R. (1979) *Biophys. J. 25*, 53a.

Dayhoff, M. O., & Barker, W. C. (1976) in *Atlas of Protein Sequence and Structure 5*, Supplement 2 (Dayhoff, M. O., Ed.) p 301, National Biomedical Research Foundation, Washington, DC.

Dayhoff, M. O., Hunt, L. T., McLaughlin, P. J., & Barker, W. C. (1972) in *Atlas of Protein Sequence and Structure 5*, Supplement 2 (Dayhoff, M. O., Ed.) p D-14, National Biomedical Research Foundation, Washington, DC.

Dayhoff, M. O., Dayhoff, R. E., & Hunt, L. T. (1976) in *Atlas of Protein Sequence and Structure 5*, Supplement 2 (Dayhoff, M. O., Ed.) National Biomedical Research Foundation, Washington, DC.

Dutton, P. L., Petty, K. M., Bonner, H. S., & Morse, S. D. (1975) *Biochim. Biophys. Acta 387*, 536–556.

Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.

Feher, G., & Okamura, M. Y. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) Chapter 19, pp 349–386, Plenum Press, New York.

Ferguson-Miller, S., Brautian, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.

Gorschein, A. (1968) *Proc. R. Soc. London, Ser. B 170*, 279–297.

Ke, B., Chaney, T. H., & Reed, D. W. (1970) *Biochim. Biophys. Acta 216*, 373–383.

Kimelberg, H. K., & Lee, C. P. (1970) *J. Membr. Biol. 2*, 252–262.

Kimelberg, H. K., Lee, C. P., Claude, A., & Mrena, E. (1970) *J. Membr. Biol. 2*, 235–251.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.

Pachence, J. M., Moser, C. C., Blasie, J. K., & Dutton, P. L. (1979) *Biophys. J. 25*, 55a.

Packham, N. K., Berriman, J. A., & Jackson, J. B. (1979) *FEBS Lett. 89*, 205.

Reed, D. W., & Clayton, R. K. (1968) *Biochem. Biophys. Res. Commun. 30*, 471.

Rosen, D. (1979) Ph.D. Thesis, Department of Biology, University of California—San Diego, La Jolla, CA.

Rosen, D., Okamura, M. Y., & Feher, G. (1979) *Biophys. J. 25*, 204a.
Steinemann, A., & Laüger, P. (1971) *J. Membr. Biol. 4*, 74–86.
Steiner, L. A., Okamura, M. Y., Lopes, A. D., Moskowitz, E., & Feher, G. (1974) *Biochemistry 13*, 1403–1410.
Straley, S. C., Parson, W. W., Mauzerall, D. C., & Clayton, R. K. (1973) *Biochim. Biophys. Acta 305*, 597.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Chapter 14, Wiley, New York.
Van Holde, K. E. (1971) *Physical Biochemistry*, Chapter 3, Prentice-Hall, New York.

Active Transport in Membrane Vesicles from *Escherichia coli*: The Electrochemical Proton Gradient Alters the Distribution of the *lac* Carrier between Two Different Kinetic States†

Dan E. Robertson,‡ Gregory J. Kaczorowski,§ Maria-Luisa Garcia,∥ and H. Ronald Kaback*

ABSTRACT: These studies document the effects of the proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline) on the kinetics of various transport systems in right-side-out membrane vesicles from *Escherichia coli*, with particular emphasis on the β-galactoside transport system. Under completely deenergized conditions (i.e., facilitated diffusion), the β-galactoside transport system exhibits a high apparent K_m for either lactose or β-D-galactopyranosyl 1-thio-β-D-galactopyranoside, and generation of $\Delta\bar{\mu}_{H^+}$ via the respiratory chain results in at least a 100-fold decrease in the apparent K_m. Furthermore, a low apparent K_m is observed when the membrane potential ($\Delta\Psi$) or the pH gradient (ΔpH) is dissipated selectively with an appropriate ionophore and when either $\Delta\Psi$ or ΔpH is imposed artifically across the membrane. Thus, either component of $\Delta\bar{\mu}_{H^+}$ is able to elicit the low apparent K_m characteristic of the energized system. A detailed series of kinetic experiments is presented in which initial rates of lactose transport were studied as a function of lactose concentration under conditions where ΔpH and/or $\Delta\Psi$ were varied systematically at pH 5.5 and 7.5. Surprisingly, the results demonstrate that the apparent K_m remains constant from about −180 to −30 mV, while the maximum velocity of transport varies to the second power with either component of $\Delta\bar{\mu}_{H^+}$ at both pHs, even though the maximum velocity is about 10-fold higher at pH 7.5 over a comparable range of $\Delta\bar{\mu}_{H^+}$ values. Since a high apparent K_m is observed under completely deenergized conditions, the findings appear to be paradoxical; however, studies carried out over an extended range of lactose concentrations demonstrate that when $\Delta\bar{\mu}_{H^+}$ is dissipated partially the system exhibits biphasic kinetics. One component of the overall process exhibits the kinetic parameters typical of $\Delta\bar{\mu}_{H^+}$-driven active transport, and the other has the characteristics of facilitated diffusion. It is apparent, therefore, that in addition to acting thermodynamically as the driving force for active transport, $\Delta\bar{\mu}_{H^+}$ alters the distribution of the *lac* carrier between two different kinetic states. The implications of this phenomenon and possible explanations are discussed. Although studied in less detail, six different amino acid transport systems respond kinetically to $\Delta\bar{\mu}_{H^+}$ and its components in a manner similar to that described for the β-galactoside transport system. Selective dissipation of either $\Delta\Psi$ or ΔpH at pH 5.5 causes a decrease in maximum velocity with little or no change in apparent K_m, and the apparent K_m values are not altered significantly when the ambient pH is increased to 7.5. Finally, the effects of $\Delta\bar{\mu}_{H^+}$ and its components on the kinetics of the lactate and succinate transport systems were studied. At pH 5.5, nigericin abolishes D-lactate transport, and valinomycin causes a 2-fold increase in maximum velocity, demonstrating complete reliance of this system on ΔpH. In contrast, valinomycin causes a 30% increase in the maximum velocity of succinate transport at pH 5.5, but nigericin inhibits by only 50%, indicating that although this transport system is biased kinetically toward ΔpH, $\Delta\Psi$ plays a significant energetic role. There is no significant change in apparent K_m with either transport system under the conditions described at pH 5.5, but each system exhibits significant increases in both apparent K_m and maximum velocity at pH 7.5, suggesting that there may be a change in the overall transport mechanisms at higher pH (i.e., an increase in proton/substrate stoichiometry).

Membrane vesicles prepared from *Escherichia coli* are essentially devoid of cytoplasmic constituents and retain the same polarity and configuration as the membrane in the intact cell (Kaback, 1971, 1974a; Owen & Kaback, 1978, 1979a,b). Furthermore, in addition to catalyzing the vectorial phosphorylation of certain sugars via the phosphoenolpyruvate–phosphotransferase system (Kaback, 1970), these vesicles catalyze the active transport of many solutes by a respiration-dependent mechanism in which chemiosmotic forces play a central, obligatory role (Kaback, 1976; Harold, 1976; Ramos et al., 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977; Boonstra & Konings, 1977; Reenstra et al., 1980). Thus, as postulated by Mitchell (1961, 1966, 1968, 1973, 1979), energy released by oxidation of certain electron donors in this experimental system leads to the generation of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] that is composed of two

† From the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received June 2, 1980.*

‡ Present address: Department of Pathology and Laboratory Medicine, Hahnemann Medical College and Hospital, Philadelphia, PA 19104.
§ Present address: Department of Biochemistry, Merck Sharp & Dohme, Rahway, NJ 07065. Fellow of the Helen Hay Whitney Foundation.
∥ Fellow of the Spanish Research Council.

[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, the electrochemical gradient of protons; $\Delta\Psi$, the membrane potential; ΔpH, the chemical gradient of hydrogen ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; TPP^+, tetraphenylphosphonium.

0006-2960/80/0419-5692$01.00/0