pH-Metric Study of Reaction Centers from Photosynthetic Bacteria in Micellular Solutions: Protonatable Groups Equilibrate with the Aqueous Bulk Phase†

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Received July 26, 1996; Revised Manuscript Received January 29, 1997⊗

ABSTRACT: Hydrogen ion equilibria of the reaction center protein from photosynthetic purple bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* dissolved in micellular solution were studied by acid−base titration to estimate the water accessibility of protonatable residues of the protein determined from structural data. The ionizable amino acids of the reaction center underwent protonation−deprotonation with protons from the interfacial layer, which, however, exchanged protons from the aqueous bulk phase. The equilibrium was described in terms of the buffering capacity of the multiphase system. The detergents decreased the proton activity coefficient (increased the buffering capacity) of the aqueous solution by a factor of 0.33 (in 0.03% Triton X-100 and LDAO) and 0.12 (0.04% dodecyl β-D-maltoside). The observed buffering capacities of the reaction center protein were large and detergent-dependent. However, corrections for proton activities made the pH dependence of buffering capacities in different detergents uniform and similar to that expected from the number and p*K* values of protonatable groups of the protein. The vast majority of protonatable amino acids of the reaction center are in protonation equilibria with the aqueous bulk phase on an extended time scale.

The reaction center (RC)[1] of photosynthetic bacteria *Rhodobacter* (*Rb.*) *sphaeroides* is a membrane-bound pigment−protein complex with three polypeptide subunits (H, L, and M). The L and M subunits are embedded in the membrane, and the hydrophilic H subunit extends into the cytosol. The absorption of light by the special pair, P (a bacteriochlorophyll dimer), initiates electron transfer to the primary stable acceptor quinone, Q_A, and further to the secondary acceptor quinone, Q_B, if available. H^+ ions from the cytoplasmic side will be bound by protonatable groups of the RC to stabilize the anionic species of semiquinones Q_A^- and Q_B^- (Maróti & Wraight, 1988a,b; McPherson et al., 1988) or directly by the secondary quinone to completely reduce Q_B to dihydroquinone (Q_BH_2) (McPherson et al., 1990). The dihydroquinone is exported from the RC and is replaced by an oxidized quinone from the pool. The binding of protons is the first step in establishment of proton electrochemical potential, the driving force for ATP formation [for reviews, see Gunner (1991), Okamura and Feher (1992, 1995), Maróti (1993), and Takahashi and Wraight (1994)).

The stoichiometry of flash-induced H^+ ion uptake has been determined directly by a variety of methods such as spectrophotometry, conductimetry, and a pH electrode in RCs from purple bacteria of *Rb. sphaeroides* (Maróti & Wraight, 1988a,b; McPherson et al., 1988), *Rhodopseudomonas* (*Rps.*) *viridis* (Maróti, 1993), and *Rhodobacter capsulatus* (Sebban et al., 1995; Maróti et al., 1995). The proton binding was explained by shifts of p*K* values of the nearby protonatable amino acid residues exposed to the influence of the negative charge on the reduced quinone (semiquinone). Because the electrostatic interaction is screened by charged residues in the protein and the surrounding water (dipol) molecules significantly, the contribution of only a few protonatable residues had to be assumed to explain the observed proton binding stoichiometry. Structure-based continuum electrostatic calculations offered further support recently (Gunner & Honig, 1992; Beroza et al., 1995; Lancaster et al., 1996).

In addition to flash light excitation, protonation of the RC protein can be evoked by lowering the pH in the dark, as well. The pH-metric method has obtained only very limited attention (Fabian et al., 1981; Kálmán et al., 1995), although the acid−base titration of water soluble proteins has become a routine tool in physical chemistry of macromolecules and has provided a testing ground for theories of electrostatic interactions in proteins (Tanford, 1966; Tanford & Roxby, 1972; Perutz, 1978; You & Bashford, 1995). It has revealed that proteins constituted the main buffering capacity of biological systems. For example, about 80% of the total buffering power of mammalian blood is due to proteins (Dawes, 1963). It has been shown that the high buffering capacity observed in the appressed portion of stacked

† This work has been supported by grants from the Hungarian Science Foundation (OTKA 17362/95 and 014954), the Hungarian Ministry of Education (53205/96/XIV), the French and Hungarian Governments (Balaton project, 7/95), and the International Human Frontier Science Program (RG-329/95 M).

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

[1] Abbreviations: *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; RC, reaction center; P, bacteriochlorophyll dimer; Q_A and Q_B, primary and secondary ubiquinone acceptors, respectively; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; Triton X-100, octyl phenol polyethylene glycol ether; maltoside, dodecyl β-D-maltoside; cmc, critical micelle concentration; β, buffering capacity.

S0006-2960(96)01860-0 CCC: $14.00

thylakoid membranes (partitions) had a mainly proteineous origin (Junge et al., 1979; Junge & McLaughlin, 1987). The property of the buffering capacity of the RC protein solubilized in micelles is of great importance as the measured values can be compared to derived data from the structure (Allen et al., 1987; Arnoux et al., 1995) and related to the availability of protonatable amino acids to the water phase. Kinetic studies have recently indicated the relevance of exposed and buried residues in the flash-induced proton uptake of the RC, as not all of the protonatable residues of the protein are exposed to the aqueous phase in the observed time frame (Takahashi et al., 1992). The dynamics of proton exchange between bulk and surface groups in different biological systems was recently reviewed (Gutman & Nachliel, 1995).

This paper addresses pH-metric aspects of changes in the protonation of residues in the RC protein from purple bacteria. There is an added complication regarding how the physical behavior of detergents, in the form of micelles or protein−detergent aggregates, affects the observed protonation−deprotonation determined from the buffering capacity of the system, thus by a different way and on a different time scale than those used for flash-induced measurements. As the RC in micellular solution is a multiphase system consisting of aqueous, detergent, and proteineous parts, the measured buffering capacity due to protonatable groups was highly sensitive to the location of these residues. From comparison of the observed pH dependence of the buffering capacity with that calculated from structural data, it was concluded that, in the thoroughly equilibrated state, the majority of protonatable groups were accessible to the water phase. A preliminary account of similar work has been presented (Kálmán et al., 1995).

MATERIALS AND METHODS

Materials. LDAO (*N*,*N*-dimethyldodecylamine *N*-oxide, stock 30%, cmc = 0.14 mM), Triton X-100 (octyl phenol polyethylene glycol ether, cmc = 0.3 mM), and maltoside (cmc = 0.12 mM) were purchased from Fluka, Serva, and Calbiochem Chemical Corp., respectively. The cmc values are referred to a 100 mM ionic strength. Buffers were citrate (disodium citrate−citric acid, Reanal) with a p*K* of 5.0, Mes [2-(*N*-morpholino)ethanesulfonic acid sodium salt, Sigma] with a p*K* of 6.1, phosphate (disodium hydrogen phosphate−sodium dihydrogen phosphate, Reanal) with a p*K* of 7.0, Tris [tris(hydroxymethyl)aminomethane, Reanal] with a p*K* of 8.0, and Bis-Tris-propane [1,3-bis[[tris(hydroxymethyl)methyl]-amino]propane, Sigma] with a pK_1 of 6.8 and a pK_2 of 9.0.

RC Preparation. Reaction centers from wild type (Ga) and carotenoid-less mutants (R-26) of *Rb. sphaeroides* and *Rb. capsulatus* were isolated in LDAO and purified as described earlier (Maróti & Wraight, 1988a; Baciou et al., 1993). The purity was checked optically by the ratio of OD(280)/OD(802) and was less than 1.30 and 1.50 for all preparations of *Rb. sphaeroides* and *Rb. capsulatus*, respectively. The concentration of RCs was determined from the 802 nm absorption using the absorption coefficient ϵ^{802} of 318 mM^{-1} cm^{-1} (McPherson et al., 1993). The RCs contained approximately 1.4 ubiquinones after purification, as determined from the charge recombination kinetics. RCs with more than 1.9 ubiquinones were prepared by addition of an excess of ubiquinone (ubiquinone-50, Sigma) at a molar ratio of 10/1. The buffer (10 mM Tris) and, for some experiments, the ionic detergent (LDAO) were removed and replaced by nonionic detergents (Triton X-100 or maltoside) after long (48 h) dialysis at 4 °C by frequent changes of the dialyzing medium. The actual concentrations of the detergents were 1.3 mM (0.03%) for LDAO, 0.47 mM (0.03%) for Triton X-100, and 0.78 mM (0.04%) for maltoside.

LM Complex Preparation. The LM complex was isolated from the RC protein with the method of Debus et al. (1985). The RCs [OD(802 nm, 1 cm) = 10] were incubated at pH 8 and 25 °C in 0.75 M $LiClO_4$, 50 mM $CaCl_2$, 10% (v/v) ethanol, 10 mM Tris, 0.1% LDAO, and 100 μM EDTA for 1 h. Precipitated H subunits were removed by centrifugation (5000*g*, 10 min). The LM polypeptide was dialyzed for 18 h at pH 8 and 4 °C against 10 mM Tris, 0.1% LDAO, and 100 μM EDTA. The concentration of LM was determined as for the RC. For pH-metric studies, the buffers were removed with the same dialyzing procedure as was done with the RC.

Optical Measurements. Absorption spectra were recorded on a Specord M40 UV-VIS spectrophotometer (Carl Zeiss Jena). Flash-induced absorbance changes were measured on a spectrophotometer of local design (Maróti & Wraight, 1988a).

Acid−Base Titration. Potentiometric titration of a concentrated (3−17 μM) solution of RCs and the LM complex was carried out in an air-tight cuvette at 295.0 ± 0.5 K under a nitrogen atmosphere. The titrant solutions (NaOH and $HClO_4$) were prepared and stored in a closed box under a nitrogen atmosphere, as well. Changes in pH were followed by a combined glass electrode (Orion, no. 91-03) and pH meter (Orion 710, precision 0.1 mV). A computer-controlled titrator was used, and establishment of equilibrium after addition of titrant was considered when the potential change of the pH electrode remained within 0.2 mV. It took from several seconds to a couple of minutes. The activity of hydrogen ions (a_H) and thus the pH (=−log a_H) were determined from the measured electromotive force (*E*) relative to the standard potential (E_0) according to the following relationship:

$$E = E_0 + \frac{RT}{F}\ln(a_H) + j_H a_H + j_{OH}\frac{K_w}{a_H} \qquad (1)$$

where j_H and j_{OH} are fitting parameters in acidic and alkaline media for the correction of experimental errors, mainly arising from the liquid-junction potential and from the possible alkaline and acidic deviations of the glass electrode [see, e.g., Gajda et al. (1995)], $K_w = a_H a_{OH}$ is the autoprotolysis constant of water ($K_w = 10^{-14.0}$ M^2 at T = 295 K; Dawes, 1963), a_{OH} is the activity of OH^- ions, and *R* and *F* are the gas constant and the Faraday constant, respectively.

Buffering Capacity and Analysis. The buffering capacity is a differential ratio of $\beta = -d[H^+]/d(pH)$, where $d[H^+]$ is the concentration of protons added to the solution and d(pH) the resultant change in pH. Ideally, the increments should be infinitesimal, but in actual practice, measurable increments permit a reasonable evaluation of β: (1) addition of a small amount (5 μL) of strong acid or base (concentrations of 5, 10, and 20 mM) to a 5 mL solution and thorough mixing and (2) derivative of the acid−base titration curve with respect to pH.

The buffering capacity of aqueous solution is due to H_3O^+ (for the sake of simplicity, H^+), OH^-, and several buffering groups with different concentrations ($[B_i]$) and equilibrium proton dissociation constants (K_i) (Junge et al., 1979; Kálmán et al., 1995):

$$\beta = \frac{2.3}{f_H}\left[a_H + \frac{K_w}{a_H} + \sum_i [B_i]\frac{K_i a_H}{(K_i + a_H)^2}\right] \qquad (2)$$

where f_H ($=a_H/[H^+]$) is the activity coefficient of H^+ ions in the solution and β is expressed in units of moles per liter of H^+ per pH. The first and second terms in the bracket refer to the contribution of H^+ and OH^- ions, respectively, and the summation in the third term should be extended to all buffers in the solution. The buffering capacity is inversely proportional to the activity coefficient of the hydrogen ions (f_H) and is linearly proportional to the concentration of the buffering groups ($[B_i]$).

If the buffering groups are distributed in two phases of different hydrogen ion activity coefficients but identical activities (pH values), the total buffering capacity will be additive in the particular relationships defined by eq 2 weighted by the volumes (V) of the phases (Junge et al., 1979; Junge & McLaughlin, 1987):

$$\beta = \frac{\beta_1 V_1 + \beta_2 V_2}{V_1 + V_2} \qquad (3)$$

Subscripts 1 and 2 and the $[B_i]$ concentrations in eq 2 refer to the two phases of the solution.

The time domain in each step of the titration was selected from seconds to several minutes to assure complete delocalization of protons in both phases; thus, a constant electrochemical potential of the proton was established throughout each phase.

Curve Fitting. Fits to data were obtained by using the program Lotus 123 on an IBM compatible personal computer.

RESULTS

Protonatable Groups in the One-Phase System. To demonstrate the effectiveness of the pH-metric method, and to check our system for protonation measurements, acid−base titrations of a single amino acid (L-histidine) and a water soluble protein with known structure (lysozyme) were carried out. The observed buffering capacity of histidine in aqueous solution could be well fitted by eq 2 with two pK values of 6.0 and 9.1 and a proton activity coefficient f_H of 1.0. Two protons were taken up per histidine as determined from integration of the buffering capacity between pH 12 and 4 (Figure 1a). The first proton with a pK of 6.0 is due to the protonation of the imidazole side chain group, and the proton with a pK of 9.1 is attributed to the protonation of the α-amino group. The pK of the α-carboxyl group is around pH 3 which is out of the investigated pH range.

The observed pH dependence of the integrated proton uptake of lysozyme is demonstrated in Figure 1b. In aqueous solution, the protein binds about six protons while the pH decreases from 10 to 5. A similar value can be deduced from earlier measurements, although there is no consensus on the reported pK values of the residues determined by different methods (Kuramitsu & Hamaguchi, 1980; Beroza et al., 1991; Bartik et al., 1994; You & Bashford, 1995). The H^+ ion activity coefficient in the solution, however, can differ from unity. The decrease of proton activity can be achieved in a one-phase system by increasing the ionic strength (100 mM NaCl, $f_H = 0.96$, Figure 2) or by mixing glycerol with the aqueous solution [50% v/v), $f_H = 0.79$, Figure 3b]. According to eq 2, the pH dependence of the buffering capacity becomes steeper compared to that of the strong acid−strong base titration. This is reflected by the

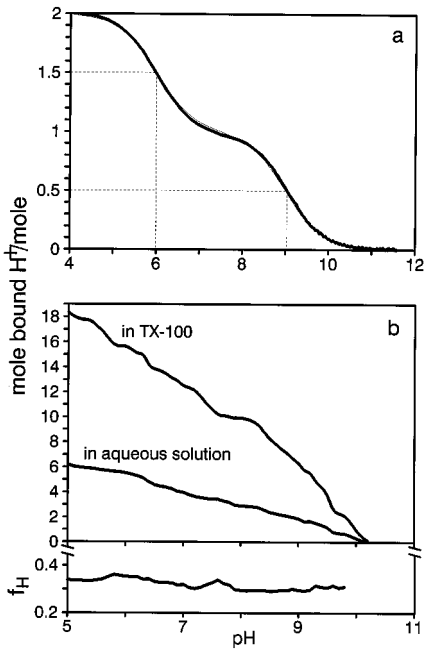

FIGURE 1: Equilibrium acid−base titration curves of water soluble amino acid L-histidine (a) and protein lysozyme (b). The observed proton uptake of L-histidine (thick line) was fitted by the sum (thin line) of two Henderson−Hasselbalch type curves of protonatable residues of the imidazole side chain group and α-amino group with pK values 6.0 and 9.1, respectively. The titration curves for lysozyme were measured both in aqueous solution and in Triton X-100 detergent between pH 5 and 10, and their ratio (the activity coefficient of H^+ ions, f_H) was also determined (bottom). Conditions: 500 μM L-histidine (a) and 40 μM lysozyme in 100 mM NaCl with and without 0.03% Triton X-100 (b).

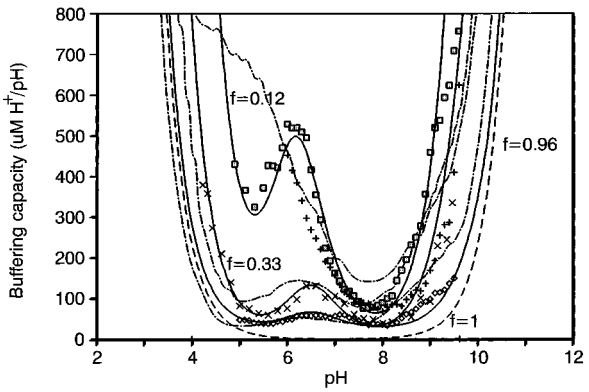

FIGURE 2: pH dependence of buffering capacity of aqueous solution of 100 mM NaCl salt (◇) and detergents 0.03% Triton X-100 (×), 0.03% LDAO (+), and 0.04% maltoside (□). The buffering capacity is determined directly from the definition [$\beta = -d[H^+]/d(pH)$, discrete points] or from the first derivative of the equilibrium pH titration (thick dotted lines). The measured data are simulated by eq 2 with fitting parameter f_H (solid thin lines). Hydrocarbonate (50 μM) with a pK of 6.4 is included as contamination. The ideal case of titration of strong acid and strong base with no hydrocarbonate contamination is also shown for comparison ($f_H = 1.0$, dashed thin line).

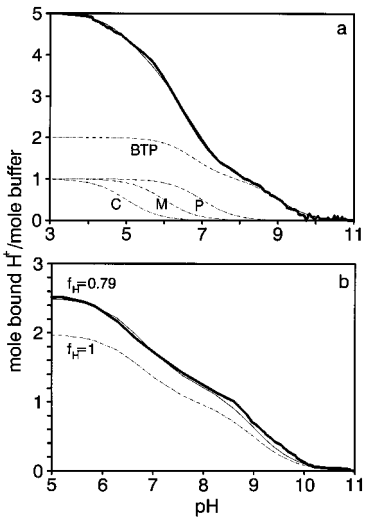

FIGURE 3: Integrated proton uptake of buffers in micellular solution (a) and in a glycerol−water mixture (b). The measured acid−base titration data (thick continuous line) are decomposed into the sum (thin continuous line) of contributions of the buffers. (a) Citrate (C, pK = 5.0), Mes (M, pK = 6.1), phosphate (P, pK = 7.0), and Bis-Tris-propane (BTP, pK_1 = 6.8 and pK_2 = 9.0) (dotted thin lines). Conditions: 200−200 μM buffers, 0.03% Triton X-100, and 100 mM NaCl. (b) Bis-Tris-propane (pK_1 = 6.8 and pK_2 = 9.0) and best fit with an f_H of 0.79 (thin line). The deviation from approximation with an f_H of 1 is also indicated (thin dotted line). Conditions: 500 μM Bis-Tris-propane, 100 mM NaCl, and 50% (v/v) glycerol.

approach of the alkaline and acidic branches of the U-shaped titration curve in the β vs pH representation (Figure 2). The actual values of proton activity coefficients can be determined from best fits of the set of the pH dependence of the buffering capacity based on eq 2. A similar tendency can be observed, and the same procedure can be carried out to determine the proton activity coefficient in micellular solutions of different detergents. f_H = 0.33 was obtained for 0.03% Triton X-100 and 0.03% LDAO and f_H = 0.12 for 0.04% maltoside (Figure 2).

Protonatable Groups in the Two-Phase System. The micellular solution is a two-phase system, and the protonatable groups can be found either in the aqueous or in the detergent phase. As the f_H values and volume fractions of the two phases are different, the observed buffering capacity of the system should depend on the participation of the protonatable residues in the phases (see eq 3). The distribution is controlled by the (physical) chemical properties of the substances in a complex way, but some general features can be established in our case.

The pH dependence of buffering capacities attributed to histidine in micellular solutions was the same as that found in aqueous solution (Figure 1a) and was independent of the types of detergent (data not shown). (The buffering capacity of the detergents, as background, was always subtracted.) The integrated proton uptake−release of a mixture of buffers spanning a wide pH range in micellular solution offered stoichiometric values and could be well fitted by the integrated form of eq 2 with an f_H of 1.0 and pK values generally accepted for these buffers in aqueous solution (Maróti & Wraight, 1988a) (Figure 3a). All these molecules were water soluble and were present in large concentrations relative to those of the detergents. The observed buffering capacity reflected the preferential distribution of the protonatable residues in the aqueous phase.

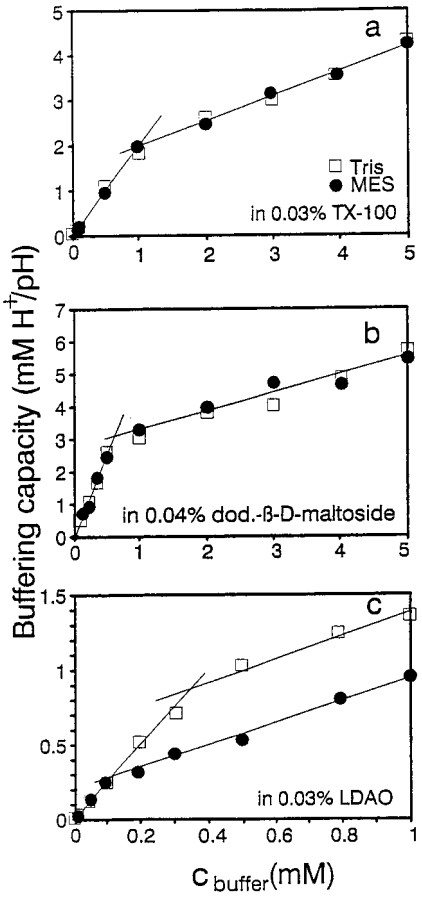

FIGURE 4: Concentration dependence of buffering capacity of cationic (Tris, □) and zwitterionic (Mes, ●) buffers in micellular solutions of nonionic [Triton X-100 (a) and maltoside (b)] and ionic [LDAO (c)] detergents. Conditions: 100 mM NaCl at pH 6.1 in Mes and at pH 8.0 in Tris buffers.

If, however, the concentration of the protonatable groups becomes much smaller than the actual concentration of the detergent, the buffering capacity will depend on the detergent and will become higher than expected from the behavior observed at high concentrations. This is demonstrated by the low concentration of lysozyme in Triton X-100 detergent (Figure 1b) and by two buffers (cationic Tris and dipolar Mes) in three detergents: in Triton X-100, in maltoside, and in LDAO (Figure 4).

Addition of detergent to the aqueous solution of lysozyme provides a titration with a global increase in the apparent protein buffering capacity (Figure 1b). The H^+ ion activity coefficient (f_H) can be determined as the ratio of the integrated proton uptake measured in water and in Triton X-100 detergent. It is constant (f_H = 0.33) on the whole observed pH range and agrees well with the value determined in Figure 2.

The concentration dependence of the buffering capacities of the buffers can be approximated by two straight lines of different slopes corresponding to the possible distribution of the buffers in the two phases (Figure 4). At low concentrations, the buffer prefers the vicinity of the micelles and saturation (break point) occurs at a concentration comparable to the actual concentration of the detergent. Unfavorable electrostatic interactions between the ionic detergent (LDAO) and polar buffers can shift the break point significantly (Figure 4c). Above the break point, the micelles have no effect on the distribution of the buffer in the aqueous

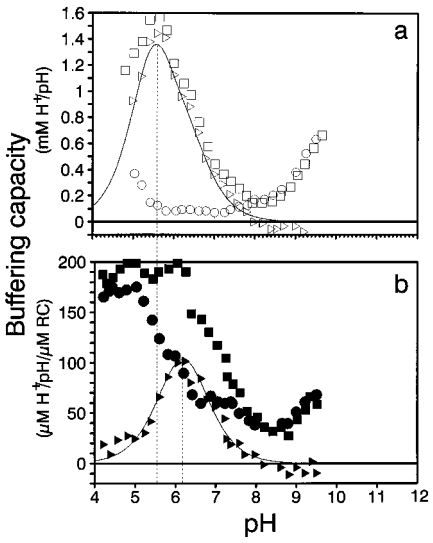

FIGURE 5: Identification of the protonatable group of ionic detergent LDAO in aqueous solution (a) and attached to the RC (b). pH dependence of buffering capacities of micellular solutions of detergents LDAO (□) and Triton X-100 (○) and their difference (tilted open triangle) in the absence of RCs (a, open symbols) and in the presence of RCs (b, filled symbols). The buffering capacities in panel b were corrected for those without RC. The best single-component fit to the difference of buffering capacities of LDAO and Triton with (pK = 6.2) and without (pK = 5.6) RCs is shown (thin lines). Conditions: 0.03% LDAO, 0.03% Triton X-100, 100 mM NaCl, and 5 μM RC.

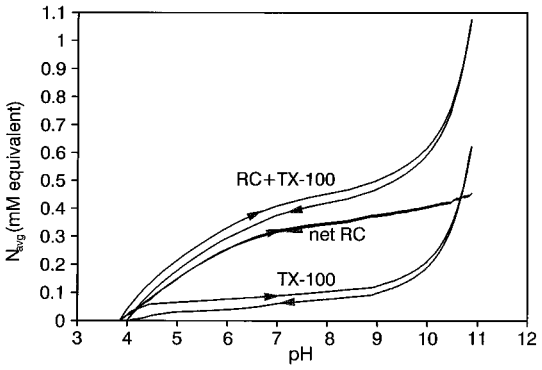

FIGURE 6: Acid−base titration curves of the RC in Triton X-100 (top), the Triton X-100 detergent (bottom), and their difference (middle). The average number of equivalents is referred to the concentration of the RC. The direction of titration is indicated by arrows. The highest pH value (pH 11.0) was the reference point for the acidic and alkaline titrations. Conditions: 9 mL sample (0.03% Triton X-100 and 100 mM NaCl with or without 5 μM RC) and 0.1024 M NaOH and 0.0915 M $HClO_4$ as titrants.

bulk phase. According to eq 2, the slopes of the increase in the buffering capacity below and above the saturation concentration of the buffer are inversely proportional to the proton activity coefficients in the micellular and bulk phases, respectively. Although the position of the break point was dependent on the types of detergent and buffer, the ratios of the slopes were very similar: 0.29, 0.31, and 0.10 for Triton X-100, LDAO, and maltoside, respectively. These values are in good agreement with the proton activity coefficients found in different detergents (Figure 2).

In titration experiments, about 50 μM hydrogen carbonate and bicarbonate groups remained as contamination in the sample (Figure 2). It can be readily identified by their pKs of 6.4 and 10.1, respectively. Although these species are water soluble, they track the proton activity coefficient characteristic to the detergent phase of the system; with the appropriate f_H value in eq 2, the complete pH dependence (including the protonation of hydrogen carbonate) can be described (Figure 2). Due to their very low concentration, they are mainly adsorbed to the micelles. Even if the group is water soluble, the micellular system can influence its protonation.

The LDAO is an ionic detergent, and the pK of its protonatable group can be determined by comparison of its buffering capacity with that of the nonionic detergent Triton X-100. In the alkaline pH region, identical buffering capacities were found for Triton X-100 and LDAO (see Figures 2 and 5a), indicating that no protonatable group was available here. However, the difference in buffering capacities of LDAO and Triton X-100 in the acidic range is not diminishing and can be approximated by the contribution of a single protonatable group with a pK of 5.6. A similar procedure can be done in the presence of the RC protein if the difference of the net buffering capacities (with or without detergent) is taken (Figure 5b). As the contribution of LDAO detergents not attached to the RC is eliminated in that way, the observed pK value of 6.2 represents the proton dissociation constant of those LDAO molecules which are in (electrostatic) interaction with the protein.

Acid−Base Titration of the RC Protein. The buffering capacity of the RC protein from strain *Rb. sphaeroides* R-26 in Triton X-100 micelles was measured as a function of concentration up to 12 μM at pH 8 (data not shown). In agreement with eq 2, a linear relationship was obtained, indicating that the change in the measured buffering capacity could be attributed to protonatable groups of the protein. As the constant part of the observed buffering capacity has a nonproteineous origin, the buffering capacity of the RC could be obtained by subtraction of the buffering capacity of micellular solution from that of the RC solution of the same detergent concentration.

A typical acid−base titration curve of the RC from *Rb. sphaeroides* in Triton X-100 is shown in Figure 6. The titration was carried out reversibly between pH 4.0 and 11.0, and small hysteresis could be observed. With the strong base, a tiny amount of sodium carbonate (pK = 10.1) was introduced into the solution that converted into hydrogen carbonate (pK = 6.4). Thus, an additional amount of titrant (acid) was required for the titration in the opposite (acidic) direction. In the difference (with or without detergent) curve, the hysteresis was reduced significantly, but some (reminiscent) hysteresis still remained which was probably due to pH-induced reversible conformational changes of the protein observed frequently in macromolecules (Tanford, 1966). It is important to note that the integrated proton uptake clearly (many times) exceeded the value expected from the number of protonatable residues in the RC. It could be expressed also in terms of buffering capacity which was more sensitive to the position (pK values) of the protonatable groups (Figure 7a). The measured buffering capacities for different species were highly detergent-dependent. The widely scattered data of RCs in different detergents show, however, a rather uniform pH pattern, if they are corrected for the proton activities of the micellular solution determined in Figure 2 (Figure 7b).

pH Titration of the LM Subunit. Acid−base titration was carried out on an active and stable LM complex obtained from RCs by dissociating the H subunit with chaotropic agent

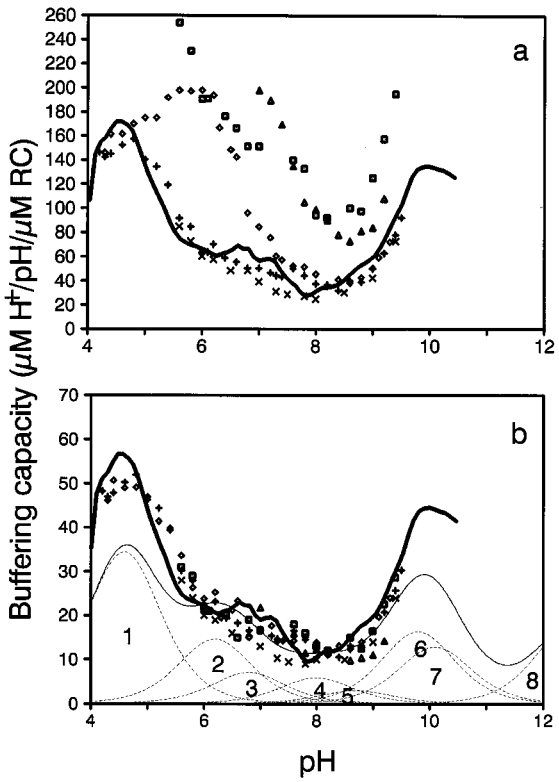

FIGURE 7: pH dependence of buffering capacity of RCs from different strains solubilized in different detergents. Observed buffering capacities (a) and buffering capacities corrected for proton activity coefficients in detergents (*f*) taken from Figure 2 (b). Symbols: *Rb. sphaeroides* R-26 in Triton X-100 (+), R-26 in LDAO (◇), R-26 in maltoside (□), 2.4.1 in Triton X-100 (×), *Rb. capsulatus* in maltoside (△), the derivative of titration (Figure 6) of *Rb. sphaeroides* R-26 in Triton X-100 (thick continuous line), contributions of the protonatable amino acid residues of the RC protein, Tris buffer as contamination and LDAO bound to RC (thin dashed lines), and their sum (thin solid line); 155 protonatable site were considered (Allen et al., 1987) with the following p*K* values expected in proteins (Cantor & Schimmel, 1980): 58 Asp and Glu (1, pK = 4.6), 12 His (3, pK = 6.8), 5 Cys (5, pK = 8.6), 28 Tyr (6, pK = 9.8), 22 Lys (7, pK = 10.1), 30 Arg (8, pK = 12.5), 25 LDAO (2, pK = 6.2, tightly bound), and 10 Tris (4, pK = 8.0) molecules. Conditions: 3−17 μM RC, and otherwise as in Figure 2.

$LiClO_4$ in the presence of mild detergent (Debus et al., 1985). Comparison of buffering capacities of the LM complex and the native RC showed that removal of the H subunit resulted in a decrease in buffering capacity throughout the pH range (Figure 8). The decrease in buffering capacity corresponded to the decrease in the numbers of protonatable groups in the LM complex compared to those in the RC.

DISCUSSION

In this work, acid−base titrations of RCs from purple bacteria were carried out which have proved to be an effective tool for studying protonation events in addition to routinely applied (spectrophotometric, conductimetric, electric, etc.) methods. Because the RC is not a water soluble but an integrated membrane protein, the evaluation of pH-metric measurements is complicated by different proton activity coefficients in different phases of the protein−detergent aqueous solution. pH titration of the protonatable amino acid residues of the RC in the pH range from 4 to 11 revealed that most of them were available for proton exchange to the aqueous phase on an extended time scale

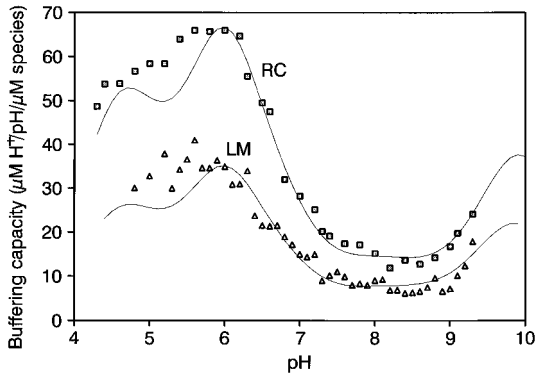

FIGURE 8: Comparison of buffering capacities of the RC and LM complex corrected for proton activity coefficients observed in detergent LDAO (f_H = 0.33, see Figure 2). The buffering capacity is expressed in terms of the unit concentration of the RC and LM complex and is decomposed into the sum of the contributions of protonatable groups: 58 and 29 Asp and Glu, 12 and 6 His, 5 and 3 Cys, 28 and 21 Tyr, 22 and 8 Lys, 25 and 25 LDAO (pK = 6.2, tightly bound), 55 and 15 LDAO (pK = 5.6, loosely bound), and 10 and 5 Tris (pK = 8.0) molecules for the RC and LM complex, respectively (thin lines). For p*K*s of amino acids, see Figure 7. Conditions: 0.03% LDAO, 100 mM NaCl, 5 μM RC, and 3 μM LM complex.

required for complete equilibration. The discussion will focus on the origin of the decreased proton activity coefficient on the micelle−water interface and the availability of protonatable residues of the RC protein to solvent.

Decrease of f_H in the Detergent Phase. H^+ ion has a 5−6 times higher molar conductivity (mobility) in water than other monovalent ions of similar sizes [see, e.g., Bockris and Reddy (1977)]. The extremely high proton mobility is due to the special mechanism of conduction by water (Grotthus mechanism). Instead of a single, highly solvated proton moving from the proton donor to the acceptor in the aqueous bulk phase, there is an effective motion of protons that involves the rearrangement of bonds in a group of water molecules. The conductivity is correlated to the activity coefficient of the H^+ ion in the solution. It is governed by the rates at which the water molecules can rotate into orientations in which they can accept or donate protons and the rate at which the protons tunnel from one end of a hydrogen bond to another. The detergents penetrate into and thus break the extended network of hydrogen bonds of water molecules. The structure can be described by the distorted hydrogen bond model (Tanford, 1973). All water molecules continue to be hydrogen-bonded to neighboring water molecules, but the intermolecular links can be bent and stretched to produce irregular and varied networks. The proportion of hydrogen-bonded clusters decreases, and the fraction of broken hydrogen bonds increases. This explains the observed dependence of the proton activity coefficient on the type and the concentration of detergents (Figures 2 and 3).

The decrease of f_H shrinks the pH range of the small buffering capacity and thus limits seriously the sensitivity of pH-metric measurements. Additionally, it has important consequences for the observed consumption of externally added H^+ ions by protonatable groups. As expressed in eq 2, protonation of a group in a phase where the proton activity coefficient is f_H requires a $1/f_H$ stoichiometric amount of added H^+ ions. This can be expressed in terms of buffering capacity, as well; the smaller the proton activity coefficient

of the phase where the protonatable residue is located, the larger will be its contribution to the observed buffering capacity of the system. On the basis of eqs 2 and 3, the measured pH dependence of the buffering capacity can be decomposed into the contribution of individual groups. In the simplest case, when only a single residue can be accounted for in the observed buffering capacity, the maximum (β_{max}) is where pH = pK and the concentration of the buffering group [B] = $^4/_{2.3}\beta_{max}f_H$.

Availability of Protonatable Groups in the RC. The measured buffering capacity of the RC showed two essential features. (1) It was much larger on the whole pH range than expected if all of the protonatable groups of the protein were exposed to the aqueous bulk phase, and (2) it depended very much on the type of detergent (Figure 7a). It should be kept in mind, however, that the micellular solution of the protein is a multiphase system and the protonatable amino acids can either be at the water−protein interface or have direct access to the water through the detergent phase or are buried in the hydrophobic interior of the protein. These positions can be characterized by appropriate f_H values. We saw that all of the groups of the protein with an f_H of 1 would not be enough to explain the observed high buffering capacity. One can argue either for impurities in the RC preparation or for many buried groups in the RC, as they have low f_H values. The different strains and preparations with different purities showed neither marked overall nor characteristic (pH-dependent) changes in buffering capacities (Figure 7). Thus, the impurities did not contain a significant amount of protonatable groups, and their contribution to the observed buffering capacity was negligible. The assumption of relatively few buried groups of small f_H values could theoretically account for the high buffering capacity but leads to apparent conflict with the observed strong detergent dependence of β. On the other hand, if all residues are supposed to have direct contact with the detergent phase, the detergent dependence of the buffering capacity would be eliminated and move reasonably close to results of model calculations (Figures 7 and 8).

Of the 155 protonatable sites resolved in the X-ray structure of *Rb. sphaeroides* (Allen et al., 1987; Arnoux et al., 1995), 58 Asp and Glu (pK = 4.6), 12 His (pK = 6.8), 5 Cys (pK = 8.6), 28 Tyr (pK = 9.8), 22 Lys (pK = 10.1), and 30 Arg (pK = 12.5) molecules were taken into account with pK values generally expected in proteins (Cantor & Schimmel, 1980). Although there are indications that some of these groups could have significantly altered pK values due to exceptionally large electrostatic interaction, the large number of residues in the RC makes the acid−base titration curves relatively insensitive to the pK shifts of only a few groups (Gunner & Honig, 1992; Beroza et al., 1995; Lancaster et al., 1996). The method sets the stage for global description of the protonation behavior of all the residues but not for some specially located groups. However, the specificity can be increased significantly if the pH-metric method is combined with selective methods as light (flash)-induced changes and/or site-directed mutagenesis of the protein (Maróti & Wraight, 1988a,b; McPherson et al., 1988; Okamura & Feher, 1992, 1995; Maróti et al., 1994, 1995; Sebban et al., 1995). In addition to the groups listed above, 10 Tris (pK = 8.0) and 25 LDAO (pK = 6.2, see Figure 5b) molecules per RC were considered in the simulation (Figure 7b). These species remained as traces in the sample because of the incomplete removal of the Tris buffer from the stock solution via dialysis and strong association of LDAO detergent with the RC. The simulation resulted in 25 LDAO molecules that were considered to be attached to the RC so firmly that they could not be replaced by other detergent molecules even after very long dialysis. In addition to this, there are LDAO molecules which are loosely associated with the RC; i.e., they can be replaced by Triton detergent after long dialysis (see Figure 5b). This number can be estimated from the measured buffering capacity and the observation that most of the detergent in the RC crystals appeared to be disordered and covered the wide hydrophobic surface around the center of the protein (Roth et al., 1989; Garavito et al., 1996). Additionally, 201 localized water molecules were identified mainly at subunit interfaces; 108 of these were located at the periplasmic side and 88 on the cytoplasmic side of the membrane, and 5 were bound in the interior of the LM complex (Deisenhofer et al., 1995). The arrangement of bound detergent and localized water molecules on the surface of the RC favors the exposure of the protonatable groups of LDAO to the aqueous phase. On the basis of Figure 5b and with f_H = 1, 175 LDAO/RC can be calculated. Together with the firmly bound 25 LDAO molecules, 200 LDAO molecules are associated with the RC. This number is in reasonable agreement with minimal solubilization concentrations determined by neutron diffraction using H_2O/D_2O contrast variation (238 LDAO/RC; Rivas et al., 1980; Roth et al., 1991) or by detergent-adsorbing beads (370 LDAO/RC at pH 6.0 and 40 LDAO/RC at pH 10.0; Hemelrijk et al., 1995).

The overlap of the calculated and (detergent-corrected) observed buffering capacities is satisfactory, and the deviation is within the error of measurement except on the rims of the pH range (Figure 7b). The difference at very low and high pH values can hardly be explained by uncertainty of the *in situ* pK values of the protonatable groups. It is more straightforward to suppose that some residues are more buried in the hydrophobic interior of the protein. As the difference is not large, few groups should be involved only. For example, the assumption of 13 Asp and Glu, 3 Tyr, and 2 Lys residues in an environment where f_H = 0.1 would result in complete elimination of the observed difference. Taking a small f_H value, even fewer groups would be required to account for the difference.

The protonation behavior of the residues in the L and M subunits has not changed significantly after removal of the H subunit; a similar procedure with proportionally less groups can be carried out with the LM complex as was demonstrated with the whole protein (Figure 8). The observed additivity of buffering capacities of the LM and H subunits indicates that the protonatable residues have a similar and rather uniform environment from the point of view of proton activity. The majority of groups are exposed to the water phase through detergents; and only a few titrating sites have direct contact with the aqueous bulk phase.

As the RC protein−detergent aggregate is the species that undergoes protonation−deprotonation, its characteristics and behavior in solution become important to understand. In solution, the RC-bound detergent appears to be distributed as a uniform band about the protein surface and contribute significantly to the degree of exposure of protonatable groups of the RC. This study showed that the physicochemical properties of the detergent layer could not be ignored when evaluating acid−base titration experiments.

ACKNOWLEDGMENT

Thanks are due to Prof. Kálmán Burger for helpful discussions.

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BI961860O