Anion Binding by Human Lactoferrin: Results from Crystallographic and Physicochemical Studies†

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ABSTRACT: The anion-binding properties of lactoferrin (Lf), with Fe^{3+} or Cu^{2+} as the associated metal ion, have been investigated by physicochemical and crystallographic techniques. These highlight differences between the two sites and in the anion-binding behavior when different metals are bound. Carbonate, oxalate, and hybrid carbonate–oxalate complexes have been prepared and their characteristic electronic and EPR spectra recorded. Oxalate can displace carbonate from either one or both anion sites of $Cu_2(CO_3)_2Lf$, depending on the oxalate concentration, but no such displacement occurs for $Fe_2(CO_3)_2Lf$. Addition of oxalate and the appropriate metal ion to apoLf under carbonate-free conditions gives dioxalate complexes with both Fe^{3+} and Cu^{2+}, except when traces of EDTA remain associated with the protein, when hybrid complexes $M_2(CO_3)(C_2O_4)Lf$ can result. The anion sites in the crystal structures of $Fe_2(CO_3)_2Lf$, $Cu_2(CO_3)_2Lf$, and $Cu_2(CO_3)(C_2O_4)Lf$, refined at 2.2, 2.1, and 2.2 Å, respectively, have been compared. In every case, the anion is hydrogen bonded to the N-terminus of helix 5, an associated arginine side chain, and a nearby threonine side chain. The carbonate ion binds in bidentate fashion to the metal, except in the N-lobe site of dicupric lactoferrin, where it is monodentate; the difference arises from slight movement of the metal ion. The hybrid complex shows that the oxalate ion binds preferentially in the C-lobe site, in 1,2-bidentate mode, but with the displacement of several nearby side chains. These observations lead to a generalized model for synergistic anion binding by transferrins.

Lactoferrin is a member of the family of binding proteins known as the transferrins (Aisen & Listowsky, 1980; Chasteen, 1983; Brock, 1985). These monomeric glycoproteins, $M_r \sim 80\,000$, play a key role in controlling the levels of free iron in biological fluids through their very tight, but reversible, binding of iron. Crystallographic studies of human lactoferrin (Anderson et al. 1987) and rabbit serum transferrin (Bailey et al., 1988) have shown that both proteins have similar bilobal structures. Within each protein there are two specific iron-binding sites, one in each lobe, located deep in a cleft between two protein domains.

A key feature of the binding properties of transferrins is the synergistic relationship between metal and anion binding; two Fe^{3+} ions are bound in the specific sites, but only if two CO_3^{2-} (or other suitable) anions are concomitantly bound. Just as other di-, tri-, and tetravalent metal ions can substitute for Fe^{3+} (Brock, 1985), so other anions can substitute for CO_3^{2-}. In a study of the binding of over 30 anions to transferrin, Schlabach and Bates (1975) concluded that synergistic anions must possess a carboxylate group and a proximal electron donor group, and obey certain steric limits. This then led to their "interlocking sites" model, whereby the anion was simultaneously bound to the metal ion (through the electron donor group) and a cationic group on the protein (through the carboxyl group).

Numerous spectroscopic studies have given evidence of direct binding of the anion to the metal. These include electron spin echo studies of Fe^{3+}-, Cu^{2+}-, and VO^{2+}-transferrins, with either carbonate or oxalate as synergistic anion (Zweier et al., 1979, 1982; Eaton et al., 1989, 1990); EPR[1] studies of VO^{2+} transferrin with various anions (Campbell & Chasteen, 1977); ^{13}C NMR studies of Fe^{3+}-, Al^{3+}-, Ga^{3+}-, and Zn^{2+}-transferrins (Harris et al., 1974; Zweier et al., 1981; Bertini et al., 1986a); and resonance Raman and EXAFS studies of anion binding to Fe^{3+}-ovotransferrin (Schneider et al., 1984). Some of these studies also suggested the presence of a neighboring cationic protein group (Zweier, 1980; Zweier et al., 1981), in accord with the "interlocking sites" model, and chemical modification experiments implicated an essential arginine side chain (Rogers et al., 1978).

Nevertheless, important questions remain. The bound carbonate ion plays a key role in metal binding and release, and direct crystallographic evidence of the metal–anion interaction is needed. The initial crystallographic studies of lactoferrin and transferrin showed electron density consistent with bidentate binding of the carbonate ion to the metal, but the limited resolution (3.2 and 3.3 Å, respectively) gave little detail. Refinement of the lactoferrin structure at 2.8-Å resolution (Anderson et al., 1989) confirmed the bidentate coordination but higher resolution is desirable.

The acceptance of a wide variety of other anions in place of carbonate raises questions of how the proteins adapt to sterically and chemically different ions. The extension of bidentate carbonate coordination to a more general model, covering other anions, and its relationship with the "interlocking sites" model, is not straightforward. For example, model building from the lactoferrin structure (Baker et al., 1990) and electron spin echo studies (Eaton et al., 1989) both suggest that oxalate binds in 1,2-bidentate fashion, while ^{13}C

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[1] Abbreviations: Lf, lactoferrin; Tf, transferrin; EPR spectroscopy, electron paramagnetic resonance spectroscopy; NTA, nitrilotriacetic acid; FeNTA, ferric nitrilotriacetate; rms, root mean square.

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NMR studies have been interpreted in terms of monodentate oxalate binding (Bertini et al., 1986a; Sola, 1990), in line with the Schlabach and Bates (1975) model. The existence of several alternate configurations, for different anions (Campbell & Chasteen, 1977) also requires structural explanation.

There is also evidence of differences between the anion sites, both within a given transferrin and between different transferrins. These differences appear much more pronounced for anions other than carbonate. Thus it has been reported that for Cu^{2+}-substituted lactoferrin and transferrin only one oxalate ion can be bound, and mixed-anion complexes, with carbonate in one site and oxalate in the other, can be prepared (Zweier & Aisen, 1977; Ainscough et al., 1983). On the other hand, Cu^{2+}-substituted ovotransferrin can accommodate two oxalate ions, with rather different spectroscopic parameters (Zweier, 1980), and distinct site inequivalence is seen for oxalate bound to Co^{2+}-substituted ovotransferrin (Bertini et al., 1986b).

Here we present high-resolution crystallographic details of the binding of both carbonate and oxalate ions to human lactoferrin (Lf), from carbonate complexes of diferric lactoferrin and dicupric lactoferrin, $Fe_2(CO_3)_2Lf$ and $Cu_2(CO_3)_2Lf$, and a hybrid carbonate–oxalate complex of dicupric lactoferrin, $Cu_2(CO_3)(C_2O_4)Lf$. The oxalate binding seen in the latter allows us to elaborate on the original "interlocking sites" model, in the light of the crystallographic data, and to advance structural explanations for some of the effects noted in previous solution studies. At the same time, we present further solution studies of oxalate binding, complementary to the X-ray studies, which confirm the inequivalence of the two sites in lactoferrin and demonstrate the quite profound differences in binding behavior which can result from the use of different metal ions or from the presence of nonsynergistic anions associated with the apoprotein.

Experimental Procedures

All glassware was soaked in nitric acid and thoroughly rinsed with doubly distilled water prior to use. All buffers were passed through a column of a chelating resin (Bio-Rad Chelex-100, 100–200 mesh, sodium form) before use to minimize contamination by adventitious metal ions.

Human apotransferrin was purchased from Sigma Chemical Co. and used without further purification. Concentrations of solutions of apo-Tf were estimated from $\epsilon_{280nm} = 83\,800$ mol^{-1} L cm^{-1} (Zweier & Aisen, 1977). Isotopically pure ^{65}Cu was obtained as ^{65}CuO from Oak Ridge National Laboratory and dissolved in concentrated HCl. All other reagents were of the highest purity commercially available.

Concentrations of Cu(II) and Fe(III) solutions were confirmed by atomic absorption spectroscopy using a GBC 905AA atomic absorption spectrophotometer. UV–visible spectra in the range 250–820 nm were measured and recorded at 25 °C on a Hewlett-Packard HP8452A diode array spectrophotometer. Electron paramagnetic resonance (EPR) spectra were recorded at 110 K, with protein concentrations of approximately 1.25×10^{-4} mol L^{-1} (for copper–lactoferrin complexes) or 2.00×10^{-4} mol L^{-1} (for iron–lactoferrin complexes), by using a Varian E-104A spectrometer equipped with a Varian E-257 variable temperature accessory. Spectral g values were calibrated with (diphenylpicryl)hydrazyl (DPPH) as a standard.

Experiments requiring rigorous exclusion of carbon dioxide or carbonate ions were manipulated under the flow of argon using standard Schlenk-line techniques (Shriver, 1969). UV spectrophotometric titrations were carried out in dry, argon-filled, 1-cm cuvettes sealed with rubber septa. EPR tubes were evacuated and back-filled with argon prior to use.

Preparation of Lactoferrin. Human lactoferrin was prepared from fresh colostrum as described previously (Norris et al., 1989). Two variations of the procedure were used. In the first, all buffers used in the isolation contained 5 mM EDTA, giving "EDTA-treated lactoferrin". The protein prepared in this way was, however, subsequently dialyzed against 5 changes of 10 mM sodium bicarbonate in 0.025 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl, over a period of 5 days. In the second procedure, no EDTA was used at any stage in the isolation, and all buffers were instead passed down a Bio-Rad Chelex-100 column to minimize contamination by adventitious metal ions. This "EDTA-free" lactoferrin was not dialyzed against bicarbonate. Both preparative procedures gave lactoferrin with a maximum iron content of 8–10% saturation, as estimated from comparison of the spectral ratios A_{280}/A_{466} and A_{412}/A_{466} (Aisen & Leibman, 1972) with those of pure, fully iron-saturated lactoferrin (ratios 20:1 to 22:1 and 0.70:1 to 0.74:1, respectively).

Metal and Anion Binding. The carbonate complexes of lactoferrin with Fe^{3+} or Cu^{2+} were prepared by a modification of previously reported methods (Ainscough et al., 1979). Stoichiometric amounts of ferric nitrilotriacetate or cupric chloride, respectively, were added to solutions of apolactoferrin (10–16 mg/mL) in 0.03 M Hepes buffer, pH 7.8, containing 0.01 M $NaHCO_3$ and 0.1 M NaCl. The protein solutions were then passed down a previously equilibrated Chelex column to remove any nonspecifically bound metal ions.

Oxalate complexes were prepared either by displacement of carbonate from $M_2(CO_3)_2Lf$ complexes or by addition of oxalate and metal ion to carbonate-free solutions of apolactoferrin. In the displacement method, solid sodium oxalate was added, in a 30-, 50-, or 100-fold molar excess, to solutions of the appropriate $M_2(CO_3)_2Lf$ complex (10–16 mg/mL) in 0.03 M Hepes buffer, pH 7.8 containing 0.1 M NaCl. In the second method, a 50-fold molar excess of solid sodium oxalate was added to the solution of apolactoferrin (10–16 mg/mL) in 0.03 M Hepes, pH 7.8, containing 0.1 M NaCl. The protein solution was transferred into a Schlenk tube and its pH was lowered to 4.1 by addition of 0.1 M HCl. The solution was then made CO_2-free by continual evacuation and flushing with argon over a period of 1–2 h. At the end of this time, the pH was restored to 8.0 by addition of CO_2-free ammonia. Metal binding was then carried out by the addition of aliquots of ferric nitrilotriacetate or cupric chloride, as before; these and all subsequent manipulations were carried out under an atmosphere of argon.

Crystallography. The structure of diferric lactoferrin, $Fe_2(CO_3)_2Lf$, has been previously reported at 2.8-Å resolution (Anderson et al., 1989) and preliminary crystallographic data have been given for both dicupric lactoferrin, $Cu_2(CO_3)_2Lf$, and the mixed-anion complex of dicupric lactoferrin, $Cu_2(CO_3)(C_2O_4)Lf$ (Smith et al., 1991).

Full details of the refinements of these structures will be published elsewhere. All were refined by restrained least-squares methods, using the program PROLSQ (Hendrickson & Konnert, 1980), with synchrotron data to 2.2, 2.1, and 2.2 Å, respectively, for $Fe_2(CO_3)_2Lf$, $Cu_2(CO_3)_2Lf$, and $Cu_2(CO_3)(C_2O_4)Lf$. In each case, data with spacings greater than 8 Å were omitted from the refinement, as were all reflections for which the intensity $I < 0.5\sigma_I$. At the start of each refinement, the metal ions, anions, and the amino acid side chains involved in binding were all omitted from the model, in order to prevent bias; they were then included, by fitting to the electron density, at the stage of the first manual rebuilding

Table I: Charge Transfer Absorption Maxima and Extinction Coefficients of Lactoferrin and Transferrin Complexes

lactoferrin/transferrin complex	λ_{max} (nm)	ϵ (mol^{-1} L cm^{-1})
$Cu_2(CO_3)_2Lf$	434	4800
$Cu_2(C_2O_4)(CO_3)Lf$	424	3400
$Cu_2(C_2O_4)_2Lf$	420	3400
$Cu_2(CO_3)_2Tf$	440	4600
$Cu_2(C_2O_4)(CO_3)Tf$	426	3200
$Cu(C_2O_4)Tf$	410	1500
$Fe_2(CO_3)_2Lf$	466	4600
$Fe_2(C_2O_4)(CO_3)Lf$	476	3400
$Fe_2(C_2O_4)_2Lf$	482	3300
$Fe_2(CO_3)_2Tf$	466	4400
$Fe_2(C_2O_4)_2Tf$	472	3300

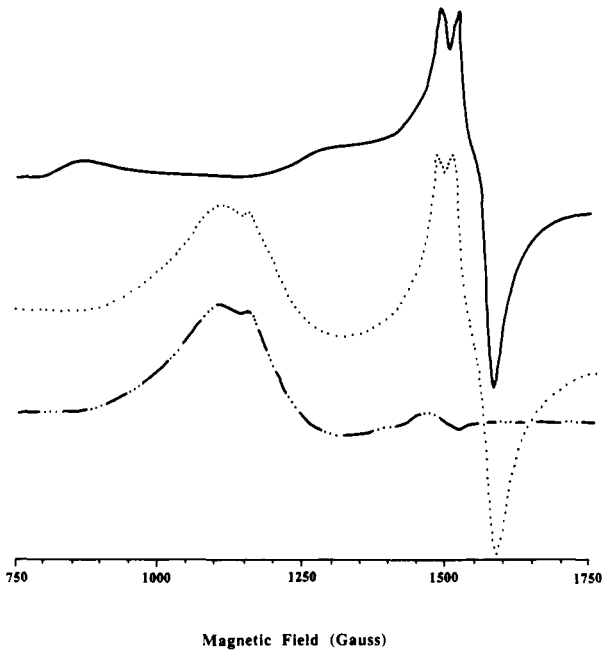

FIGURE 1: EPR spectra of carbonate and oxalate complexes of diferric human lactoferrin, recorded at 110 K and a microwave frequency of 8.990 GHz. Solutions were in 0.03 M Hepes buffer, pH 7.8, containing 0.1 M NaCl. Spectra are (—) $Fe_2(CO_3)_2Lf$; (···) $Fe_2(CO_3)(C_2O_4)Lf$; (-···-) $Fe_2(C_2O_4)_2Lf$.

of the structure (usually after 10–20 cycles of least squares refinement).

During the refinements, loose restraints (σ = 0.05 Å) were placed on the metal–ligand distances to prevent fluctuations arising from the less-than-optimal resolution of the data. The initial target distances were those derived from the first fitting of the metal ion and anion to the electron density, but they were then periodically updated as the refinements proceeded. Protein bond distances and angles were all restrained to standard values, but with the geometry relaxed and then retightened at intervals to allow movement from false minima. The structures were regularly examined on an Evans and Sutherland PS300 interactive graphics system, using the program FRODO (Jones, 1978), and solvent molecules (asumed to be water) were added to the model where they appeared consistently in difference maps and made structural sense in terms of their interactions with surrounding structure.

RESULTS

Carbonate Complexes. The carbonate complexes of diferric and dicupric lactoferrin, $Fe_2(CO_3)_2Lf$ and $Cu_2(CO_3)_2Lf$, gave characteristic visible absorption maxima at 466 and 434 nm, respectively (Table I). For these and other anion complexes, however, their EPR spectra provided the more sensitive identification. The EPR spectrum of $Fe_2(CO_3)_2Lf$ (Figure 1) has the unique $g' = 4.3$ resonance characteristic of diferric transferrins, indicating an approximately rhombic zero-field tensor ($E/D \approx 1/3$). That of $Cu_2(CO_3)_2Lf$ (Figure 2) is a typically axial Cu(II) spectrum ($g_\parallel = 2.314$, $g_\perp = 2.060$, $A_\parallel = 150$ G) as reported previously (Ainscough et al., 1980).

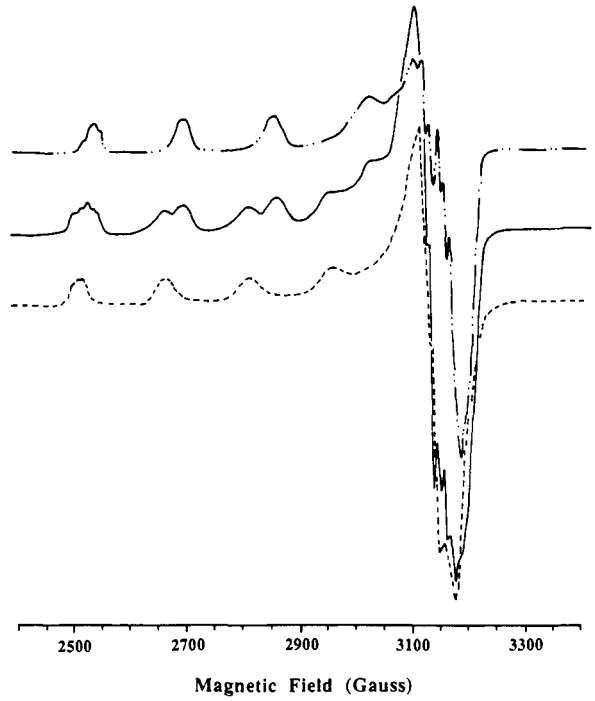

FIGURE 2: EPR spectra of carbonate and oxalate complexes of dicupric human lactoferrin. Conditions are as for Figure 1. Spectra are (-···-) $Cu_2(CO_3)_2Lf$; (—) $Cu_2(CO_3)(C_2O_4)Lf$; (···) $Cu_2(C_2O_4)_2Lf$.

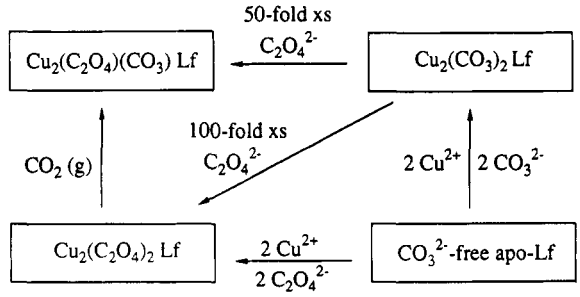

FIGURE 3: Reaction scheme for preparation and interconversion of carbonate, oxalate, and hybrid carbonate–oxalate complexes of dicupric lactoferrin.

Oxalate Complexes of Cu^{2+}. For dicupric lactoferrin, oxalate complexes could be prepared either by reaction with apolactoferrin in carbonate-free conditions or by displacement of carbonate from $Cu_2(CO_3)_2Lf$ (Figure 3).

Titration of apolactoferrin with Cu^{2+}, in carbonate-free conditions, in the presence of a 50-fold molar excess of oxalate, at pH 7.8, showed clearly that 2 mol of Cu^{2+} are bound per mole of protein (Figure 4). The resulting yellow complex had an intense visible absorption maximum at 420 nm and a weaker d–d band at 725 nm (ϵ = 350 mol^{-1} L cm^{-1}). The EPR spectrum (Figure 2) with $g_\parallel = 2.345$, $g_\perp = 2.070$, and $A_\parallel$ = 142 G is similar to but not identical to that of $Cu_2(CO_3)_2Lf$. Titration of apolactoferrin with oxalate, in carbonate-free conditions, in the presence of 2 equiv of Cu^{2+}, at pH 7.8, showed that 2 mol of $C_2O_4^{2-}$ are bound per mole of protein (Figure 4), giving the same complex and establishing the formulation as $Cu_2(C_2O_4)_2Lf$.

Addition of a 30- or 50-fold molar excess of oxalate to the carbonate complex, $Cu_2(CO_3)_2Lf$, caused the displacement of carbonate from one of the two specific sites, giving the hybrid

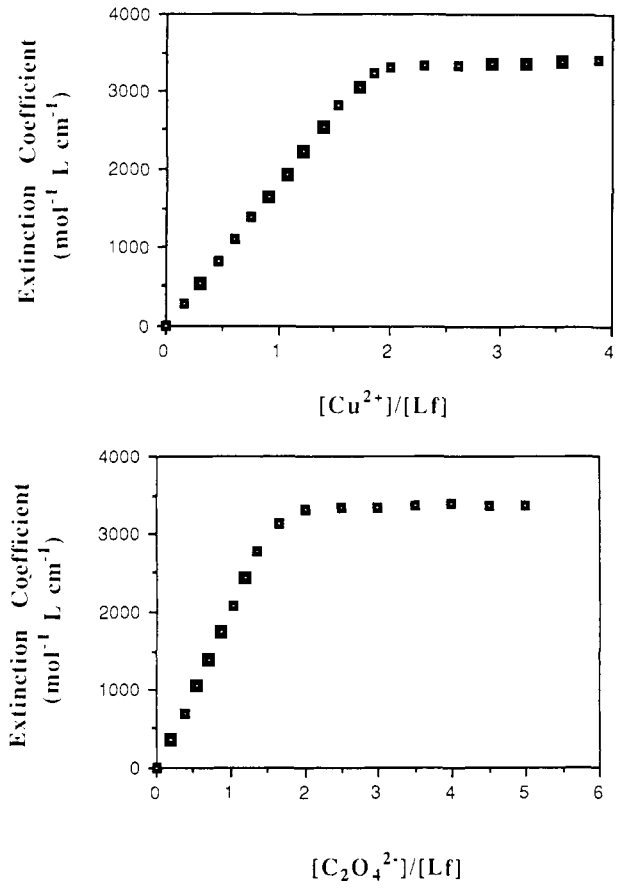

FIGURE 4: Metal- and anion-binding titrations with carbonate-free human apolactoferrin. Protein solutions were made up in 0.03 M Hepes buffer, pH 7.8, containing 0.1 M NaCl. (a, top) Addition of Cu^{2+} to lactoferrin in the presence of excess oxalate. (b, bottom) Addition of $C_2O_4^{2-}$ to lactoferrin in the presence of 2 molar equivalents of Cu^{2+}. Essentially identical results are obtained with Fe^{3+} as the added metal ion.

species $Cu_2(CO_3)(C_2O_4)Lf$. This was the complex which was analyzed crystallographically. It showed a shift in the visible absorption maximum to 424 nm, together with a shift in the d–d band from 670 nm (ϵ = 630 mol^{-1} L cm^{-1}) to 700 nm (ϵ = 560 mol^{-1} L cm^{-1}). The EPR spectrum is easily recognizable as an overlap of the spectra of $Cu_2(CO_3)_2Lf$ and $Cu_2(C_2O_4)_2Lf$ (Figure 2), in which the superhyperfine structure on the low field line comprises two independent triplets superimposed to form a five-line resonance.

At a 100-fold molar excess of oxalate, both carbonate ions were displaced from $Cu_2(CO_3)_2Lf$ to give the dioxalate complex $Cu_2(C_2O_4)_2Lf$. This contrasts with our earlier experiments (Ainscough et al., 1983) which showed that oxalate concentrations >100-fold molar excess caused dissociation of Cu^{2+} from $Cu_2(CO_3)_2Lf$ as the bis(oxalato) complex $Cu(C_2O_4)_2^{2-}$; the difference is probably attributable to the use of phosphate buffer (rather than Hepes as in the present study) since phosphate ions appear to promote metal-ion release from lactoferrin.

The mixed-anion complex could also be prepared by displacement of oxalate from $Cu_2(C_2O_4)_2Lf$. Thus a solution of $Cu_2(C_2O_4)_2Lf$, if exposed to atmospheric CO_2 for approximately 4 weeks, shows changes in both the electronic and EPR spectra characteristic of $Cu_2(CO_3)(C_2O_4)Lf$, e.g., λ_{max} shifted from 434 to 424 nm. The hybrid complex is stable under ambient conditions for at least 3 months. With higher carbonate concentrations, displacement was more rapid; in 0.03 M Hepes containing 0.01 M $NaHCO_3$ and 0.1 M NaCl, pH 7.8, $Cu_2(C_2O_4)_2Lf$ was converted to $Cu_2(CO_3)(C_2O_4)Lf$ over a period of 2 days, while with 0.1 M $NaHCO_3$ both oxalate ions were displaced, to give $Cu_2(CO_3)_2Lf$.

Oxalate Complexes of Fe^{3+}. For diferric lactoferrin we were unable to prepare oxalate complexes by displacement of carbonate from $Fe_2(CO_3)_2Lf$, even with addition of up to 200-fold molar excess of oxalate. The dioxalate complex $Fe_2(C_2O_4)_2Lf$ could, however, be prepared from apolactoferrin by addition of 2 mol of Fe(III) to a carbonate-free solution of the protein containing a 50-fold molar excess of oxalate at pH 7.8. This complex is purple-red (λ_{max} 482 nm) compared with the orange-red carbonate complex (λ_{max} 466 nm). Spectrophotometric titrations, similar to those in Figure 4, carried out in the absence of CO_3^{2-}, clearly indicated a required stoichiometry of 2 mol of Fe(III) and 2 mol of $C_2O_4^{2-}$ per mole of protein. Because NTA is a synergistic anion, these experiments required that Fe(III) be added as a citrate complex rather than FeNTA. The EPR spectrum of $Fe_2(C_2O_4)_2Lf$ is markedly different from that of $Fe_2(CO_3)_2Lf$ (Figure 1) but similar to that reported for the transferrin complex, $Fe_2(C_2O_4)_2Tf$ (Aisen et al., 1967).

A mixed-anion complex, $Fe_2(CO_3)(C_2O_4)Lf$, could also be formed by exposure of a carbonate-free solution of $Fe_2(C_2O_4)_2Lf$ to atmospheric CO_2; over a period of weeks, the absorption maximum shifted from 482 to 476 nm, after which no further change occurred. The EPR spectrum of this complex comprised two resonances, centered around g' = 4.3 and g' = 6.1, characteristic of carbonate and oxalate binding, respectively.

Transferrin Complexes. The formation of a dioxalate complex of copper lactoferrin contrasted with previous studies showing that serum transferrin bound only one oxalate with Cu^{2+} as the associated metal ion (Zweier & Aisen, 1977). We therefore carried out a spectrophotometric titration of apotransferrin with oxalate, under argon, in the presence of 2 mol of Cu^{2+} per mole of transferrin. This confirmed that only one oxalate could be bound, with the formation of $Cu(C_2O_4)Tf$. On the other hand, a dioxalate complex, $Fe_2(C_2O_4)_2Tf$, resulted from titration of apotransferrin wth oxalate in the presence of Fe(III). This had a slightly higher energy optical absorption maximum than its lactoferrin analogue (Table I). Its EPR spectrum matched that given by Aisen et al. (1967).

Anion Competition. Addition of either Cu(II) or Fe(III) to solutions of apolactoferrin, containing 0.1 M NaCl and equal concentrations (0.01 M) of sodium bicarbonate and sodium oxalate, pH 7.8, gave very different results. For Fe(III) the carbonate complex $Fe_2(CO_3)_2Lf$ was formed when 2 mol of Fe(III) were added, while for Cu(II) the hybrid complex, $Cu_2(CO_3)(C_2O_4)Lf$ resulted.

Effect of EDTA Treatment. The anion-binding behavior of lactoferrin was not entirely reproducible, depending on whether EDTA had been used in its preparation or not. On several occasions, the EDTA-treated apolactoferrin, despite extensive dialysis against sodium bicarbonate, followed by CO_2-removal, could be induced to bind oxalate only in one site. This was true irrespective of whether the metal ion was Fe^{3+} or Cu^{2+}. On the other hand, carbonate could always be bound in both sites.

Crystallographic Analyses. The results of crystallographic refinement of the various metal- and anion-substituted lactoferrins are summarized in Table II. The three structures, $Fe_2(CO_3)_2Lf$, $Cu_2(CO_3)_2Lf$, and $Cu_2(CO_3)(C_2O_4)Lf$, all at high resolution, give crystallographic R factors less than 0.20 and have an estimated coordinate error of 0.2–0.3 Å. Full reports of these structures will be given elsewhere. Here we concentrate on the details of the anion sites and their impli-

Table II: Crystallographic Refinement of Lactoferrin Complexes

structure	protein atoms	ions	water molecules	res[a] (Å)	refs[b]	R^c	rms Δ^d (Å)
$Fe_2(CO_3)_2Lf$	5322	2 Fe^{3+} 2 CO_3^{2-}	431	2.2	38047	0.176	0.020
$Cu_2(CO_3)_2Lf$	5321	2 Cu^{2+} 2 CO_3^{2-}	308	2.1	43525	0.196	0.018
$Cu_2(CO_3)(C_2O_4)Lf$	5323	2 Cu^{2+} 1 CO_3^{2-} 1 $C_2O_4^{2-}$	228	2.2	43043	0.198	0.020

[a] Maximum resolution. [b] Number of reflections used in refinement ($I > 0.5\sigma_I$). [c] Crystallographic R factor, $R = \sum||F_o| - |F_c||/\sum|F_o|$. [d] rms Δ = root-mean square deviation of bond lengths from standard values.

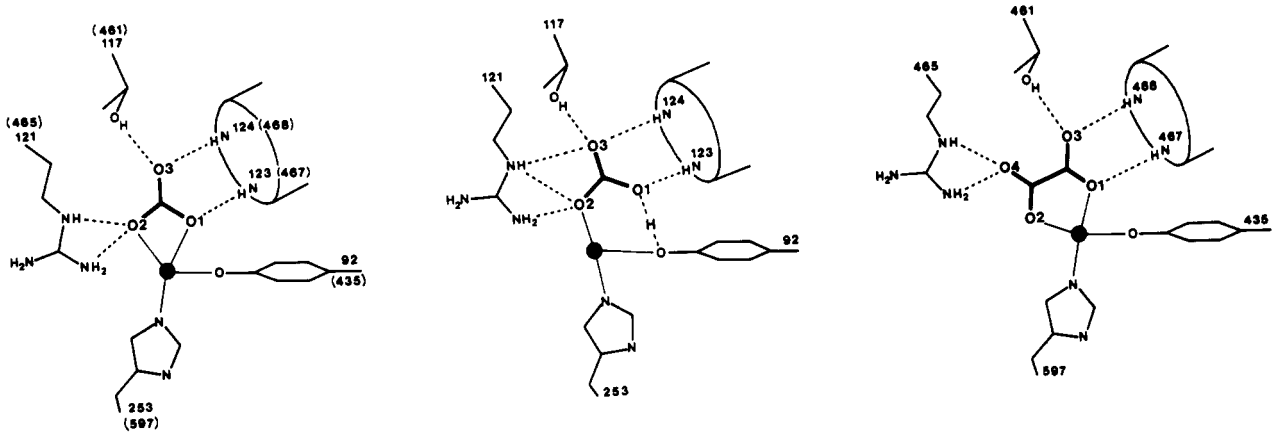

FIGURE 5: Schematic diagram showing the anion interactions with the protein and the metal ion in (a, left) the bidentate carbonate site in each lobe of $Fe_2(CO_3)_2Lf$ and the C-lobe of $Cu_2(CO_3)_2Lf$; (b, middle) the monodentate (bi)carbonate site in the N-lobes of $Cu_2(CO_3)_2Lf$ and $Cu_2(CO_3)(C_2O_4)Lf$; and (c, right) the oxalate site in the C-lobe of $Cu_2(CO_3)(C_2O_4)Lf$.

cations for anion binding by transferrins generally. We note first, however, that the overall protein structure is not changed significantly by metal or anion substitution, except as noted below, and in each case the metal ion remains bound to the same four protein ligands, Asp 60 (395 in the C-lobe), Tyr 92 (435), Tyr 192 (528), and His 253 (597).

Carbonate Binding in $Fe_2(CO_3)_2Lf$. High-resolution refinement has not significantly changed the description of the anion sites given for diferric lactoferrin at 2.8-Å resolution (Anderson et al., 1989). The carbonate ion bridges between the metal ion, to which it binds in bidentate mode, and two positively charged groups on domain 2 of each lobe of the protein, i.e., the N-terminus of helix 5 and the side chain of an arginine residue (Arg 121 in the N-lobe, Arg 465 in the C-lobe). The network of interactions which binds each carbonate ion appears close to optimal (see Figure 5a). One oxygen, O(1), binds to iron and also accepts a hydrogen bond from the peptide NH of residue 123 (467) at the N-terminus of helix 5; the second, O(2), binds to iron and accepts a hydrogen bond from Arg 121 (465); while the third, O(3), forms two hydrogen bonds, with the peptide NH of residue 124 (468) and the hydroxyl group of Thr 117 (461). The arrangement is the same in both anion sites, although the interactions are somewhat tighter in the C-lobe than in the N-lobe; in the latter the hydrogen bond O(3)...N(124) is 3.3 Å, compared with 2.9 Å in the C-lobe.

Carbonate Binding in $Cu_2(CO_3)_2Lf$. In dicupric lactoferrin, $Cu_2(CO_3)_2Lf$, the carbonate binding is generally similar to that in $Fe_2(CO_3)_2Lf$. There are differences in detail between the N- and C-terminal sites, however, and in the metal–carbonate interactions. In the C-terminal site the carbonate ion makes the same hydrogen bonds with the protein as in the C-lobe of the diferric protein. Again the metal coordination is bidentate, although in this case it is asymmetric [Cu–O(1) is slightly longer than Cu–O(2)].

In the N-terminal site, however, the carbonate ion is rotated 20°, compared with the diferric structure, so that its hydrogen-bonding pattern is slightly charged (Figure 5b). At the same time, the copper atom is displaced 1.0 Å from the iron position; the result is that the carbonate ion binds in monodentate fashion to the copper in this site, through O(2), and the copper coordination becomes square pyramidal. In fact, closer analysis of interactions in this site (Smith et al., 1992) suggests that the form of the anion may be HCO_3^- rather than CO_3^{2-}, with a hydrogen bond linking the anion to Tyr 92 (Figure 5b).

Carbonate and Oxalate Binding in $Cu_2(CO_3)(C_2O_4)Lf$. In the hybrid complex, $Cu_2(CO_3)(C_2O_4)Lf$, the oxalate ion occupies the C-terminal site, where it is bound to copper in a symmetrical, 1,2-bidentate mode (Figure 5c). Its interactions with the protein are very similar to those by carbonate, involving the helix 5 N-terminus, Arg 465, and Thr 461. In the present model O(1) is bound to copper and receives a hydrogen bond from 467 NH, O(3) is hydrogen bonded to both 468 NH and Thr 461 $O_{\gamma 1}$, O(4) is hydrogen bonded to Arg 465 N_ϵ and $N_{\eta 2}$, and O(2) is bound only to the copper atom. The larger size of the oxalate ion, compared with carbonate, does however cause local rearrangement beyond the anion site; the side chain of Arg 465 is displaced ~2 Å away from the anion and the neighboring Tyr 398 side chain ~1.5 Å. These appear to be the only changes resulting from the introduction of the larger anion, however. In the N-terminal site of this hybrid complex, the carbonate ion is bound exactly as in the N-terminal site of $Cu_2(CO_3)_2Lf$.

Discussion

The present results emphasize the synergistic relationship between metal- and anion-binding in transferrins, through the differences in anion binding seen for Fe^{3+}-and Cu^{2+}-substituted lactoferrin. Further, the crystallographic analyses give defi-

nitive descriptions of the binding modes of carbonate and oxalate and of the common site they occupy in the various complexes.

The details of carbonate binding to diferric transferrins are now well established. The model presented here for diferric lactoferrin is essentially identical to that obtained in the recent structure analysis of the N-terminal half-molecule of rabbit transferrin (Sarra et al., 1990) and is consistent with the pattern of density at the iron sites seen in the lower resolution diferric transferrin structure (Bailey et al., 1988). The exquisite fit of the anion between protein and metal ion explains the high specificity for Fe^{3+} and carbonate in transferrins. The full hydrogen-bonding potential of the carbonate ion is used as it is locked in place by the interactions with groups at or near the N-terminus of helix 5; these hydrogen bonds have close to optimal geometry, with distances of 2.7–3.0 Å and N–H...O angles near 180°. The bidentate coordination to iron also completes a six-coordinate, distorted octahedral geometry which is common for Fe(III) complexes.

The bidentate coordination and the hydrogen-bonding pattern both suggest that the form of the anion is carbonate rather than bicarbonate. This conclusion is also in accord with NMR studies (Zweier et al., 1981; Bertini et al., 1986a). The protein groups which bind the carbonate ion include an arginine side chain, which should be positively charged below pH ~12, and the helix N-terminus, which carries a partial positive charge, estimated at ~0.5+ (Hol et al., 1978). Thus the role of the anion, which is believed to bind first (Zweier et al., 1981; Cowart et al., 1982), is to neutralize this positive charge (which might otherwise repel an approaching metal ion) and also to partially prepare the binding site.

Differences between the N- and C-lobe sites appear minimal when Fe^{3+} and CO_3^{2-} are bound, but become accentuated when Cu^{2+} and/or oxalate are substituted. This is seen in the structure of $Cu_2(CO_3)_2Lf$, where small movements of the metal ion and anion lead to monodentate anion binding and a square pyramidal metal stereochemistry in the N-lobe, but bidentate coordination and distorted octahedral geometry in the C-lobe (Smith et al., 1992). The main difference between the two sites seems to be that the hydrogen-bonding interactions in the N-lobe are somewhat looser, allowing more room for alternative configurations.

The monodentate anion coordination in the N-lobe of dicupric lactoferrin, together with the apparent hydrogen-bonding network (Figure 5b), suggests that the form of the anion here may be bicarbonate rather than carbonate. A change of this kind could explain the observation that in transferrin three protons are released for each Fe^{3+} ion bound (probably from deprotonation of the bicarbonate ion and two tyrosine ligands) but that only two protons are released for each Cu^{2+} ion bound (Gelb & Harris, 1980). It could also model the kind of changes that might occur if protonation of the CO_3^{2-} anion is a first step in iron release, as postulated by various authors (Aisen et al., 1973; Williams & Woodworth, 1973; Sarra et al., 1990). Protonation would disrupt the anion hydrogen bonding in the diferric protein and movement of the anion and a change to monodentate coordination could lead toward breakup of the iron site.

The oxalate binding studies show that dioxalate complexes form very easily in the absence of competing anions. Thus dioxalate complexes of both Fe^{3+} and Cu^{2+} can be made using stoichiometric quantities of oxalate, providing the apolactoferrin is carbonate-free and no trace of EDTA is present. In air, however, or when complexes are prepared by displacement, one oxalate binds quite readily when Cu^{2+} is the metal ion, but it requires much higher concentrations of oxalate before it can compete with carbonate to bind at the second site. With Fe^{3+} as metal ion, oxalate cannot compete with carbonate for either anion site at concentrations below 100-fold molar excess of oxalate. The competition experiments, using equal concentrations of carbonate and oxalate, show most strikingly the synergistic relationship between metal and anion binding and the inequivalence of the two sites. Under these conditions, Fe^{3+} binds carbonate preferentially over oxalate in both sites whereas Cu^{2+} preferentially binds oxalate over carbonate in one site (presumably the C-site) and carbonate over oxalate in the other.

The structure of the hybrid complex, $Cu_2(CO_3)(C_2O_4)Lf$, shows that it is the C-terminal site in which oxalate binds preferentially when Cu^{2+} is the metal ion. This is contrary to our original predictions (Baker et al., 1990), and the reasons for the preference are not immediately obvious. There is certainly as much room for oxalate in the N-lobe site as in the C-lobe, and the apolactoferrin structure (Anderson et al., 1990) and other evidence suggest that the N-lobe is more flexible and might thus be more amenable to anion substitution. The most likely explanation is that it is the precise detail of the metal–anion–protein complex at each site which matters. The combination of Fe^{3+} and CO_3^{2-} is highly favorable at both sites, sterically and geometrically, and the anion is not easily substituted by oxalate. When Cu^{2+} is the metal ion, the N-lobe site provides a more favorable copper geometry (square pyramidal) than the C-lobe, and perhaps it is for this reason that the C-lobe is more easily substituted by oxalate.

The oxalate-binding preferences for lactoferrin mirror those for copper-substituted ovotransferrin, to which oxalate binds preferentially in the C-terminal site, and for which dioxalate complexes are obtained using carbonate-free apo-ovotransferrin but monooxalate complexes when prepared in air (Zweier, 1980). In contrast, serum transferrin binds only one oxalate with Cu^{2+}, in the N-terminal site (Zweier & Aisen, 1977).

An intriguing feature of the spectra of the lactoferrin complexes is that substitution of oxalate for carbonate shifts the absorption maximum of the charge-transfer band, assigned to a phenolate (π) → metal d charge transfer transition (Ainscough et al., 1980), to higher energy (434 to 420 nm) when Cu^{2+} is the metal ion but to lower energy (466 to 482 nm) when Fe^{3+} is the metal ion. In view of the variation in geometry around the metal for the carbonate and oxalate complexes, spectral comparisons should be made with caution. However, the observations are consistent with the different nature of the metal d-type acceptor orbitals involved in the charge transfer transition and the greater ligand field strength of the oxalate ligand relative to carbonate (Moeller, 1982; Gray, 1965). Hence for the d^9 copper(II) complexes, where the transition is assigned as a phenolate (π) → Cu(d_σ*) type, the shift to higher energies on going from $Cu_2(CO_3)_2Lf$ to $Cu_2(C_2O_4)_2Lf$ reflects a destabilization of the d_σ* orbitals resulting from stronger $C_2O_4^{2-}$ to Cu^{2+} σ interactions. In contrast, for the d^5 iron (III) complexes, where the charge transfer transition is assigned as a phenolate (π) → Fe(d_π*) type, the results indicate that it is the difference in the π donor properties of the two anions that influences the relative energies of the visible absorption bands. In this case the shift of the charge transfer band to lower energies on oxalate substitution points to the d_π* orbitals for the $Fe_2(CO_3)_2Lf$ complex being relatively destabilized and implies that it is CO_3^{2-} to Fe^{3+} π interactions that are the stronger.

Substitution of carbonate by oxalate in lactoferrin (and transferrin) complexes of both iron and copper results in a

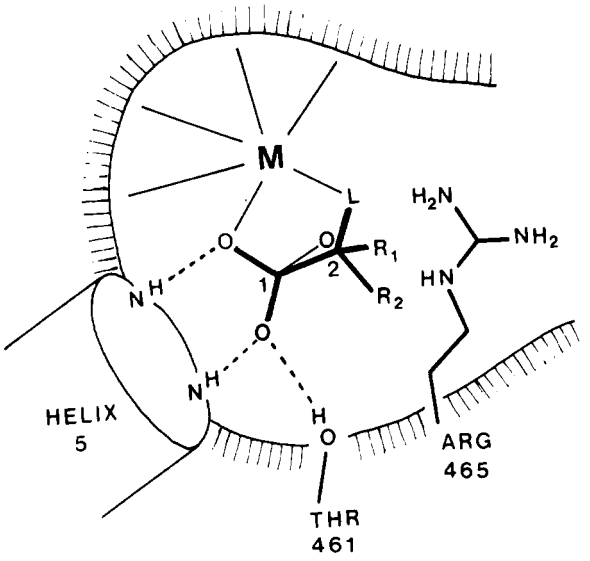

FIGURE 6: Generalized model for anion binding to transferrins, based on crystal structure analyses of carbonate- and oxalate-substituted lactoferrin. Binding of a general anion (thick lines) is compared with carbonate (thin lines). Interaction with the protein is primarily through the carboxylate group (carbon 1); interactions with the arginine side chain depend on the substituents on carbon 2.

decrease, by over 1000 mol^{-1} L cm^{-1}, in the molar absorption coefficients (Table I). The intensity of a ligand to metal charge transfer transition is proportional to the square of the overlap of the orbitals involved (Douglas & Hollingsworth, 1985), in this case phenolate (π) and metal (d) types. Thus the decrease in the overlap indicates that the replacement of the carbonate ion by the more sterically demanding oxalate has somehow weakened the tyrosine–metal interactions. Such slight changes would not be detected by X-ray crystallography, but the results are consistent with the point made earlier for the hybrid complex, $Cu_2(CO_3)(C_2O_4)$Lf, that the oxalate ion does cause changes beyond the anion-binding site.

The 1,2-bidentate coordination of the oxalate ion was predicted from electron spin echo spectroscopy (Eaton et al., 1989) and from model building (Baker et al., 1990) but disagrees with suggestions from other spectroscopic studies (Bertini et al., 1986a; Sola, 1990). Comparison of the oxalate and carbonate binding in these complexes leads to a reappraisal of the classic Schlabach and Bates (1975) "interlocking sites" model for anion binding. The anion does indeed bridge between the metal ion and positively charged groups on the protein, as in that model. However, the common feature of all the synergistic anions identified by Schlabach and Bates is that all have both a carboxyl group and, on a proximal carbon atom, a potential electron donor ligand L. By analogy with the carbonate and oxalate sites described here, we suggest that the primary feature of anion binding to transferrins is the hydrogen bonding of the carboxyl group of the anion to the N-terminus of helix 5. The anion then coordinates the metal through L and one carboxylate oxygen (Figure 6).

Anions larger than CO_3^{2-} force the arginine side chain to move away (this is possible because of the solvent-filled interdomain cavity beyond). Whether the arginine hydrogen bonds to an anion, as for carbonate and oxalate, will depend on the nature of the substituents on the proximal carbon. This is probably a major factor in the strength of anion binding, and the favorable interactions of oxalate with the arginine side chain probably account for the fact that it is the only anion whose binding affinity approaches that of carbonate. Differences in the interaction of this arginine may also explain the differences in binding modes inferred for some anions from spectroscopic studies. In one such study, of anion binding to vanadyl(IV)–transferrin (Campbell & Chasteen, 1977), two classes of anion conformation were identified; interestingly, one class comprised all those which could not hydrogen bond to the arginine, as in Figure 6, and the other class those which could. Moreover, transition between the two classes of conformation was dependent on the ionization of a protein group of pK ~10.0 (Chasteen et al., 1977), presumably Arg 121 (465).

Finally, we note that it is necessary to be extremely careful when performing and interpreting anion-binding experiments such as those reported here, if other anions, whether synergistic or not, have been used in the preparation of the protein. EDTA is a poor synergistic anion for transferrins (Schlabach & Bates, 1975) but apparently associates strongly with the apoprotein, being difficult to remove by dialysis. Our observations that on occasions only one oxalate could be bound to "EDTA-treated lactoferrin", suggest that EDTA binds preferentially to the N-lobe. This conclusion comes from (i) the spectral similarity between the hybrid $Cu_2(CO_3)(C_2O_4)$Lf complex prepared from the EDTA-treated protein and that characterized crystallographically, and (ii) structural differences between the two lobes of apolactoferrin (Anderson et al., 1990); the N-lobe cleft opens more easily and has one extra cationic group, Lys 301, which may help to bind EDTA and whose equivalent in the C-lobe, Asn 644, is uncharged.

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Registry No. Carbonate, 3812-32-6; oxalate, 144-62-7.

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Construction and Characterization of a Mutant of *Rhodobacter sphaeroides* with the Reaction Center as the Sole Pigment-Protein Complex†

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ABSTRACT: A strain of *Rhodobacter sphaeroides* has been constructed in which the photosynthetic reaction center is the sole pigment-protein complex. The strain, named RCO1, is capable of photoheterotrophic growth and possesses assembled and functional reaction centers which can undergo photochemical charge separation and are reduced by electrons derived from the cytochrome bc_1 complex. The circular dichroism and linear dichroism of reaction centers in membranes from strain RCO1 are similar to those described previously for reaction centers isolated in detergent solution. A second strain, named RCLH11, which is devoid of the peripheral LH2 antenna complex has also been constructed. A description of the properties of these strains is presented.

Rhodobacter sphaeroides (*Rb. sphaeroides*) is a facultatively photosynthetic purple (non-sulfur) bacterium which has a relatively simple membrane-bound photosystem. The photosynthetic reaction center is closely associated with an LH1 antenna complex, which in turn is surrounded by a second variable antenna complex, LH2 (Aagaard & Sistrom, 1972). All three pigment-protein complexes are located within extensive invaginations of the cytoplasmic membrane. The antenna complexes, each of which consists of a network of interconnected pairs of polypeptides (α and β) which bind bacteriochlorophyll and carotenoid, are responsible for the capture of light and funnelling of excitation energy to a "special pair" of bacteriochlorophyll molecules located within the reaction center, initiating photochemistry. The resulting charge separation oxidizes the special pair and reduces first a bacteriopheophytin and then the primary quinone (Q_A).

The reaction center of the closely related bacterium *Rhodopseudomonas viridis* was the first integral membrane protein

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