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High-Resolution Proton Magnetic Resonance Study of Porcine Colipase and Its Interactions with Taurodeoxycholate†

Tadeusz Wieloch,* Bengt Borgström, Karl-Erik Falk, and Sture Forsén

ABSTRACT: A high-resolution 270-MHz proton NMR study of procine colipase I has been performed, and the resonances in the aromatic region of the spectrum have been assigned to amino acid residues by pH titration and decoupling experiments. The apparent pK_a values of the three tyrosines were calculated to be 10.2, 10.3, and 11.8 with one of the tyrosines having properties of a "buried" residue. A tentative assignment to the amino acid residues in the primary sequence of colipase will be discussed. The effects of taurodeoxycholate (TDC) and a positively charged deoxycholate derivative on the aromatic region of the colipase NMR spectrum indicate that all tyrosines and one histidine are affected by the bile-salt binding, suggesting that the TDC molecules bind near these residues to a hydrophobic region on colipase. Measurements and calculations on the line width of the C(18) methyl group resonance suggest that the line-width increase of this resonance upon interaction of TDC with colipase to a large extent can be explained as due to the slower tumbling of the TDC molecules bound to colipase.

The hydrolysis of the dietary triglycerides in the intestinal lumen is mainly carried out by the enzyme pancreatic lipase with its cofactor colipase. The cofactor function of colipase is to bring the lipase molecule to the substrate surface in the presence of bile salts (Borgström & Erlanson, 1973). The colipase molecule has been purified (Mayliē et al., 1971; Erlanson & Borgström, 1972) and its primary and secondary structure characterized (Charles et al., 1974; Erlanson et al., 1974). The colipase molecule studied in the present investigation is composed of 100 amino acid residues (Figure 1). Among these, three are tyrosines, two are phenylalanines, and two are histidines. NMR[1] has proved to be a useful tool in studies of changes in the microenvironment of macromolecules upon ligand binding and in studies of the dynamics of molecular interactions (e.g., Dwek, 1973; Wütrich, 1977). In order to gain information on structural features of the colipase molecule and to interpret the interaction between colipase and TDC on the molecular level, a high-resolution proton NMR study has been performed with special reference to effects observed on the tyrosines, phenylalanines, and histidines of

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[1] Abbreviations used: CD, circular dichroism; cmc, critical micellar concentration; NMR, nuclear magnetic resonance; TDC, taurodeoxycholate; UV, ultraviolet; DSS, 2,2-dimethylsilapentane-5-sulfonate.

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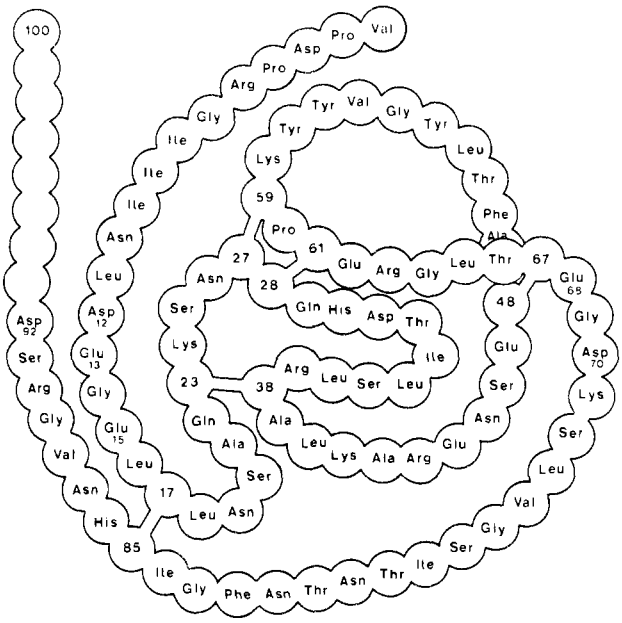

FIGURE 1: The primary sequence of porcine colipase (Charles et al., 1974; Erlanson et al., 1977).

colipase and the C(18) methyl and $-CH_2SO_3^-$ methylene group of TDC.

Experimental Section

Materials. Porcine pancreatic colipase I was purified as described (Erlanson et al., 1973) and found pure by disc electrophoresis. TDC and tritiated TDC were synthetized in the laboratory as described (Norman, 1955; Hofmann et al., 1968). The positively charged bile-salt molecule was synthesized in the laboratory. All solutions were made up in D_2O (Ciba Geigy) with a sodium chloride concentration of 150 mM. In the TDC binding studies 5 and 10 mM phosphate buffers were used. In the dialysis experiments Spectrapor 1 dialysis membranes (Spectrum Medical Industries Inc., Los Angeles) with a molecular weight cutoff of 6000–8000 were used.

Sample Preparation. Since some of the backbone-NH protons of colipase exchange slowly with the solvent D_2O, the colipase solutions were heated at 45 °C for about 15 min and cooled before spectral accumulation.

TDC titrations were performed by addition of small aliquots of a 100 mM TDC solution to the NMR tube. Colipase titrations were performed by dilution of a TDC solution of initially high colipase concentration with a similar solution containing no colipase.

NMR Measurements. Spectra (270 MHz) were run on a Bruker WH-270 NMR spectrometer equipped with a Nicolet 1085 24K computer with FT facilities. NMR tubes (5 mm) were used. The spectra were accumulated at a temperature of 30 °C. An internal deuterium lock was used. The chemical-shift values are quoted downfield from DSS. In the colipase and TDC titration experiments tetramethylammonium chloride was used as an internal reference.

pH Measurements. pH was measured with a combined glass electrode, and the samples were transferred from the NMR tube to a glass vessel before measurement. pH was varied by addition of small aliquots of 1 M KOD and 1 M DCl. All pH and pK_a values quoted are pH_{app} and pK_{app}.

Equilibrium Dialysis. The equilibrium dialysis experiment was performed in a three-chamber equilibrium dialysis cell system (Robinson & Tanford, 1975) with a Spectrapor 1 membrane between the chambers. The cells were rotated continuously during equilibration. Samples from the chambers were taken every second day to follow the equilibration. TDC was allowed to dialyze from the middle chamber to a chamber containing buffer and to another chamber containing buffer and 0.15 mM colipase. Samples (50 μL) were taken and counted in a Packard scintillation counter. The equilibration continued for 7–10 days. To avoid bacterial growth all solution contained 0.02% sodium azide.

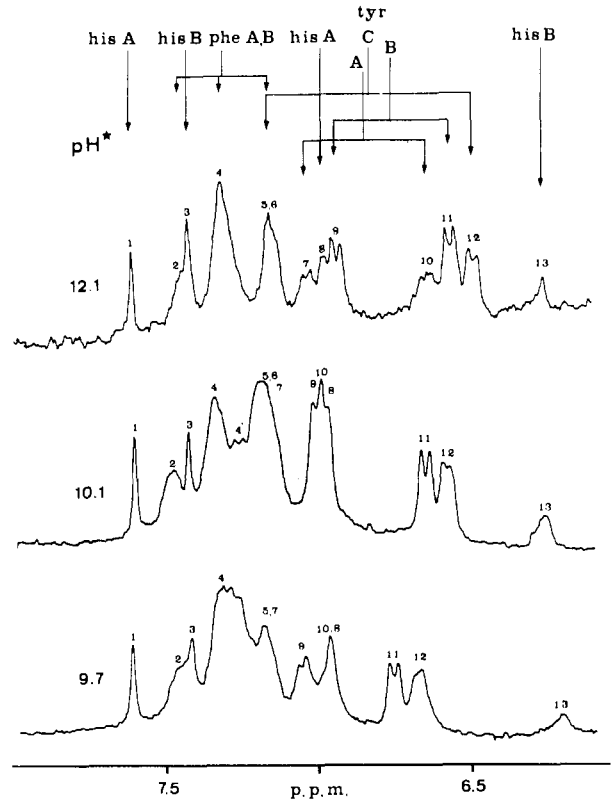

FIGURE 2: A 270-MHz proton NMR spectrum of colipase showing the aromatic region (δ_{obsd} 6–9) at different pH values. The protein concentration was $\simeq$2 mM in D_2O and 150 mM NaCl. The spectra are results of 500 scans. Temperature was 30 °C. The assignments are presented in Table I.

Results and Discussion

A presentation of the proton NMR spectrum of colipase and an investigation of the histidine residues in colipase have recently been reported (Cozzone, 1976; Wieloch & Falk, 1978). In the present investigation we shall assign the resonances in the aromatic region of the colipase NMR spectrum based on the pH dependence of their chemical-shift values and on decoupling experiments. Figure 2 shows the aromatic region of the colipase NMR spectrum at some different pH values. The chemical-shift values of the resonances in the spectra were plotted as a function of pH, and the data points were fitted by a least-squares procedure to the Hill equation

$$\delta_0 = \delta_A \frac{[H^+]^n}{[H^+]^n + K_a^n} + \delta_B \frac{K_a^n}{[H^+]^n + K_a^n}$$

with δ_A, δ_B, and K_a as variable parameters and $n = 1$ (Figure 3). By this procedure individual pK_a values of the three tyrosines could be obtained (Table I).

Histidines. The two histidine C(2) and C(4) protons (resonances 1, 8 and 3, 13, His-A and -B, respectively) in colipase have earlier been assigned to histidine residues with pK_a values of 6.9 and 7.8 (Wieloch & Falk, 1978). In the same investigation it was concluded that the histidine having a pK_a value of 6.9, His-B in Figure 2, is positioned near a tyrosine

Table I: Assignment of the Resonance in the Aromatic Region of the Proton NMR Spectrum of Colipase[a]

resonance	pK_a value	assignment to protons in amino acid residues	tentative assignment to residues in the structure of colipase
1	7.8	histidine C(2)	surface histidine in the proximity of negatively charged groups, possibly His-30
2		phenylalanine	
3	6.9	histidine C(2)	a histidine in the vicinity of a tyrosine; see Figures 3 and 6; His-86
4		phenylalanine	
5		phenylalanine	
6	10	tyrosine (meta)	surface tyrosine in the vicinity of a histidine (resonances 3 and 13); Tyr-56 or -57
7	11.8	tyrosine (meta)	due to the large line width of the resonance and the high pK_a value, this resonance is assigned to a buried tyrosine residue in the structure of colipase; Tyr-53
8	7.8	histidine C(4)	see resonance 1
9	10.4	tyrosine (meta)	surface tyrosine; Tyr-56 or -57
10	11.8	tyrosine (ortho)	see resonance 7
11	10.2	tyrosine (ortho)	see resonance 9
12	10.3	tyrosine (ortho)	see resonance 6
13	6.9	histidine C(4)	see resonance 3

[a] The numbers in the table refer to the labeled resonances in Figure 2. The apparent pK_a values were calculated by a least-squares fitting of the chemical-shift values.

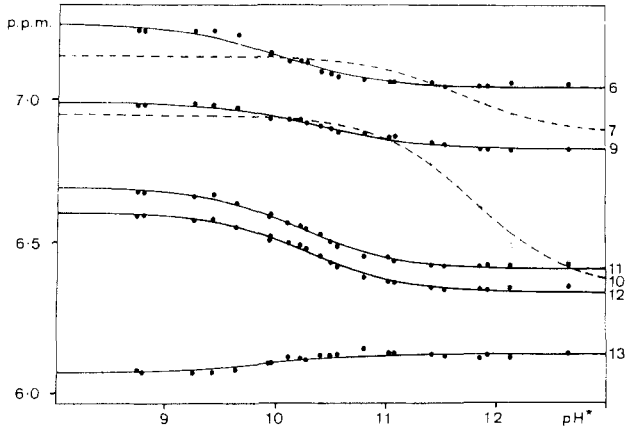

FIGURE 3: The chemical shift of the tyrosine ortho and meta proton resonances and a C(4) proton resonance of a histidine of colipase as a function of pH. The chemical-shift values of the tyrosine represent the midpoint of the resonances. The pK_a values were calculated by use of a least-squares fit to the data points as described in the text. The curves are labeled as indicated in Figure 2.

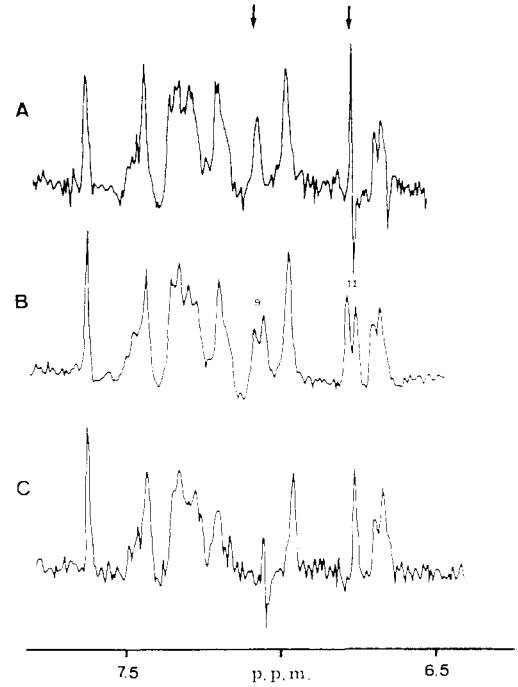

FIGURE 4: The decoupling of the two Tyr-B doublets. (A) Irradiation at 6.74 ppm causes decoupling at 7.05 ppm. (B) Undecoupled spectrum. (C) Decoupling at 7.05 ppm causes decoupling at 6.74 ppm. The resolution is enhanced by use of the convolution difference method (Campbell et al., 1973).

residue, Tyr-C (resonances 6 and 12). As is seen in Figure 3 the His-B C(4) proton, resonance 13, moves downfield in the pH range 9.5–10.5, which would be expected in view of the proximity of this residue to the Tyr-C residue. The assignment of the two histidines to residues in the primary sequence of colipase is in progress. A tentative assignment has earlier been made assigning His-A to His-30 and His-B to His-86 (Wieloch & Falk, 1978).

Tyrosines. In the present investigation we have determined the pK_a values of the tyrosine residues by a fit of the chemical-shift values of the ortho proton resonances. Thus the pK_a value of Tyr-C was found to be 10.3. We also observe a slight increase in the line widths of the Tyr-C resonances at pH values around the pK_a, indicative of a slower proton exchange than normally observed for free tyrosine molecules.

Resonances 9 and 11 are assigned to a second tyrosine residue, Tyr-B (Figure 2). The pK_a value was calculated to be 10.2 based on the chemical-shift changes of the ortho proton resonance. The area of each peak corresponds to two protons. Figure 4 shows the decoupling of Tyr-B resonances as a pair of mutually collapsing doublets. The splitting of the resonances is of the order of 8 Hz. The resonances are well resolved and the pK_a value is that normally found for a tyrosine residue. These findings indicate that resonances 9 and 11 arise from a freely rotating tyrosine residue probably on the surface of colipase. Resonances 7 and 10 are assigned to the third tyrosine, Tyr-A. The pK_a value was found to be 11.8, an unusually high pK_a value. The difference in chemical shift of the ortho protons of the protonated and deprotonated Tyr-A was found to be 0.57 ppm, which should be compared to 0.27 ppm for Tyr-B and -C. A conformational change of colipase accompanying the ionization of this tyrosine, possibly arising

from the disruption of a hydrogen bond to, for example, a lysine, might be the explanation of this effect. Another feature of this tyrosine is the large line width of the resonances, which indicates that the aromatic ring rotates slowly, on the NMR scale, around the C_B–C_γ bond (e.g., Wütrich & Wagner, 1975; Campbell et al., 1976). In view of other investigations of tyrosines in proteins we assign these resonances to a tyrosine residue "buried" in the structure of colipase. The data from the present investigation are not sufficient to unambigously assign the tyrosine resonances to specific amino acid residues in colipase. A tentative assignment can, however, be made by examining the primary structure of colipase (Figure 1). The three tyrosines are positioned within a pentapeptide, amino acids 53–57, of an amino acid sequence of colipase, which can be characterized as increasingly hydrophobic toward the N-terminal end and would thus be expected to be buried in the protein.

Cys-Ala-Phe-Thr-Leu-Tyr-Gly-Val-Tyr-Tyr-Lys
50 55

In view of the mobility, pK_a values, and proton exchange the following tentative assignment can be made

Tyr-A = Tyr-53 Tyr-B, Tyr-C = Tyr-56, Tyr-57

Phenylalanines. The nontitrating amino acid groups exhibiting NMR resonances in the aromatic region are phenylalanines, tryptophans, and nonexchangeable amide and hydroxyl protons. Colipase lacks tryptophan and we can assume that all backbone protons exchange with deuterons. The remaining resonances, 2, 4 (4′), and 5, can thus be attributed to the 10 protons of the two phenylalanines. Due to the large line width and thus lack of fine structure, decoupling experiments were unsuccessful, and assignments to the individual phenylalanines could not be made. In the pH range 10–12, however, small shifts of resonances 2 and 4′ indicate that a conformational change might accompany the deprotonation of the tyrosines and/or the lysines.

A summary of the assignments, including a tentative assignment to specific residues in the primary structure of colipase, is presented in Table I. The assignment of resonances to specific amino acid residues in the primary structure in colipase is in progress by use of chemical modification and proteolytic degradation procedures.

Interaction with Bile Salts. Although the binding of TDC to colipase probably has no physiological importance (Donnér, 1977), it is still interesting from other points of view. By a close NMR study of the effects of TDC binding to colipase as seen in the colipase NMR spectrum, some information concerning the structural arrangement of the amino acids can be obtained. Also, it has been shown that bile salt decreases the binding between lipase (Donnér et al., 1976; Patton et al., 1978) and colipase, suggesting a competition between bile salts and lipase for the same binding site on colipase. An identification of the bile-salt binding area on colipase can thus indicate a possible lipase binding site.

The interactions between colipase and TDC have been studied by various methods. Equilibrium dialysis, gel filtration (Borgström & Donnér, 1975), and ultracentrifugation studies (Charles et al., 1975b) have shown that TDC binds to colipase at concentrations near the cmc of TDC and that the binding numbers were close to those of TDC micelles. UV spectroscopy (Charles et al., 1975a) and circular dichroism (Donnér et al., 1976) have been used to show that the aromatic residues of colipase are involved in the binding of TDC. The CD studies (Donnér et al., 1976) also showed that no major conformational changes of colipase take place when TDC is bound. It has been suggested that the TDC–colipase interaction is comparable to bile-salt micelle formation (Borgström & Donnér, 1976).

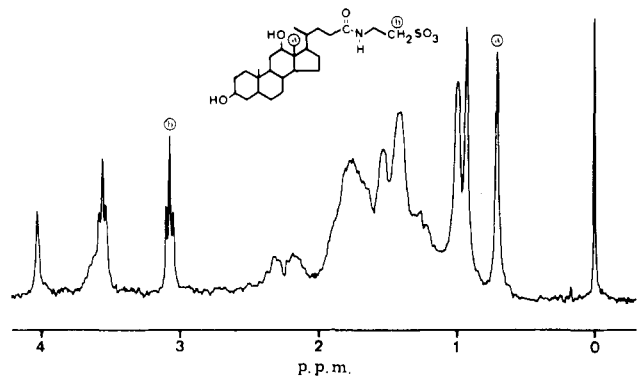

FIGURE 5: A 270-MHz proton NMR spectrum of TDC. The two resonances studied are (a) the angular methyl group (Small et al., 1969) and (b) the $-CH_2SO_3^-$ methylene group (assigned from chemical-shift tables).

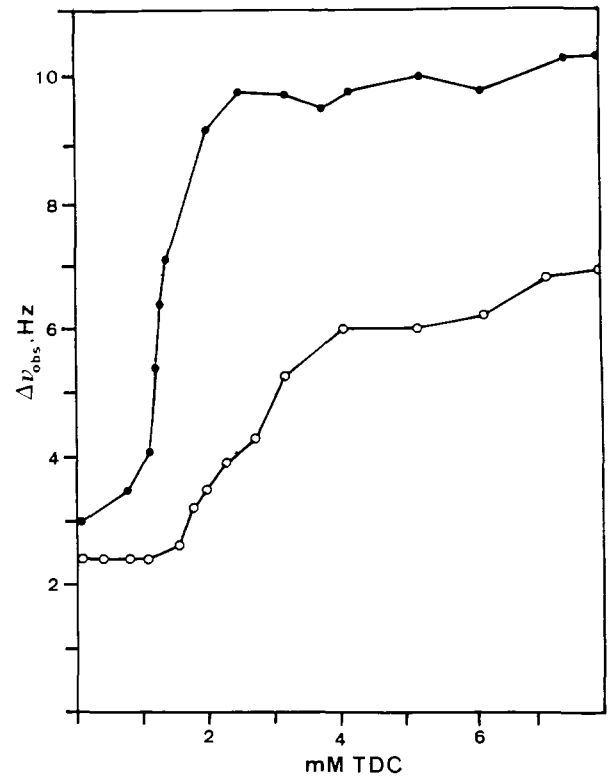

FIGURE 6: The line width of the angular C(18) methyl group as a function of TDC concentration in the absence (○) and presence (●) of 0.15 mM colipase. Phosphate buffer (10 mM), pH 7.0, 150 mM NaCl in D_2O. Temperature was 30 °C.

Observed Effects in the TDC Proton NMR Spectrum. We have studied this interaction by investigating the effects observed in the TDC and colipase proton NMR spectra. Thus Figure 5 shows a proton NMR spectrum of TDC. The resonances studied are the angular C(18) methyl group and the $-CH_2SO_3^-$ methylene group.

Figure 6 shows the line width of the C(18) methyl groups in the presence and absence of 0.15 mM colipase. The steep increase in line width at about 1 mM clearly indicates that binding of TDC to colipase takes place. The increase in line width in the absence of colipase is a manifestation of TDC micelle formation.

As the line shape of the resonances was found to be Lorentzian and as the line width increases continuously, we assume that we are confronted with a fast-exchange situation. Thus

Table II: Equilibrium Dialysis Data of the Binding of TDC to Colipase in a 10 mM Phosphate Buffer, pH 6.8, and 150 mM NaCl, Using a Three-Chamber Dialysis Cell[a]

TDC_t (mM)	fraction bound (%)
0.9	4
1.1	7
1.5	22
2.2	25
3.0	26

[a] The middle chamber contained a pure TDC solution; the other two contained a 0.15 mM colipase and a buffer solution, respectively.

the observed line width $\Delta\nu_{obsd}$ of the resonance is a weighted average of the line width in the bound $\Delta\nu_b$ and in the free state $\Delta\nu_f$

$$\Delta\nu_{obsd} = P_f\Delta\nu_f + P_b\Delta\nu_b \quad (1)$$

where P_f and P_b are fraction-free and -bound TDC, respectively. By use of values of P_b and P_f obtained from equilibrium dialysis (Table II) and by use of $\Delta\nu_{obsd}$ and $\Delta\nu_f$ values for the methyl signal in Figure 6, $\Delta\nu_b$ was calculated to be of the order of 25 Hz, for a concentration range of 0–3 mM TDC. By assuming the radius of colipase to be 17 Å (Erlanson & Borgström, 1972) and by using the theory of Woessner (Woessner, 1962; Navon & Lanir, 1972), the line width of the bound TDC C(18) methyl group was calculated to be 5 Hz, assuming the internal motion of the methyl group to be essentially free.

The differences in line width of 20 Hz in comparison to the experimental value of 25 Hz can be explained either as an additional increase in the rotational correlation time τ_c of the TDC–colipase complex or by dipolar interactions between the methyl protons and the protons on colipase. The former explanation, however, most likely makes the largest contribution the difference in $\Delta\nu_b$. The molecular weight of the colipase–TDC complex is of the order of 20 000 (Charles et al., 1975a), twice that of colipase, and consequently the larger τ_c of bound TDC should have a considerable effect on $\Delta\nu_b$. An additional effect is cross correlation between the pairs of interacting spins in the bound TDC methyl group (Werbelow & Marshall, 1973; Kalk & Berendsen, 1976). However, a full theoretical analysis of these latter effects in an exchanging system as is the case here is beyond the scope of this paper.

In order to study the TDC–colipase aggregates at low TDC/colipase ratios, solutions of 5 and 10 mM TDC at pH 7.0 and 150 mM NaCl and with initially high colipase concentration were stepwise diluted with solutions containing similar concentrations of TDC. The changes of the line width of the $-CH_2SO_3^-$ proton resonance were measured and plotted as $\Delta\nu_{obsd}$ vs. colipase concentration (Figure 7). In an ultracentrifugation study performed at 4 mM TDC and with varying concentrations of colipase, it was observed that large TDC–colipase complexes (Charles et al., 1975b) including more than one colipase molecule were formed. The steep increase of $\Delta\nu_{obsd}$ of the $-CH_2SO_3^-$ methylene proton resonance in Figure 7 at high colipase concentration might thus be an effect of the formation of these large aggregates, and the latter is probably also an explanation of the general broadening of all resonances in the aromatic region of the colipase spectrum, observed in the presence of TDC at pH values below 9 (Figure 9). It is thus important to choose the right pH for the TDC–colipase experiments where the TDC/colipase ratio is less than $\simeq$10 and the TDC concentration is above the cmc.

Effects Observed in the Aromatic Region. In order to study the binding effects of TDC on the aromatic region of the

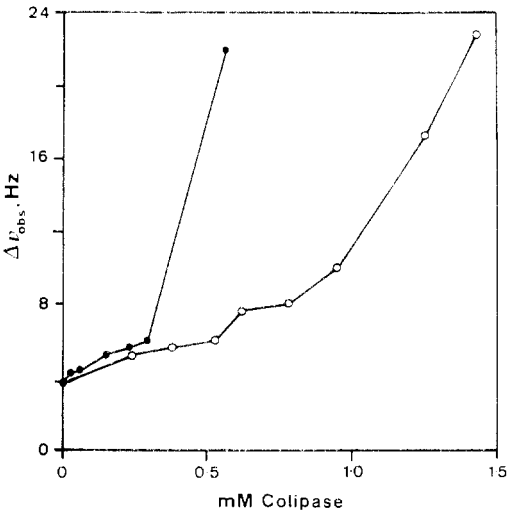

FIGURE 7: The line width of the $-CH_2SO_3^-$ methylene group as a function of colipase concentration at 5 mM TDC (●) and 10 mM TDC (○). Experimental conditions are as in Figure 6.

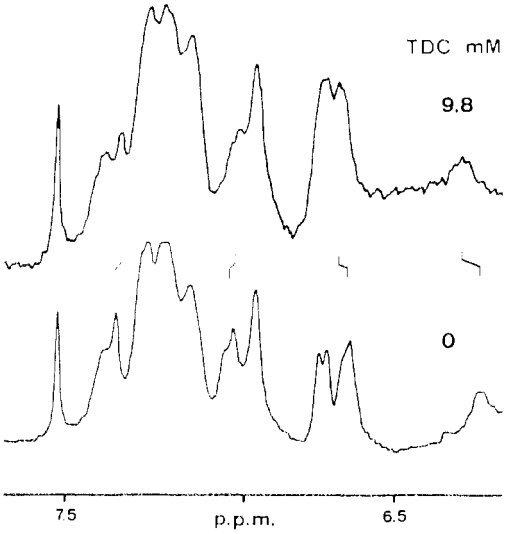

FIGURE 8: The aromatic region of the colipase proton NMR spectrum in the absence (lower) and presence (upper) of 9.8 mM TDC. The solution was made up in a 10 mM phosphate buffer, pH 9.8, 150 mM NaCl in D_2O. Temperature was 30 °C. The spectra are results of 1000 scans.

colipase proton NMR spectrum we performed the experiments at pH 9.8 and 150 mM NaCl.

Figure 8 shows the aromatic region of the colipase NMR spectrum in the absence and presence of 8.7 mM TDC.

The addition of TDC causes shifts of the His-B and Tyr-B meta and Tyr-C ortho proton resonances. The effects of the Tyr-A resonances, if any, are not detectable due to overlapping of other resonances. No shifts of the His-A and the phenylalanine residue resonances were observed.

The observed shifts of the Tyr-C and -B resonance can be interpreted as a conformational change of colipase caused by the binding of TDC or an interaction of TDC with the residues corresponding to the perturbed resonances. CD measurements (Donnér et al., 1976), however, did not show any gross conformational changes though shifts in the aromatic region were observed. UV spectroscopic studies (Charles et al., 1975a) also indicate that tyrosine residues are affected by the TDC binding. The observed effects on the tyrosine resonances in the present investigation would thus seem to indicate a direct TDC interaction with the tyrosine residues, possibly involving a small local conformational change. This is further confirmed

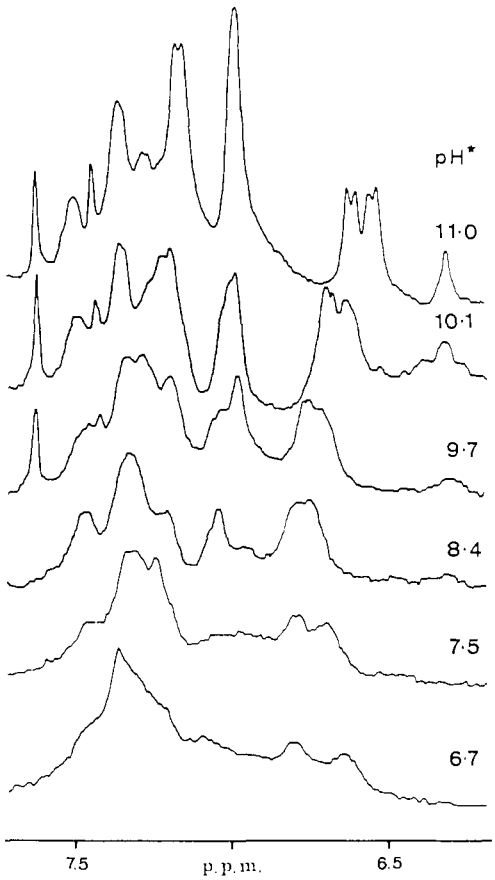

FIGURE 9: The aromatic region of colipase at different pH values and at a TDC concentration of 8.7 mM. The solutions were made up in a 150 mM NaCl solution in D_2O. Temperature was 30 °C.

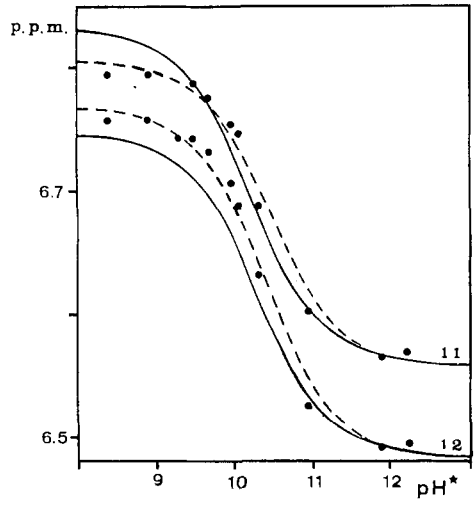

FIGURE 10: The chemical-shift values of the ortho proton resonances of Tyr-B and Tyr-C (resonances 11 and 12 in Figure 2). The solid graph corresponds to the least-squares fit to resonances 11 and 12 in Figure 3. The dashed graph is a fit to the data points obtained in the presence of 8.7 mM TDC.

by a study of the effects of pH (Figures 9 and 10).

At pH 8.5 and in the presence of 8.7 mM TDC the ortho proton resonances of Tyr-B and -C are shifted 0.09 ppm upfield and 0.04 ppm downfield, respectively, compared to the chemical-shift values in the absence of TDC. As the pH is raised the resonances are shifted upfield due to deprotonation of the tyrosine hydroxyls. However, as the pH approaches the pK_a value(s) of the tyrosine(s) the chemical shift approaches the values observed in the absence of TDC, and these are essentially equal at pH values above 11. The signals also

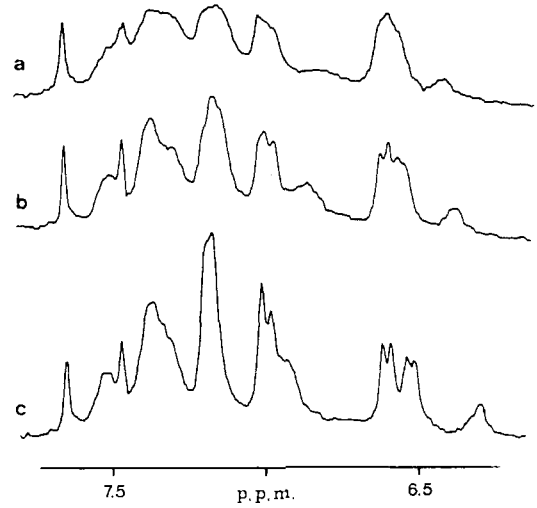

FIGURE 11: The aromatic region of the colipase proton NMR spectrum in the presence of (a) 8 mM bile-salt cation (see text), (b) 5.9 mM bile-salt cation, and (c) a pure colipase solution at pH 11.5; 2 mM colipase solution in D_2O with 150 mM NaCl.

become better resolved, indicating that no large fraction of TDC could be bound. The mutual effects observed on Tyr-B and -C and His-B resonances indicate that these three residues are closely arranged in space and thus support the tentative assignment of Tyr-B and -C to Tyr-56 and Tyr-57. The observed decrease in binding at high pH is most probably due to the repulsion between the tyrosine anions and the sulfate groups of TDC. To study this effect a positively charged deoxycholate derivative was synthesized, where the $-C(=O)NHCH_2CH_2SO_3^-$ sequence (Figure 5) was replaced by a trimethylamino group, $-CH_2N^+(CH_3)_3$. Figure 11 shows the effects of an increasing concentration of this positive bile-salt molecule on the aromatic region at pH 11.5. The Tyr-A signal shifts upfield 0.2 ppm and a general broadening of the spectrum takes place. The Tyr-B, Tyr-C, and His-B resonances also shift. The repulsive effects between the tyrosine-56 and -57 anions and the sulfate groups of TDC suggest that the observed shifts of the NMR resonances of these residues are due to the interaction with the TDC side chain, possibly involving hydrogen bondings to the tyrosines, while the steroid nucleus of TDC most probably would interact with other residues on colipase by hydrophobic interactions. In view of the earlier discussion on the structural features of colipase, such TDC binding region could have the following tentative arrangement

-Leu-Cys-Leu-
-Phe-Gly-Ile-Cys-His-Asn-Val-Gly-
-Tyr-Val-Gly-Tyr-Tyr-

As was stated earlier the bile salts seem to compete with lipase for the same site on colipase (Donnêr et al., 1976; Patton et al., 1978). The region of colipase described above might thus be a part of the lipase binding site. Further investigations on the latter interaction are in progress using proton and carbon NMR methods.

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Proton Nuclear Magnetic Resonance Studies of *Rhodospirillum rubrum* Cytochrome c_2†

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ABSTRACT: *Rhodospirillum rubrum* cytochrome c_2 was studied by proton nuclear magnetic resonance at 220 MHz. Assignments were made to the resonances of heme *c* by double-resonance techniques and by temperature-dependence studies. The aromatic resonances of Trp-62 and Tyr-70 of ferrocytochrome c_2 were identified by spin-decoupling experiments. The resonances of the Met-91 methyl group of the ferri- and ferrocytochromes were assigned by saturation-transfer experiments. The assignments are compared to those made for cytochromes *c*. A pH titration showed that the methionine methyl resonance of ferricytochrome c_2 shifted with a p*K* of 6.25 and disappeared above pH 9. No histidine CH resonances that titrated normally over the neutral pH range were observed in the spectrum of either oxidation state of the protein. The possible origins of the ionizations at pH 6.25 and 9 are discussed.

The cytochromes c_2 of photosynthetic bacteria are small, momeric, monoheme proteins that are structurally similar to the well-known mitochondrial cytochromes *c*. In the nonsulfur purple photoheterotroph *Rhodospirillum rubrum*, the cytochrome c_2 is believed to function as the electron donor to bacteriochlorophyll (Smith et al., 1973).

The heme group of cytochrome c_2 is attached to the polypeptide chain via condensation of the two-carbon side chains with the sulfur atoms of cysteines-14 and -17. The heme iron is involved in coordinate bonds with two other amino acid residues, His-18 and Met-91 (Salemme et al., 1973). The iron exists in either of two oxidation states, diamagnetic Fe^{2+} or low-spin Fe^{3+}. The midpoint potential at neutral pH is 310 mV (Pettigrew et al., 1978), some 60 mV higher than that of the mitochondrial cytochrome. Despite the striking similarity of the X-ray structure of the mitochondrial and *R. rubrum* cytochromes, they differ not only in midpoint potential but also in their reactivity with purified preparations of mitochondrial cytochrome oxidase and reductase (Davis et al., 1972; Errede & Kamen, 1978) and *Pseudomonas aeruginosa* nitrite reductase (Yamanaka, 1972).

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