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An Enzyme Kinetics and ^{19}F Nuclear Magnetic Resonance Study of Selectively Trifluoroacetylated Cytochrome *c* Derivatives†

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ABSTRACT: The reaction of cytochrome *c* with ethyl thioltrifluoroacetate was carried out under conditions which led to the selective trifluoroacetylation of a small number of the 19 lysines. The mixture of derivatives was separated by ion-exchange chromatography and four different derivatives with well-resolved ^{19}F nuclear magnetic resonance (NMR) spectra were obtained. Peptide mapping techniques indicated that one of these derivatives contained a single trifluoroacetyl group at lysine 22, and another derivative was singly labeled at lysine 25. The trifluoroacetylated lysine 22 derivative was fully active toward both succinate-cytochrome *c* reductase (EC 1.3.99.1) and cytochrome oxidase (EC 1.9.3.1) while the trifluoroacetylated lysine 25 derivative was fully active toward the reductase, but had a threefold greater Michaelis constant in the cytochrome oxidase reaction. This supports the hypothesis that the cytochrome oxidase binding site is located in the heme crevice region, and that Lys-25 is important in the binding. ^{19}F NMR spectra of the cytochrome *c* derivatives bound to phospholipid vesicles were obtained. The reasonably narrow line widths (35-65 Hz) and good sensitivity of the trifluoroacetyl resonances indicated that they might be useful probes for the interaction of cytochrome *c* with intact mitochondria.

Although a wide variety of techniques have been used to study the reduction of cytochrome *c* by cytochrome *c* reductase and its oxidation by cytochrome oxidase, little is known about the mechanisms of these processes at the molecular level. The location of the reaction sites on cytochrome *c* for cytochrome *c* reductase and cytochrome oxidase is the subject of some controversy, particularly as to whether the sites are the same or different. A number of chemical modification and antibody binding studies indicate that the binding sites might be different (Takano et al., 1973; Margoliash et al., 1973; Smith et al., 1973) while Salemme et al. (1973) have suggested that both the oxidase and the reductase bind at the same site on the front of cytochrome *c* over the heme crevice. We report here on the preparation and characterization of several cytochrome *c* derivatives, two of which contained a single specifically trifluoracetylated lysine residue. We studied the effects of these modified lysine groups on the reactivity of cytochrome *c* with the reductase and the oxidase to characterize the reaction sites

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on cytochrome *c*. We also studied the ^{19}F NMR[1] spectra of these derivatives, both in aqueous solution and bound to phospholipid vesicles.

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Company. Ethyl thioltrifluoroacetate was obtained from Pierce Chemical Company. Tos-PheCH_2Cl-treated trypsin was obtained from Worthington Biochemical Corp. Sodium cholate and TMPD were obtained from Sigma.

Preparation of Trifluoroacetylated Cytochrome c Derivatives. Cytochrome *c* (250 mg) was dissolved in 2 ml of 0.14 M phosphate (pH 7.2) and adjusted to pH 8.0 with 1 M NaOH. Ethyl thioltrifluoroacetate (10 μl) was added to the rapidly stirred solution at room temperature and the pH was continuously maintained at 8.0 by addition of 1 M NaOH with a micrometer syringe. After 1 h, base consumption had ceased, and the solution was passed through a small Bio-Gel P-4 column equilibrated with 0.03 M ammonium phosphate buffer (pH 7.2) to remove ethyl mercaptan and trifluoroacetate. The cytochrome was then applied to a 2 × 70 cm Bio-Rex 70 (200–400 mesh) column equilibrated with 0.14 M ammonium phosphate (pH 7.2). The column was eluted with 0.14 M ammonium phosphate buffer at 25 ml/h. The fractions for each peak were pooled (from half-height to half-height), concentrated on a small column of Bio-Rex 70, eluted in a small volume by a buffer containing 0.5 M NaCl, 0.1 M phosphate (pH 7.0), and finally desalted by passing through a small Bio-Gel P-4 column equilibrated with 0.02 M sodium phosphate buffer (pH 7.0). The fractions were repurified on a 1.5 × 100 cm Bio-Rex 70 (200–400 mesh) column and concentrated and desalted as above.

Peptide Mapping. Cytochrome *c* at a concentration of 10 mg/ml in 0.05 M Tris buffer (pH 7.5) was digested with Tos-PheCH_2Cl-treated trypsin (0.5 mg/ml) at 37 °C. The hydrolysis was stopped after 3 h by freezing in dry ice and lyophilization. The cytochrome *c* derivatives were digested with trypsin following the same procedure.

The separation of peptides from the tryptic hydrolysates was carried out on a modified Phoenix amino acid analyzer using a 0.9 × 23 cm Aminex A-5 resin column operated at 52 °C, following a modification of the method of Benson et al. (1966). A 250-μl solution of tryptic hydrolysate containing 4 to 10 mg of cytochrome *c* was adjusted to a pH of 3.5 and applied to the column previously equilibrated with a 0.05 M pyridine–acetic acid buffer at pH 3.3 (64.5 ml of pyridine and 1114 ml of concentrated acetic acid diluted to 16 l.). The first developer was a 17-h linear gradient of 540 ml of the 0.05 M buffer and 540 ml of 0.5 M pyridine–acetic acid buffer at pH 4.9 (645 ml of pyridine and 573 ml of concentrated acetic acid diluted to 16 l.). This was followed by a second, 8-h linear gradient of 259 ml of the 0.5 M buffer and 259 ml of 3 M pyridine–acetic acid buffer at pH 5.3 (967.2 ml of pyridine and 340 ml of concentrated acetic acid diluted to 4 l.), and finally, by 300 ml of the limiting 3 M buffer. The flow rate was maintained at 60 ml/h throughout the analysis. The column eluent was stream split, 70% going to a fraction collector, and 30% going through the amino acid analyzer for ninhydrin detection and automatic plotting. The pyridine–acetate buffer was removed from the peptide containing fractions by vacuum evaporation. Paper chromatography of some of the peptides was carried out using the method of Margoliash (1962).

Amino Acid Analysis. The analyses were carried out on a modified Phoenix amino acid analyzer using a 0.9 × 62 cm Durrum DC-1A resin column, following the Durrum single-column methodology. Samples were hydrolyzed with 6 N hydrochloric acid in evacuated sealed tubes at 110 °C. The hydrolysis time for the dinitrophenyl derivatives was 16 h, and for all other compounds 24 h.

Dinitrophenylation. The dinitrophenylation of native cytochrome *c* and its trifluoroacetylated derivatives was conducted in 67% ethanol, following the method of Sanger as modified by Fanger and Harbury (1965).

Nuclear Magnetic Resonance. ^{19}F NMR spectra were obtained on a Bruker HFX 90 spectrometer operating at 84 MHz, using a proton internal lock on H_2O. A Nicolet NMR-80 Fourier transform accessory was used to accumulate the free induction decays and perform the Fourier transform, 5 mm o.d. NMR tubes were used. Both trifluoroacetate and the H_2O proton lock were used as internal references, and the two were in agreement at a constant temperature. For the reduced spectra, ascorbate was added to reduce cytochrome *c*, and the derivative was passed through a Bio-Gel P-4 column to remove ascorbate. The extent of reduction was checked spectroscopically. Proton NMR spectra were obtained at 90 MHz in the Fourier transform mode using techniques similar to those described by Redfield and Gupta (1971).

Visible Absorption Spectra and Redox Potential. The visible absorption spectra of the cytochrome *c* derivatives were obtained on a Cary 14 spectrophotometer. The redox potential of the derivatives was measured by the method of Wada and Okunuki (1969).

Enzyme Kinetics. The derivatives used for enzyme kinetics studies were used without lyophilization to prevent formation of polymers reported by Feinberg and Brautigan (1975). The source of both succinate-cytochrome *c* reductase and cytochrome oxidase activities was a cytochrome *c* depleted Keilin–Hartree preparation prepared as described by Smith and Camerino (1963). For the reductase assays, the Keilin–Hartree preparation (20 mg of protein/ml) was treated with 5% cholate (1 mg/mg of protein), allowed to stand 10 min, and then diluted with buffer as described by Takemori and King (1964). The Keilin–Hartree preparation (0.02 mg of protein/ml) was preincubated for 4 min in 5 mM succinate, 1 mM KCN, and 0.1 M potassium phosphate (pH 7.3) to obtain complete inhibition of the oxidase activity. The initial rate of reduction was measured at 550 nm after addition of ferricytochrome *c* (1.0 to 10.0 μM), using a Cary 14 spectrometer.

The cytochrome oxidase activity was measured using two different techniques. In the first, the initial rate of oxidation was measured spectroscopically at 550 nm after addition of the cholate-treated Keilin–Hartree preparation (0.005 mg of protein/ml) to a solution containing 0.02 M phosphate (pH 7.3) and ferrocytochrome *c* (1 to 10 μM). The oxidase activity was also measured polarographically with a Gilson Model KM Clark electrode cell using the ascorbate–TMPD system (Ferguson-Miller et al., 1976). Assays were run in 50 mM potassium Mops (pH 7.8), 200 mM sucrose, 10 mM sodium ascorbate (from a stock solution of 0.5 M sodium ascorbate containing 1 mM EDTA), and 1 mM TMPD. The low baseline rate of oxygen consumption measured after addition of the Keilin–Hartree particles (0.01 mg of protein/ml) was sub-

[1] Abbreviations used are: TFA, trifluoroacetyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; TNP, trinitrophenyl; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; Tris, tris(hydroxymethyl)-aminomethane; Mops, morpholinopropanesulfonic acid; ATP, adenosine 5′-triphosphate.

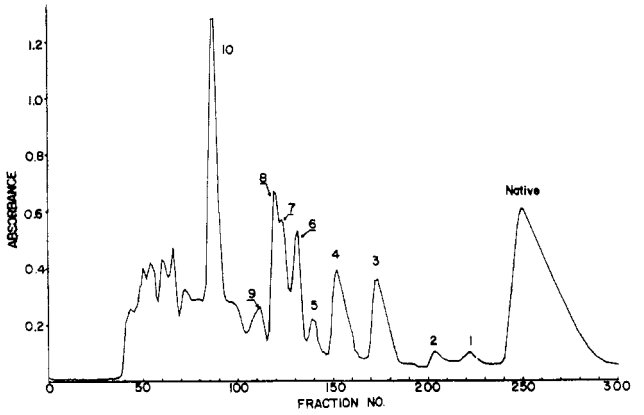

FIGURE 1: Chromatogram of 250 mg of trifluoroacetylated cytochrome *c* on a 2 × 70 cm Bio-Rex 70 column as described in the text. The column was eluted with 0.14 M ammonium phosphate, pH 7.2, at a flow rate of 25 ml/h, at room temperature. The fraction size was 4 ml. The absorbances were taken at 430 nm.

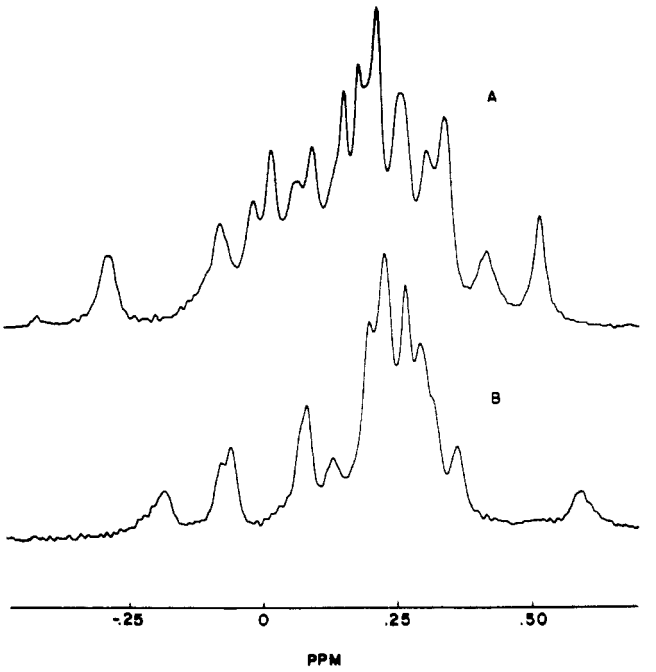

FIGURE 2: ^{19}F NMR spectra of totally trifluoroacetylated cytochrome *c* in the oxidized state (A) and the reduced state (B). Both spectra were obtained by taking the Fourier transform of 4000 two second free induction decays of 2×10^{-3} M cytochrome *c* in 0.02 M sodium phosphate, pH 7.2. The chemical shifts are measured in ppm from 10^{-2} M trifluoroacetate in 0.02 M sodium phosphate, pH 7.2.

stracted from the rates measured in the presence of various concentrations of cytochrome *c* (0.03 to 1.5 μM).

Cytochrome c–Phospholipid Complexes. Complexes of cytochrome *c* with total mitochondrial phospholipid were prepared by the method of Kimelberg et al. (1970). The phospholipids were extracted from beef heart mitochondria with 4:1 chloroform–methanol, redissolved in pure chloroform, and purified by acetone precipitation. Complexes were prepared by adding 40 ml of a solution containing 1 μmol of cytochrome *c*, 15 mM KCl, and 5 mM phosphate (pH 7.5) to 90 μmol of dried phospholipids and shaking for 10 min. The complex recovered by centrifugation at 30 000*g* for 15 min contained about 70:1 mol ratio of phospholipid to cytochrome *c*.

Results

Preparation of Trifluoroacetylated Cytochrome c Derivatives. The reaction of ethyl thioltrifluoroacetate with cytochrome *c* was studied with the objective of trifluoroacetylating the ε-amino group of a small number of lysines as selectively as possible. We found that the best way to increase the selectivity of the reaction was to lower the pH from 10.0 to 8.0, and also to use a very small amount of ethyl thioltrifluoroacetate. The resulting mixture of derivatives was purified on a 2 × 70 cm Bio-Rex 70 cation exchange column (Figure 1).

It was extremely important to concentrate the cytochrome *c* solutions to small volumes and reduce the salt concentration to low levels before lyophilization was used to concentrate and store the derivatives. A small Bio-Gel P-4 column was used to desalt small volumes (typically 2 ml) of concentrated cytochrome *c* solutions. Peaks 3, 4, 6, and 10 re-chromatographed on Bio-Rex 70 as single peaks at their original positions.

^{19}F NMR Spectra of Cytochrome c Derivatives. The ^{19}F NMR spectra of totally trifluoroacetylated cytochrome *c* prepared by the method of Fanger and Harbury (1965) is shown in Figure 2. There are 14 partially resolved peaks in the oxidized spectrum, some with a considerable amount of overlap. If the well-resolved, outer peaks are taken as unity, integration of the entire spectrum yields a total of 19 ± 1, thus accounting for all the lysines, with no evidence of any additional modification. There is a significant change in the spectrum upon reduction (Figure 2B). We also obtained ^{19}F NMR spectra of derivatives 3, 4, 6, and 10 in both oxidized and reduced states (Figure 3 and Table I). In some cases, the chemical shifts were significantly changed upon reduction. The ^{19}F NMR spectra indicate that derivatives 3 and 6 are singly labeled while derivatives 4 and 10 are either doubly labeled or are composed of two unresolved singly labeled derivatives. The small peaks in the ^{19}F NMR spectra of derivatives 3 and 6 were due to small amounts of cytochrome *c* labeled at other positions. They were not removed by re-chromatography on Bio-Rex 70.

TABLE I: Chemical Shifts of Trifluoroacetylated Cytochrome *c* Derivatives.[a]

Derivative	σ (Oxidized)	σ (Reduced)
3	+0.191	+0.273
4	−0.076	+0.113
	+0.217	+0.194
6	+0.142	+0.313
10	+0.149	+0.215
	+0.198	+0.171

[a] Chemical shifts in parts per million (+ is upfield) from 10 mM trifluoroacetate in 20 mM phosphate buffer, pH 7.2. The cytochrome *c* derivatives were at a concentration of 2 mM in phosphate buffer, pH 7.2. The temperature was 22 °C.

Peptide Mapping and Chemical Characterization. Since the trifluoroacetylamide bond is rather easily hydrolyzed, hydrolysis in 6 M HCl at 110 °C followed by amino acid analysis yields the same amino acid composition for the derivatives as for native cytochrome *c*. The number of lysines trifluoroacetylated was estimated by reacting the derivative with fluorodinitrobenzene followed by amino acid analysis. Those lysines trifluoroacetylated would be protected from reaction with fluorodinitrobenzene and, hence, would appear

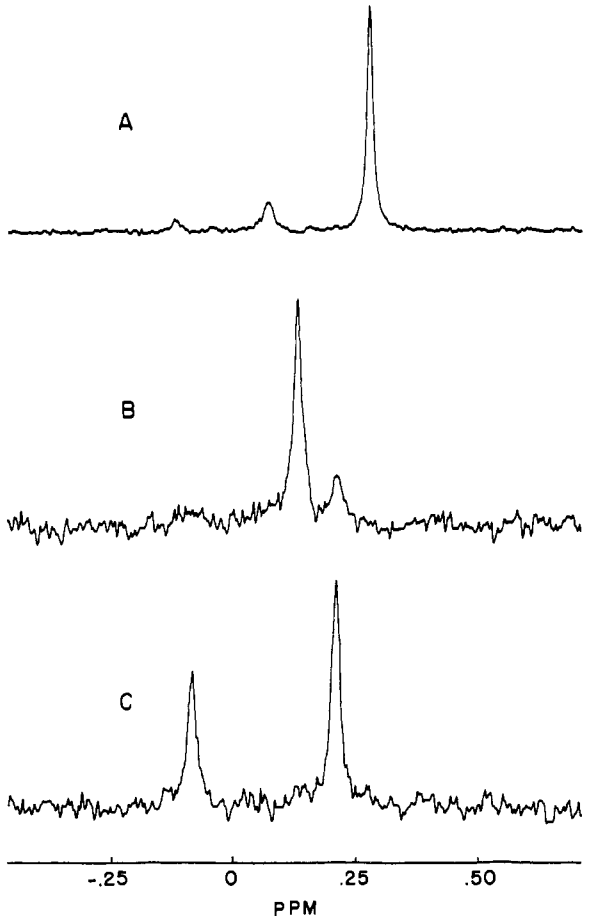

FIGURE 3: ^{19}F NMR spectra of TFA-cytochrome *c* derivatives in 20 mM sodium phosphate (pH 7.2). (A) Derivative 3 (3 mM) in the reduced state; (B) 1 mM derivative 6 in the oxidized state; (C) 1 mM derivative 4 in the oxidized state. Spectrum A was the Fourier transform of 1000 two second free induction decays, and spectra B and C were the Fourier transforms of 2000 two second free induction decays.

TABLE II: Results of Treatment with Fluorodinitrobenzene.

Derivative	Lysine[a]	Arginine[b]
Cytochrome *c*	0.20	2.0
Totally labeled cytochrome *c*	18.70	2.0
3	1.02	2.0
4	0.85	2.0
6	1.10	2.0
10	1.21	2.0

[a] Average of three analyses each. [b] Taken as reference.

TABLE III: Ferricytochrome *c* Proton NMR Chemical Shifts.[a]

CH_3 Group[b]	Native	Fraction 10	19-TFA
Met 80	−24.3	−24.3	−24.1
Heme ring 4	+35.3	+35.3	+35.3
Heme ring 2	+32.4	+32.4	+32.4
Heme ring 1 (or 3)	+10.1	+10.1	+10.5
Heme ring 1 thioether bridge	−2.7	−2.7	−2.6
Heme ring 2 thioether bridge	−2.4	−2.5	−2.5

[a] In parts per million from 2,2-dimethyl-2-silapentanesulfonate. Spectra obtained at 90 MHz in D_2O, pD = 7.5, T = 23 °C. [b] Assignments from Redfield and Gupta (1971).

in the amino acid analysis as native lysine. The results of this analysis are shown in Table II.

Peptide mapping was carried out on a 0.9 × 23 cm column of Aminex A-5 resin using a modified Phoenix amino acid analyzer with stream splitting. The pyridine–acetate gradients were adjusted until all but two of the 24 peptides of the tryptic hydrolysate of native cytochrome *c* were well resolved (Figure 4). Each peak was identified by amino acid analysis with reference to the known sequence of horse heart cytochrome *c* (Margoliash, 1962). The only peptides which were not resolved were 89–91 and 6–7 which eluted at the same position. These peptides were separated by paper chromatography. In the peptide map of derivative 3 peptide 23–25 and the heme peptide 14–22 both decreased substantially, and a new heme peptide eluted somewhat earlier. Amino acid analysis of this new heme peptide indicated that it now comprised residues 14–25, indicating that lysine 22 was trifluoroacetylated, thus protecting the peptide bond from tryptic cleavage. In the peptide map of derivative 6, peptides 23–25 and 26–27 both decreased substantially, and a single new peptide appeared at 1161 ml with an amino acid composition consistent with 23–27, thus indicating that lysine 25 was trifluoroacetylated.

Peptide mapping results on derivatives 4 and 10 were less conclusive. In the peptide map of derivative 4, several peaks decreased in amplitude by small amounts, and several new peaks appeared which were too small to obtain reliable amino acid analysis results. In the peptide map of derivative 10, peptide 88–90 was reduced in amplitude by about 50%, and a new peptide appeared at 847 ml with amino acid composition consistent with a peptide 87–90, indicating that lysine 87 was trifluoroacetylated. No other significant changes were observed, however.

Since it appeared likely that some of the trifluoroacetylated lysines in derivatives 4 and 10 were labile under the conditions of tryptic hydrolysis, these conditions were studied, particularly with regard to pH. pH 7.5 was the lowest pH that could be used for the hydrolysis without affecting the peptide map of native cytochrome *c*.

Visible Absorption Spectra, Proton NMR Spectra, and Redox Potential. No detectable differences were observed between the visible absorption spectra of native cytochrome *c* and the trifluoroacetylated derivatives in either redox state. The absence of any change in the conformation-sensitive 695-nm band indicates that the heme environment is unmodified in the derivatives and that no polymeric material was present. There were no significant differences between the proton chemical shifts of the contact shifted methyl groups of native ferricytochrome *c* and those of the derivatives, except for a slight change in the heme ring 1 methyl resonance of totally trifluoroacetylated cytochrome *c* (Table III). The redox potentials of derivatives 3, 6, and 10 were identical with that of native cytochrome *c* to within experimental error (±5 mV). These experiments indicate that trifluoroacetylation does not cause any change in the heme environment of cytochrome *c*, but do not rule out small local changes in protein conformation.

Enzyme Kinetics. The cytochrome oxidase activity of the derivatives was studied in two different assay systems, the ascorbate–TMPD polarographic system at low cytochrome *c* concentrations and the spectroscopic system at higher cytochrome *c* concentrations. In the ascorbate–TMPD system, at

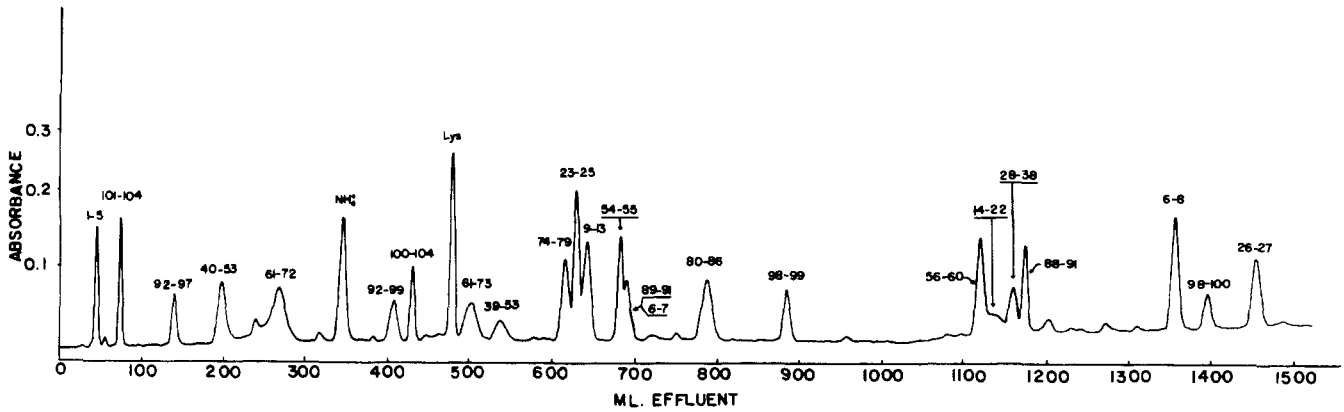

FIGURE 4: Elution diagram of a 10-mg tryptic hydrolysate of native horse heart cytochrome *c* on a 0.9 × 23 cm Aminex A-5 column, as described in the text. The column was eluted first with a 1080-ml linear pH gradient from pH 3.3 (0.05 M pyridine–acetic acid buffer) to pH 4.9 (0.5 M pyridine–acetic acid buffer), then with a 518-ml linear concentration gradient from 0.5 M buffer to 3 M pyridine–acetic acid buffer (pH 5.3), and finally with 300 ml of 3 M buffer. The identification of each peak as the peptide indicated by the number is discussed in the text.

TABLE IV: Enzymatic Activity of Trifluoroacetylated Cytochrome *c* Derivatives.

		Reductase Act.		Oxidase Act.[a]	
Fraction	Modified Lysine	K_m (μM)	V_{max} (% Native)	K_m (μM)	V_{max} (nmol of O_2 min^{-1})
Native		2.1 ± 0.3	100	0.051	27.3
3	22	2.0 ± 0.3	100 ± 5	0.057	27.8
6	25	1.9 ± 0.3	104 ± 5	0.140	24.6
10[b]	87 + ?	2.8 ± 0.3	105 ± 5	0.127	20.4

[a] Measured in the ascorbate–TMPD system. [b] Derivative 10 contains one TFA group at Lys-87, and a second TFA group at an unidentified lysine.

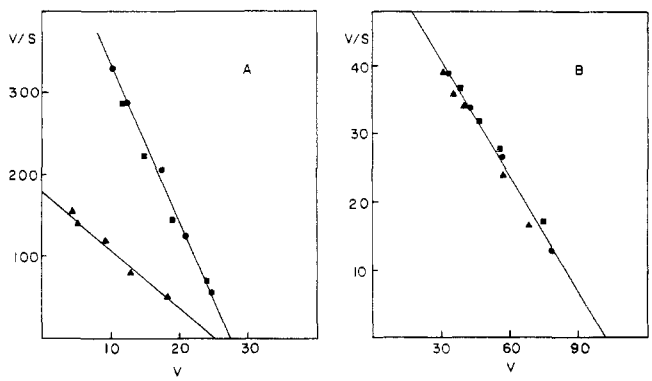

FIGURE 5: Activities of TFA-cytochrome *c* derivatives in the cytochrome *c* oxidase (A) and succinate–cytochrome *c* reductase (B) systems. In A, the velocity (v) is expressed in nanomoles of O_2 per minute, and s is in μM concentrations of cytochrome *c*. In B, the rate of reduction (v) was followed at 550 nm and is expressed as micromoles of cytochrome *c* per minute × 10^2, and s is in μM concentrations of cytochrome *c*. Activities are shown for native cytochrome *c* (●), derivative 3 (■), and derivative 6 (▲). Reaction conditions are described in the text.

cytochrome *c* concentrations (0.03 to 1.5 μM) for which only the high affinity phase of the reaction was observed (Ferguson-Miller et al., 1976) Eadie–Hofstee plots of the oxidase kinetics of native cytochrome *c* were linear and gave an apparent K_m value of 0.05 μM. It was found that derivative 3 had nearly the same reactivity with the oxidase as native cytochrome *c*, while the apparent K_m value of derivative 6 was about 2.8 times that of native cytochrome *c* (Figure 5A, Table IV). In the spectroscopic assay system in the presence of 20 mM phosphate (pH 7.3), the K_m values of both derivatives 3 and 6 were found to be identical with that of native cytochrome *c* (5 μM). Evidently, the low affinity reaction measured in this assay system is not very sensitive to the surface topology of cytochrome *c*.

We studied the enzyme kinetics of the reduction of ferricytochrome *c* by the succinate-cytochrome *c* reductase activity of a Keilin–Hartree preparation, inhibiting the cytochrome oxidase activity with cyanide. Nicolls et al. (1972) have found that the inhibition of cytochrome oxidase by cyanide is relatively slow, and only takes place in the presence of reducing substrates. We found that a four minute preincubation of the Keilin–Hartree preparation with KCN in the presence of succinate completely inhibited the oxidase activity. Eadie–Hofstee plots of the initial reduction rates of the cytochrome *c* derivatives were found to be linear for cytochrome *c* concentrations ranging from 1 to 10 μM (Figure 5B). Both the TFA-Lys-22 derivative and the TFA-Lys-25 derivative were completely active toward the reductase, while derivative 10 had a somewhat larger K_m.

^{19}F NMR Studies of Cytochrome c–Phospholipid Complexes. ^{19}F NMR spectra of insoluble phospholipid–cytochrome *c* complexes were obtained at 84 MHz using 5-mm NMR tubes. T_1 values were measured using the progressive saturation method, which works very well for these samples, since T_1 is so much larger than T_2. Figure 6 shows the ^{19}F NMR spectrum of TFA-Lys-22-cytochrome *c* in the absence of phospholipid (Figure 6A), in the insoluble phospholipid complex (Figure 6B), and in a complex subjected to ultrasonic radiation for 1 min using the microprobe of a Branson sonifier at maximum intensity (Figure 6C). The linewidth, T_1 value,

TABLE V: ^{19}F NMR Parameters for TFA-Cytochrome *c*–Phospholipid Complexes.

Fraction	Conditions	Δ (ppm)[a]	Linewidth (Hz)	T_1 (s)
3	Unsonicated, oxidized	+0.059	65	0.55 ± 0.05
3	Sonicated, oxidized	+0.083	37	0.36 ± 0.04
6	Unsonicated, oxidized	−0.047	40	0.51 ± 0.05
6	Sonicated, oxidized	−0.047	40	0.59 ± 0.06
10	Unsonicated, oxidized	−0.154	43	0.35 ± 0.04
10	Sonicated, oxidized	−0.142	37	0.37 ± 0.04

[a] Chemical shift relative to uncomplexed derivative (upfield shift is positive).

and chemical shift difference (Δ) between the complexed form and the uncomplexed form are given in Table V for derivatives 3, 6, and 10.

Discussion

Since the binding interaction of cytochrome *c* with both cytochrome *c* reductase and cytochrome oxidase is known to involve the positively charged lysines on cytochrome *c*, one way to study the location of the binding sites is to measure how modification of specific lysine groups affects the reactivity of cytochrome *c* with the reductase and the oxidase. Specific trifluoroacetylation is an attractive method because it does not appear to cause any general protein conformational changes in cytochrome *c* and the resulting derivatives can be used for ^{19}F NMR studies of the binding interactions of cytochrome *c* as well as for enzyme kinetics studies. A change in the oxidase or reductase activities due to trifluoroacetylation would indicate either that the ε-amino group of the unmodified lysine played some specific role in the reaction, such as electrostatic binding, or that the trifluoroacetylated lysine was oriented in such a way as to sterically interfere with the electron-transfer reaction.

We have selectively trifluoroacetylated cytochrome *c* at a pH value where only lysine residues with abnormally low pK_a values would be expected to react. Two of the derivatives have been unambiguously shown by fluorodinitrobenzene analysis, peptide mapping, and ^{19}F NMR to each contain a single trifluoroacetylated lysine. Derivative 3 has a label at lysine 22 at the bottom right side of cytochrome *c* (Takano et al., 1973, Figure 10) while derivative 6 has a label at Lys 25 to the lower right of the heme crevice. The results were inconclusive, however, for derivatives 4 and 10. The ^{19}F NMR spectra indicated that each of these derivatives was either doubly labeled or composed of two singly labeled derivatives. The fluorodinitrobenzene analysis was consistent with the latter possibility for both derivatives. This seems to be the most likely explanation for derivative 4, but derivative 10 would seem to pass through the ion-exchange column too rapidly to be composed to two singly labeled derivatives. The peptide mapping results definitively indicated that some of the trifluoroacetylated lysines in derivatives 4 and 10 were labile under the conditions of the tryptic hydrolysis. If these trifluoroacetylated lysines were also labile under the conditions of the fluorodinitrobenzene assay, derivative 10 could very well be doubly labeled. One of these labels is lysine 87, at the top left of cytochrome *c*. This lability is somewhat puzzling since totally trifluoroacetylated cytochrome *c* yields the expected 19 lysines (18.7) in the fluorodinitrobenzene analysis, and is not cleaved at any of the lysines by trypsin (Fanger and Harbury, 1965). It is possible that a nearby unlabeled lysine might catalyze the hydrolysis of a trifluoroacetylated lysine under the conditions of tryptic

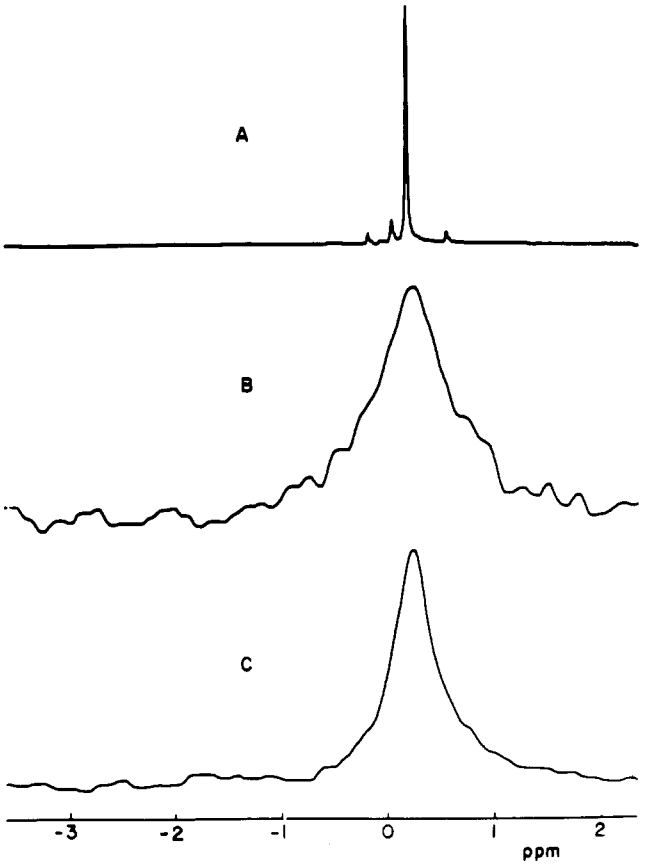

FIGURE 6: ^{19}F NMR spectra of cytochrome *c* complexed to phospholipid membranes. (A) Derivative 3 (4×10^{-3} M) (Lys-22), without phospholipid. (B) Derivative 3 (8×10^{-4} M) complexed to 6×10^{-2} M mitochondrial phospholipids, suspended in H_2O. Spectrum is Fourier transform of 20 000 two second scans. (C) Complex prepared as in B, and then sonicated as described in text. Spectrum is Fourier transform of 20 000 one second scans. NMR tubes (5 mm) were used for all spectra.

hydrolysis. The trifluoroacetylated lysines of derivatives 4 and 10 were completely stable at pH 7.0 in the absence of trypsin and fluorodinitrobenzene, however.

Ferguson-Miller et al. (1976) have recently devised a very sensitive assay for studying the cytochrome *c* oxidase reaction of Keilin-Hartree particles at low cytochrome *c* concentrations. They found that, in the absence of inhibitory anions such as phosphate and ATP, the kinetics were biphasic with the high afinity phase having an apparent K_m of 5×10^{-8} M, and the low affinity phase having a K_m of 5 to 20 times this value, depending on the preparation. We have found that the low affinity phase of the reaction was not apparent at cytochrome *c* concentrations up to 1.5 μM. Cytochrome oxidase kinetic

studies at low cytochrome *c* concentrations indicated that Lys-25 was involved in binding to cytochrome oxidase, while Lys-22 probably was not. We considered how small concentrations of impurities would affect the enzyme kinetic results since ^{19}F NMR indicated that derivative 3 contained about 15% impurity consisting of other singly labeled derivative, while derivative 6 contained about 20% impurity. Since the V_{max} values of both derivatives were nearly the same as that of native cytochrome *c*, the presence of cytochrome *c* inhibitors (species with low K_m but low V_{max}) could be ruled out. A more realistic situation would be if the impurities had the same V_{max} as native, but K_m values ranging from that of native (0.05 μM) to infinity. It can be readily shown that the measured K_m value of a mixture of two substrates with the same V_{max} will be $1/K_m = f_A/K_A + f_B/K_B$, where $f_A + f_B$ are the mole fractions of species A and B, and K_A and K_B are the Michaelis constants of species A and B. For derivative 6, where the measured K_m is 0.14 μM, the true value K_A for TFA-Lys-25 could range from 0.11 to 0.25 μM, depending on whether impurity B present at 20% had an infinite K_B, or K_B = 0.05 μM, respectively. We conclude that Lys-25 makes a significant contribution to the electrostatic binding to cytochrome oxidase, while Lys-22 does not.

Since derivatization of Lys-13 with NBD (Margoliash et al., 1973), TNP (Wada and Okunuki, 1969), or TFA (Staudenmayer et al., 1976) led to a four- to sixfold increase in K_m, it is apparent that Lys-13 is also important in the electrostatic binding to cytochrome oxidase. The most likely location of the cytochrome oxidase binding site is therefore probably at the heme crevice region (Takano et al., 1973, Figure 10), and it would seem reasonable that, in addition to lysines 13 and 25, lysines 27 and 79 would also be involved in the binding.

Since both derivatives 3 and 6 were fully active in the succinate-cytochrome *c* reductase reaction, it would seem unlikely that either Lys-22 or Lys-25 is involved in reductase binding. It should be noted, however, that the reductase assay used is open to some question since it shows almost no ionic strength dependence (Smith et al., 1974). The high affinity coupled polarographic reductase assay developed by Ferguson-Miller et al. (1976) cannot be used for these derivatives since some of them have reduced oxidase activity. Our results therefore support the hypothesis that the reductase and oxidase binding sites are distinct, but provide no information on the detailed mechanism by which electrons are transferred to and from cytochrome *c*.

The rather large range in the ^{19}F chemical shifts of the chemically identical trifluoroacetylated lysines is indicative of the differing environments on the surface of cytochrome *c*. We have found ^{19}F NMR to be a very useful analytical tool in characterizing the derivatives and testing their purity since none of the derivatives we have so far prepared have identical chemical shifts in both oxidized and reduced states. The chemical shift changes observed upon oxidation could be due either to a conformational change which modifies the van der Waals interaction of the ring current shift, or to a paramagnetic shift due to the unpaired spin on the iron in the oxidized state (Millett and Raftery, 1972).

Binding of the trifluoroacetylated cytochrome *c* derivatives to mitochondrial phospholipid vesicles caused a broadening of the ^{19}F NMR resonances of the trifluoromethyl groups. The line widths (35–65 Hz) were, however, surprisingly narrow for insoluble complexes, and the relatively large T_1 values (0.5 s) indicated that there was a significant amount of anisotropic motion. Seiter and Chan (1973) have shown that in a system containing isolated CH_3 groups in which the CH_3 group can rotate freely around its symmetry axis, but in which the symmetry axis itself can only undergo very slow rotation, the NMR spectrum will consist of a very narrow central resonance containing 50% of the total intensity, and a very broad continuum extending out to either side of the central resonance containing the other 50% of the intensity. The trifluoromethyl group of the labels studied here will approximate such a motion if the cytochrome *c* molecule is undergoing slow rotation in the membrane ($\tau_c \approx 10^{-7}$ s), and the label itself does not undergo a great deal of intramolecular motion. The rotation about the symmetry axis of the CF_3 group would be expected to be unhindered in almost all situations. In this case interaction between the fluorine nuclei will not make any appreciable contribution to the T_1 value and line width of the sharp central peak of the CF_3 group. The relaxation will be determined predominantly by dipolar interactions between the fluorine and protons on cytochrome *c*, phospholipids, or water molecules. Since T_1 is so much larger than T_2, the motion of these protons relative to the fluorine group must be quite slow.

There was a considerable amount of variation in the ^{19}F NMR parameters of the different labels, indicating that the environments in the complex are quite different. Sonication had a significant effect on the line width of the Lys-22 label, indicating some change in environment, but not on that of Lys-25 or Lys-87. None of the heme methyl proton resonances of ferricytochrome *c* were observable in phospholipid complexes, due to excessive line broadening (Pitts, 1973). Evidently the decreased rotational motion in the complex gave rise to this broadening since the heme methyl groups have no internal degrees of freedom except for rotation about their symmetry axis. It is very encouraging that reasonably narrow ^{19}F NMR spectra of the trifluoroacetylated cytochrome *c* derivatives can be obtained at low concentrations in the membrane state. With the use of 13-mm sample tubes it is possible to obtain good-quality, ^{19}F NMR spectra of these complexes at cytochrome *c* concentrations down to 0.1 mM which is the concentration of cytochrome *c* in intact mitochondria.

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Effects of Pressure upon the Fluorescence of the Riboflavin Binding Protein and Its Flavin Mononucleotide Complex†

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ABSTRACT: The effect of pressure in the range of 10^{-3}–10 kbars upon the ultraviolet fluorescence of the riboflavin binding protein and the fluorescence of its complex with flavin mononucleotide has been studied. The fluorescence spectrum of the isolated protein showed a reversible red shift of 12 nm (1000 cm^{-1}) at high pressure, indicating the reversible exposure of the tryptophan to solvent. From the pressure dependence of the visible fluorescence of the protein–flavin complex in the region of 1–4 kbars the volume change in dissociation of the protein–ligand complex was estimated to be +3.3 ml/mol. A very sharp increase in fluorescence—up to 30-fold of the low-pressure value—takes place in the region 5–8 kbars. This increase is due to release of the flavin from the complex and is assigned to pressure denaturation of the protein. The midpoint, $p_{1/2}$, of this transition was found at 6.5 kbars and the change in volume, ΔV, in the reaction (native-to-denatured) was calculated to be −74 ml/mol. Addition of up to 30% methanol results in a progressive decrease both in ΔV and $p_{1/2}$, in agreement with the concept that hydrophobic bonding stabilizes the native structure.

The reversible pressure denaturation of proteins was first demonstrated by Brandts et al. (1970) who reported changes in the absorption of the aromatic residues of ribonuclease A subjected to pressures up to 3.4 kbars. Hawley (1971, 1975) and Zipp and Kauzmann (1973) have made similar observations on chymotrypsinogen and metmyoglogin, respectively. In all cases, the pressure-induced reversible denaturation of the protein was followed by ultraviolet or visible absorption spectrophotometry. In general fluorimetric techniques offer much higher sensitivities than absorption measurements. The lower protein concentration required for fluorescence spectroscopy permits working under practically ideal thermodynamic conditions that minimize any anomalous effects due to aggregation of protein molecules. Absorption measurements are restricted to the determination of band position and absorbancy. Fluorescence measurements can include observation of fluorescence lifetimes and polarization, which together with band position and emission intensity can provide wider information than the absorption observations. In this paper we restrict ourselves to observations of intensity and spectral distributions of the emission by the protein itself and by a specific fluorophore, flavin mononucleotide (FMN),[1] which is in binding equilibrium with the specific protein, the riboflavin binding protein (RBP) from hen's egg white. By these means we are able to characterize the changes in equilibrium between ligand and protein as pressure is raised, as well as demonstrate the phenomenon of pressure denaturation, and calculate the characteristic denaturation pressure and the change in volume in this reaction.

Materials and Methods

Riboflavin binding protein from Leghorn egg white was prepared according to Becvar (1973). Physical homogeneity of the protein was ascertained by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The dialyzed protein was stored in polycarbonate bottles and frozen at −15 °C until use. Concentrations of protein solutions were determined from the absorption coefficient of 4.9×10^4 M^{-1} cm^{-1} at 281 nm (Becvar, 1973). Flavin mononucleotide from Sigma Chemical Co. was purified on DEAE-cellulose columns according to Massey and Swoboda (1963). The FMN concentration was determined from the absorbance of 375 nm assuming an absorption coefficient of 10.4×10^3 M^{-1} cm^{-1}. L-Tryptophan was purchased from Nutritional Biochemicals. *N*-Bromosuccinimide (NBS) was obtained from the Fisher Scientific Co. and recrystallized from water before use. Methanol was spectro-quality from Matheson Coleman and Bell. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer was prepared from Trizma base (Sigma). Other chemicals used were reagent quality and were used without further purification.

Choice of Buffer. The pH of buffer solutions changes under pressure. Tris-HCl has been shown to be almost pressure insensitive (Neuman et al., 1973). The pH of a Tris-HCl buffer decreases by less than 0.2 unit over a pressure range of 6.3 ki-

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[1] Abbreviations used are: FMN, flavin mononucleotide; RBP, riboflavin binding protein; NBS, *N*-bromosuccinimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; AMP, adenosine 5′-monophosphate.