Some Properties of a Cytochrome *c*–Mixed Mitochondrial Phospholipid Complex†

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ABSTRACT: A complex of cytochrome *c* with mixed mitochondrial phospholipids has been investigated as a model for cytochrome *c in vivo*. The stability and exchange properties of the complex were investigated using [^{14}C]monocarboxymethylmethionine cytochrome *c*. The complex was intact between pH 5 and 8, but exchange of cytochrome *c* with the complex was observed at pH 5 and 6. Reduction of the complex markedly diminished or eliminated the exchange, implying that lipid binding strength is dependent on the oxidation state of the protein. The complex exhibited greater rates of reduction and oxidation in the absence of added reductant or oxidant than did cytochrome *c*, but the enhanced rates largely resulted from elevated levels of superoxide radical or peroxide arising from ultrasonication of the complex. In the NADH–cytochrome *c* reductase assay the complex was equivalent to cytochrome *c* as a substrate, while in the cytochrome oxidase assay it was apparently a slightly better substrate than cytochrome *c*. Chemically modified cytochromes *c* retaining net positive charge at pH 5 complexed phospholipid, while modified cytochromes *c* with a net negative charge did not. The retention of a positive charge on the modified cytochrome *c* is a requisite for activity in the cytochrome oxidase assay. The enhanced susceptibility of the ferricytochrome *c*–phospholipid complex to denaturation by urea indicates that complexing to phospholipid weakens the heme crevice of ferricytochrome *c*. Studies of carbon monoxide binding to the ferrocytochrome *c* complex indicate that reduction of the complex overcomes this weakening.

Knowledge of the environment of cytochrome *c in vivo* is relevant to understanding the structure–function relationships of this protein and ultimately the mechanism of mitochondrial electron transfer. Numerous proposals that cytochrome *c* is complexed to phospholipid *in vivo* (*e.g.*, Ambe and Crane, 1959; Machinist *et al.*, 1962; Green and Fleischer, 1963) prompted us to investigate a complex of cytochrome *c* and mixed mitochondrial phospholipids as a model for endogenous cytochrome *c*. In previous papers we have reported on the preparation, characterization and some properties of the complex (Ivanetich *et al.*, 1973; Kaminsky *et al.*, 1973). We report here on our continued studies of the physical properties of the complex and our investigations into the functional properties and stability of the complex.

Experimental Section

Materials

Horse heart cytochrome *c* (Grade I, 95% pure, 0.425% Fe) purchased from Miles Seravac, Maidenhead, England, was oxidized with potassium ferricyanide and reduced with either sodium ascorbate or sodium dithionite and the oxidant or reductant was removed by chromatography on a Sephadex G-25 column (0.9 × 18 cm). Cytochrome oxidase, isolated from fresh beef heart by the method of Yonetani (1967), was solubilized in a 0.1% solution of Tween-80 and assayed by the method of Minnaert (1961). Mitochondria were prepared from fresh beef heart by differential ultracentrifugation using the method of Marinetti *et al.* (1958). Bovine erythrocuprein (superoxide dismutase) was obtained from Miles Laboratories Inc. NADH–cytochrome *c* reductase was prepared from beef heart mitochondria by the method of Hatefi and Rieske (1967). Sodium deoxycholate was Merck bacteriology grade. Total beef heart mitochondrial phospholipid and its complex with cytochrome *c* were prepared and purified as previously described (Ivanetich *et al.*, 1973). The reduced complex was prepared in the same manner as the oxidized but with purified ferrocytochrome *c*. The complex was routinely dispersed in water or buffer by ultrasonication or solubilized in 0.025 M sodium deoxycholate. Cytochrome *c* was monocarboxymethylated at methionine-65 by the method of Stellwagen (1968) with a reaction time of over 80 hr. Bromoacetic acid was obtained from British Drug Houses and [1-^{14}C]bromoacetic acid (55 Ci/mol) from the Radiochemical Centre, Amersham, England. Dicarboxymethylmethionine cytochrome *c* was prepared by the method of Schejter and Aviram (1970). Water was distilled and deionized.

Methods

The stability and cytochrome *c* exchange of the cytochrome *c*–phospholipid complex were investigated using [^{14}C]CM_1 cytochrome *c*.[1] The ultrasonically dispersed complex (5.2–6.4 μM) was incubated with the same concentration of [^{14}C]-CM_1 cytochrome *c* (5.2–6.4 μM) under varying conditions of pH, temperature, ionic strength, oxidation state, urea, specific ions, and time. The complex was removed from the incubation medium by centrifugation at 2000*g*. The supernatant was decanted, filtered through a 0.45 μ Millipore filter and its spectrum was scanned from 490 to 570 nm. The complex was washed twice with the incubation buffer. In experiments incorporating high concentrations of urea, centrifugation at 15,000*g* was required to sediment the complex. The

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[1] Abbreviations used are: CM_1 cytochrome *c*, monocarboxymethylmethionine cytochrome *c*; TMPD, tetramethyl-*p*-phenylenediamine.

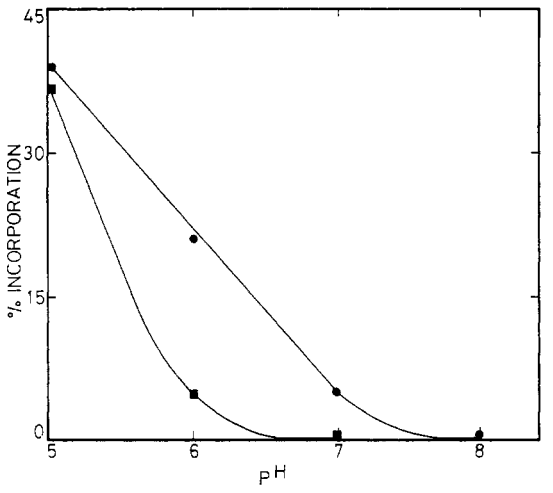

FIGURE 1: The exchange of [^{14}C]CM_1 ferricytochrome *c* with (●) the ferricytochrome *c*–phospholipid complex and (■) the CM_1 ferricytochrome *c*–phospholipid complex as a function of pH; room temperature, complex and cytochrome *c* (5.2–6.4 μM).

pellet was then solubilized and decolorized (Makin and Lofberg, 1966) and the radioactivity was determined in a Nuclear Enterprises 8310 automatic $\beta\gamma$ spectrometer. Appropriate control experiments were performed in all cases. Our criterion for exchange was the incorporation of ^{14}C into the complex, together with an unchanged supernatant CM_1 cytochrome *c* + cytochrome *c* concentration. An increase in the supernatant CM_1 cytochrome *c* + cytochrome *c* concentration was assumed to indicate disruption of the complex.

The reduction of cytochrome *c* and its phospholipid complex by mitochondrial NADH–cytochrome *c* reductase was monitored spectrally at 550 nm at 38° by the method of Hatefi and Rieske (1967) without added phospholipid. In some experiments, 0.15 M KCl was present. The reaction was initiated by the addition of 10 μl of enzyme suspension (8.1 mg of protein/ml). Zero-order rates were calculated from the slope of the linear portion of the tracing. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

Oxidation of sodium deoxycholate solutions of ferrocytochrome *c* or its phospholipid complex was monitored spectrophotometrically at 550 nm. For these studies zero time was taken at formation of the complex and at the beginning of chromatography (removal of reducing agent) in the case of ferrocytochrome *c*. Pseudo-first-order rate constants were calculated from plots of log $(A_t - A_\infty)$ *vs.* time. In some experiments where reduction of the samples in the absence of added reducing agent was evident, 100 μl of erythrocuprein (10 mg/ml) was added to 2.5 ml of the reaction solution. Erythrocuprein (superoxide dismutase) was assayed by the method of Keele *et al.* (1971).

Assays of cytochrome *c* or ultrasonically dispersed complex as substrates for purified cytochrome oxidase and mitochondrial cytochrome oxidase were performed with a Yellow Springs Instrument Clark type oxygen electrode and a W and W 1100 strip chart recorder. Assay solutions contained the following concentrations of reagents in a 2.9-ml final volume: 0.7×10^{-5} to 1.4×10^{-5} M cytochrome *c* or ultrasonicated complex, 6.0×10^{-3} M sodium phosphate, 3.6×10^{-3} M potassium citrate, 6.0×10^{-5} M EDTA, 6.2×10^{-4} M TMPD, and 4.6×10^{-3} M sodium ascorbate. Assays were performed at pH 5.2, 6.2, and 7.1. The concentrations of cytochrome *c* and complex were identical in each experiment. In all experiments, the reaction was initiated by the addition of 0.1 ml of mitochondrial suspension or of approximately 10^{-5} M cytochrome oxidase. TMPD was present to facilitate reduction of the ferricytochrome *c* or its complex (Sun and Crane, 1969). Zero-order rates were calculated for the reaction before and after the addition of oxidase or mitochondria. These rates were corrected for oxygen consumption in the absence of cytochrome *c* or complex.

Electrophoresis experiments were performed using a Pleuger power supply type CVC-D operated at 400 V in 0.017 M sodium phosphate–0.004 M sodium deoxycholate (pH 7.1). The cytochrome *c* was located visually and with 0.2% Amido Black in 2% acetic acid. Phospholipid was stained with the Molybdenum Blue reagent (Skipski and Barclay, 1969).

The lysine residues of cytochrome *c* were modified by guanidination (Hettinger and Harbury, 1964), trifluoroacetylation (Fanger and Harbury, 1965), trinitrophenylation (Wada and Okunuki, 1969), and acetylation by a modification of the method of Wada and Okunuki (1968). Acetic anhydride (10 μl; twofold excess over lysine residues) was added to ferricytochrome *c* (25 mg) in 2.0 ml of half-saturated sodium acetate at 0°. The reaction mixture was stirred vigorously, and the pH was maintained at 7.2 with 2 M sodium hydroxide. The reaction was complete within 3 min, and protein was then desalted on a Sephadex G-25 column (0.9 × 18 cm) and subsequently chromatographed on a DEAE-cellulose column (2.5 × 10 cm) with stepwise elution by 0.02, 0.05, and 0.20 M sodium phosphate (pH 7.4).

The lysine content of the modified cytochromes *c* was determined by amino acid analysis on a Beckman Model 120C amino acid analyzer after hydrolysis of the protein in redistilled constant-boiling hydrochloric acid at 110° for 24 hr. In the case of acid-labile lysine derivatives, the protein was treated with 2,4-dinitro-1-fluorobenzene prior to hydrolysis (Fanger and Harbury, 1965). Ninhydrin assays (Habeeb, 1966) were performed on the acetylated protein and agreed closely with the amino acid analyses.

The extinction coefficients of the 695-nm absorbance band of the ferricytochrome *c* derivatives were determined in 0.05 M sodium phosphate buffer (pH 7.0). Solutions were filtered through 0.45 μ Millipore filters prior to the spectral studies. The concentrations of cytochrome *c* and its derivatives were determined using the absorbance of the pyridine hemochrome [E^{1cm}_{550nm} 2.91×10^4 (Norton, 1958)].

The influence of varying concentrations of urea on the conformation of cytochrome *c* and its phospholipid complex was monitored by determining the induced Soret band absorbance changes. These changes are reported as $\Delta\epsilon/\epsilon_0$ where $\Delta\epsilon$ is the difference in the extinction coefficients of the Soret band before and after the addition of urea and ϵ_0 is the extinction coefficient of the free or complexed cytochrome *c* in the absence of urea.

The interaction of carbon monoxide with the reduced complex in the presence or absence of 8 M urea was investigated using an anaerobic cuvet fitted with a serum cap. The complex was reduced with an excess of dithionite and introduced into the anaerobic buffer through the serum cap. The spectrum of the reduced complex was determined, oxygen-free carbon monoxide was bubbled through the solution for 5 min, and the spectrum of the carbon monoxide complex was recorded at intervals. All spectral studies were performed with a Unicam SP1800 spectrophotometer and SP 1805 program controller.

Results

The stability of the cytochrome *c*–phospholipid complex was investigated using ^{14}C-labeled cytochrome *c*. The exchange of [^{14}C]CM_1 cytochrome *c* with the ultrasonically dispersed complex of cytochrome *c* and phospholipid (eq 1) was markedly affected by pH (Figure 1). At pH 5 and 6 considerable exchange occurred, while at pH 7 and 8 little or no exchange was observed, even when the incubation temperature was raised to 37°. No net disruption of the ultrasonically dispersed complex was noted over this pH range, but the complex was almost completely disrupted above pH 9. When the complex was constituted with unlabeled CM_1 cytochrome *c*, exchange with [^{14}C]CM_1 cytochrome *c* (eq 2) was diminished relative to exchange with the cytochrome *c* complex (eq 1). All subsequent experiments involved exchange of [^{14}C]CM_1 cytochrome *c* with complexed unmodified cytochrome *c*.

ferricytochrome *c*–phospholipid + [^{14}C]CM_1 ferricytochrome $c \rightleftharpoons$ [^{14}C]CM_1 ferricytochrome *c*–phospholipid + ferricytochrome *c* (1)

CM_1 ferricytochrome *c*–phospholipid + [^{14}C]CM_1 ferricytochrome $c \rightleftharpoons$ [^{14}C]CM_1 ferricytochrome *c*–phospholipid + CM_1 ferricytochrome *c* (2)

ferrocytochrome *c*–phospholipid + [^{14}C]CM_1 ferrocytochrome $c \rightleftharpoons$ [^{14}C]CM_1 ferrocytochrome *c*–phospholipid + ferrocytochrome *c* (3)

At pH 5 exchange reached approximately 50% of its final value after 2-min incubation and its equilibrium value after about 30 min. Incubation of the complex for 3 hr at pH 5 resulted in incorporation of activity into the complex in excess of the maximum for complete exchange and a decrease in the cytochrome *c* + CM_1 cytochrome *c* concentration in the supernatant to below its initial level. Apparently after long incubation periods additional cytochrome *c* is bound to the complex.

The addition of 0.15 M KCl to the incubation medium at pH 7.4 resulted in total disruption of the complex, while low concentrations of KCl (0.04 M) produced neither disruption of the complex nor exchange. The presence of 0.04 M $MgCl_2$ or 0.04 M $CaCl_2$ in the incubation medium completely disrupted the complex.

Exchange at pH 5 was decreased by approximately 45% in the presence of either sodium ascorbate or sodium dithionite (eq 3). At pH 6 these reducing agents completely prevented exchange.

Appreciable reduction of the ultrasonically dispersed complex occurred at 38° at pH 8.3 in the absence of added reducing agent. The rate of reduction was diminished if the complex was ultrasonicated under nitrogen or if superoxide dismutase was present. The sodium deoxycholate solubilized complex was also reduced in the absence of added reducing agent at room temperature, although at a much slower rate than the ultrasonicated complex. This reduction was eliminated by the addition of superoxide dismutase (Figure 2). The slight increase in the absorbance in the lower curve results from an increase in turbidity.

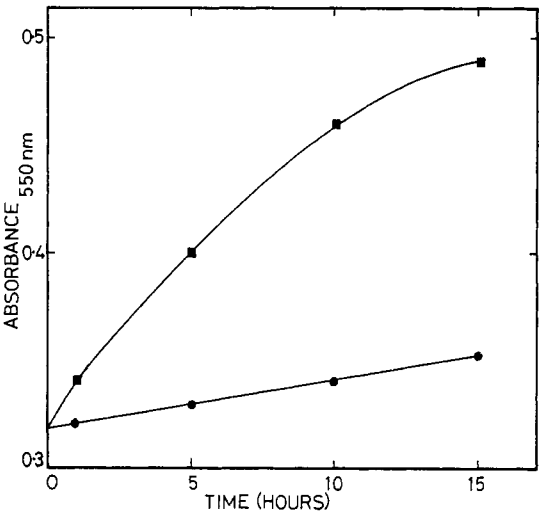

FIGURE 2: Effect of superoxide dismutase on the rate of reduction of cytochrome *c*–phospholipid complex solubilized in 0.025 M sodium deoxycholate (pH 8.3) in the absence of added reductant with (●) and without (■) superoxide dismutase, complex 30 μM, room temperature.

The rate of reduction of cytochrome *c* by NADH–cytochrome *c* reductase is unaffected by the presence of 0.15 M KCl. In the case of the complex, the rate of reduction is

TABLE I: Reduction of Cytochrome *c* and Its Phospholipid Complex with NADH–Cytochrome *c* Reductase.

Substrate	Rate of Reduction ($\Delta A_{550\ nm}$/sec)
3.7×10^{-5} M cytochrome *c*	$2.9 \times 10^{-3} \pm 0.06 \times 10^{-3}$
4.8×10^{-5} M cytochrome *c*	$3.05 \times 10^{-3} \pm 0.15 \times 10^{-3}$
4.8×10^{-5} M cytochrome *c* + 0.15 M KCl	$3.02 \times 10^{-3} \pm 0.14 \times 10^{-3}$
3.7×10^{-5} M complex	$3.37 \times 10^{-3} \pm 0.11 \times 10^{-3}$
3.7×10^{-5} M complex + 0.15 M KCl	$3.78 \times 10^{-3} \pm 0.08 \times 10^{-3}$

TABLE II: Oxidation of Ferrocytochrome *c* and the Ferrocytochrome *c*–Phospholipid Complex.[a]

Substrate	pH	Rate of Non-enzymic Oxidation[b,c]	Rate of Enzymic Oxidation[c,d]	
			Purified Oxidase[e]	Mitochondrial Oxidase[f]
Cytochrome *c*	5.2	3	125	22
Complex[g]	5.2	7	52	4
Cytochrome *c*	6.2	3	104	61
Complex[h]	6.2	18	136	16
Cytochrome *c*	7.1	1	60	52
Complex[h]	7.1	13	74	24
Cytochrome *c*–0.15 M KCl	7.1	3	50	
Complex–0.15 M KCl	7.1	15	33	

[a] Oxygen consumption assayed with an oxygen electrode. [b] Corrected for background rate in absence of cytochrome *c* or complex. [c] Rate relative to the rate of nonenzymic oxidation of cytochrome *c* at pH 7. [d] Corrected for nonenzymic oxidation. [e] Corrected for variations in oxidase concentration. [f] Concentration of endogenous cytochrome oxidase unknown. [g] Complex aggregates. [h] Complex ultrasonically dispersed.

TABLE III: Oxidation of Ferrocytochrome *c* and Solubilized Ferrocytochrome *c*–Phospholipid Complex in the Absence of Added Oxidizing Agent.

Compound	Solvent	Reductant	% of Reaction by Slow Path	Oxidation Rate Constant (min^{-1})	Oxidation Rate Rel to Cytochrome *c*
Cytochrome *c*	0.025 M NaCl, pH 8.3	Ascorbate or dithionite	96 ± 3	$3.6 \times 10^{-4} \pm 0.1 \times 10^{-4}$	
Cytochrome *c*	0.025 M SDC,[a] pH 8.3	Ascorbate or dithionite	96 ± 3	$4.5 \times 10^{-4} \pm 0.2 \times 10^{-4}$	1
Cytochrome *c*	0.5 mM EDTA	Fe^{2+}	83[b]	1.2×10^{-4} [b]	
Cytochrome *c*	0.05 M sodium phosphate, pH 7.5	Fe^{2-}	74[b]	4.6×10^{-4} [b]	
Complex	0.025 M SDC[a]	Ascorbate	52 ± 7	$1.3 \times 10^{-2} \pm 0.05 \times 10^{-2}$	29
Complex	0.025 M SDC[a]	Dithionite	52 ± 7	$6.0 \times 10^{-3} \pm 0.6 \times 10^{-3}$	13

[a] Abbreviation used is: SDC, sodium deoxycholate. [b] Calculated from Figures 1 and 4 of Taborsky (1972).

slightly greater than that of cytochrome *c* but slightly less than that of the KCl-disrupted complex (Table I). The results of studies of the oxidation of ferrocytochrome *c* and ultrasonicated or solubilized ferrocytochrome *c*–phospholipid complex in the absence of added oxidizing agent are presented in Tables II and III. The oxidation of the ultrasonically dispersed complex in the absence of adding oxidizing agent was monitored with an oxygen electrode. Between pH 5 and 7, the ultrasonicated complex was oxidized at a 2- to 13-fold faster rate than cytochrome *c* (Table II). Oxidation of ferrocytochrome *c* and of sodium deoxycholate solubilized ferrocytochrome *c*–phospholipid complex in the absence of added oxidizing agents was studied spectrophotometrically at pH 8.3 at room temperature (Table III). Treatment of the kinetic data with zero-, first-, or second-order rate equations yielded nonlinear plots. In the case of the first-order treatment, the nonlinear plot was biphasic and could be resolved into two linear portions (Figure 3). We have interpreted these plots in terms of a fast and a slow reaction. For the cytochrome *c*–phospholipid complex solubilized in deoxycholate the proportion of the oxidation reaction proceeding *via* the slow pathway is about half. The rates calculated for the slow reaction varied with the type of reductant initially used to reduce the cytochrome *c* (Table III).

For cytochrome *c* in solutions of sodium deoxycholate the portion of the oxidation reaction proceeding *via* the slow pathway exceeded 90% and the rates of oxidation were independent of the type of reductant initially used. Comparison of the rate constants for the slow phase of the oxidation reaction indicates that the deoxycholate solubilized ferrocytochrome *c* complex is 13- to 30-fold more oxidizable than ferrocytochrome *c* in deoxycholate (Table III). Low-voltage electrophoresis experiments indicate that the complex is intact when solubilized in 0.004 M sodium deoxycholate–0.017 M phosphate (pH 7.1).

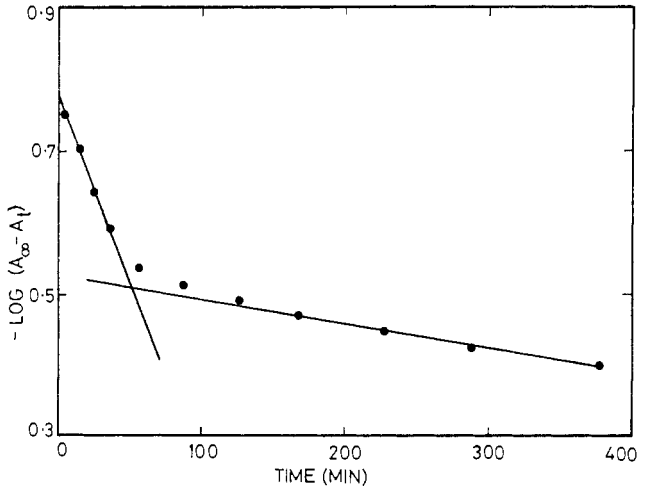

FIGURE 3: First-order plot of the nonenzymic oxidation of sodium deoxycholate solubilized ferrocytochrome *c*–phospholipid complex. Complex 28 μM, 0.025 M sodium deoxycholate, pH 8.3, room temperature. Absorbance changes measured at 550 nm.

The results of attempts to compare cytochrome *c* and its dispersed phospholipid complex as substrates for cytochrome oxidase are shown in Table II. The rates of enzymic oxidation have been corrected for rates of oxidation observed in the absence of enzyme. At pH 5 where the complex is known to aggregate (Sun and Crane, 1969), it is less active with isolated cytochrome oxidase than is cytochrome *c*. At pH 6 and 7, however, the dispersed complex exhibits greater activity as a substrate for the oxidase than does cytochrome *c*. The addition of 0.15 M KCl to the reaction mixture at pH 7 slightly decreases the activity of cytochrome *c* but markedly decreases that of the complex. When mitochondria were used in the assay as the source of cytochrome oxidase the complex was oxidized one-half to one-fifth as rapidly as cytochrome *c* (Table II).

The results of attempts to correlate phospholipid binding properties of chemically modified cytochromes *c* with their activities in the cytochrome oxidase assay are shown in Table IV. The extinction coefficients at 695 nm of the modified preparations are included in the table and are used to indicate the degree of integrity of the heme crevice region of the protein (Schejter and George, 1964; Sreenathan and Taylor, 1971). Unmodified, guanidinated, mono- and dicarboxymethylated, trinitrophenylated, and trisuccinylated cytochromes *c* all form complexes with mixed mitochondrial phospholipids. Of this group, only the modified proteins having a native conformation in the heme crevice region are active with cytochrome oxidase. Where modification of the lysine residues of the protein resulted in the introduction of a net negative charge onto the protein, no complex was formed. Amino acid analyses of the modified cytochromes *c* are presented in Table V.

A comparative study of the influence of urea on the conformations of cytochrome *c* and the cytochrome *c*–phospholipid complex was made. The influence of varying concentrations of urea on the Soret absorbance band of free and complexed ferricytochrome *c* is shown in Figure 4. The Soret absorbance band of cytochrome *c* undergoes minor changes in 6 M urea but markedly greater changes from 6 to 8 M urea.

TABLE IV: Properties of Chemically Modified Cytochrome *c*.

Cytochrome *c* Deriv	Residues Modified	Complex Formation with Phospholipid[a]	Act. with Cytochrome Oxidase[b] (%)	$\epsilon_{695\ nm}$ (%)
Unmodified	0	Yes	100[c]	100
Carboxymethylated	1 Met	Yes	100	100
Carboxymethylated	2 Met	Yes	0[d]	0
Trinitrophenylated	5 Lys	Yes	3	5
Guanidinated	18 Lys	Yes	≥100	>100
Succinylated	3 Lys	Yes	40	100
Succinylated	16 Lys	No	0[d]	100
Trifluoroacetylated	18 Lys	No	0[e]	100
Acetylated	18 Lys	No	0[d]	<30

[a] Based on observation of precipitation of complex. [b] Spectrophotometric assay, pH 5.8, 0.1 M phosphate buffer. [c] Activity of unmodified cytochrome *c* assigned as 100%. [d] Autoxidizable. [e] Fanger and Harbury (1965).

In contrast the Soret band of the complex attains its maximum change by approximately 3 M urea. The complex, dispersed in concentrations up to 11.5 μM, was shown by exchange studies to be stable in 8 M urea at pH 7 for over 2 hr.

The interaction of carbon monoxide with ferrocytochrome *c* and the ferrocytochrome *c* complex was investigated in the presence and absence of urea. Carbon monoxide did not affect the Soret band of the ferrocytochrome *c* complex in aqueous buffer at pH 7. In 8 M urea under anaerobic conditions, however, the extinction coefficient of the Soret band of the ferrocytochrome *c* complex was appreciably increased by the addition of carbon monoxide. This increase occurred slowly over a period of 2 hr (Figure 5).

TABLE V: Amino Acid Analyses of Cytochrome *c* and Derivatives.[a]

Compound[b]	Amino Acid Analyses[c] Lys	Dnp-Lys	Har	No. of Residues Modified[d]
Cyt *c*	18.8			0.0
Cyt *c* + N_2phF	0.8	17.3		0.0
Acetylated cyt *c* + N_2phF	18.3	0.0		18.3
Trifluoroacetylated cyt *c* + N_2phF	18.0	0.0		18.0
Guanidinated cyt *c*	0.2		19.0	19.0
Succinylated cyt *c* + N_2phF	2.6	15.3		2.6
Succinylated cyt *c* + N_2phF	15.8	1.1		15.8
Trinitrophenylated cyt *c*	14.4			4.6[e]

[a] Each result represents the average of at least two analyses. [b] Abbreviations used are: cyt *c*, cytochrome *c*; Dnp, dinitrophenyl; N_2phF, 1-fluoro-2,4-dinitrobenzene. [c] Based on arginine = 2.0. [d] Prior to N_2phF treatment. [e] Spectral assay (Wada and Okunuki, 1969).

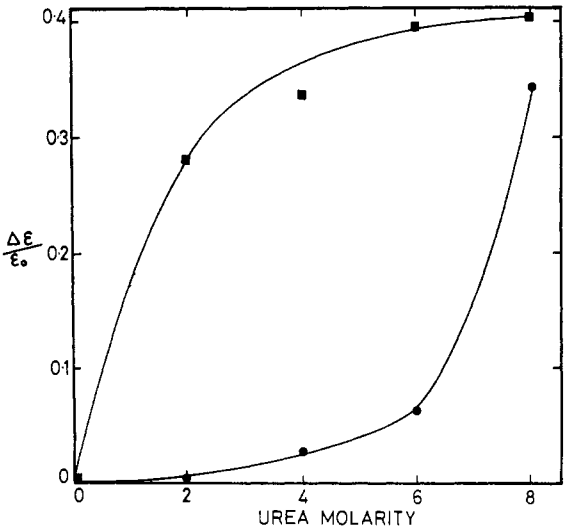

FIGURE 4: The influence of urea on the extinction coefficients of the Soret absorbance band of (●) cytochrome *c* and (■) cytochrome *c*–phospholipid complex; pH 7.0, cytochrome *c* and complex 5 μM, room temperature.

Discussion

Our studies involving the exchange of [^{14}C]CM$_1$ cytochrome *c* with the phospholipid-complexed cytochrome *c* indicate that the ultrasonically dispersed complex is stable between pH 5 and 8, although exchange occurs at pH 5 and 6 in the presence of free cytochrome *c* (eq 1). The complete disruption of the complex by 0.15 M KCl, low concentrations of divalent cations or high pH supports previous proposals that ionic interactions are essential in maintaining the lipoprotein complex (Das *et al.*, 1962; Das and Crane, 1964). The stability of the complex in 8 M urea suggests that hydrophobic or hydrogen bonds have little or no involvement in the binding of mixed mitochondrial phospholipid to cytochrome *c* in the complex.

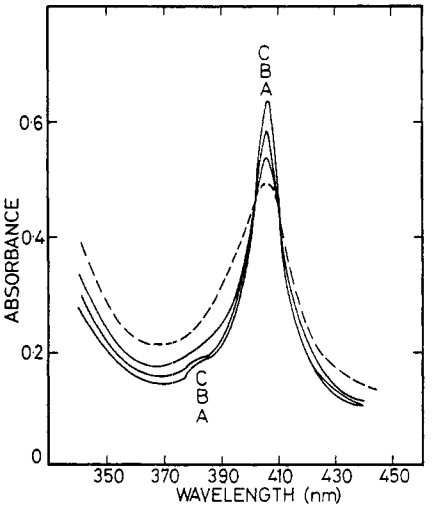

FIGURE 5: Absorption spectra of dispersed cytochrome *c*–phospholipid complex (- - -) and cytochrome *c*–phospholipid complex with carbon monoxide (—); A, B, C, scanned at 0, 15, and 30 min after addition of carbon monoxide. Solutions in 8 M urea–0.1 M phosphate buffer (pH 7.0).

We have previously demonstrated that ionic salts and other reagents used to extract cytochrome *c* from the mitochondria also disrupt the cytochrome *c*–phospholipid complex *in vitro* (Ivanetich *et al.*, 1973). The facile disruption of the complex by ionic salts may lead one to question the possibility of the existence of a cytochrome *c*–phospholipid complex *in vivo*. It has been proposed, however, that the ionic strength of the outer surface of the inner mitochondrial membrane, the presumed location of cytochrome *c in vivo*, is low enough to permit integrity of a cytochrome *c*–phospholipid complex *in vivo* (Vanderkooi *et al.*, 1973b).

The differences in exchange of [^{14}C]CM_1 cytochrome *c* observed with complex constituted with CM_1 cytochrome *c* (eq 2) instead of cytochrome *c* (eq 1) may reflect slight alterations in the conformation of cytochrome *c* following carboxymethylation at methionine-65. Slight conformational changes have, in fact, been reported following monocarboxymethylation of cytochrome *c* (Stellwagen, 1968). Alternatively, the differential in exchange (eq 2 *vs.* 1) may indicate that one or more sites in the region of methionine-65, which is located on the surface of the protein (Takano *et al.*, 1971; Dickerson *et al.*, 1971), are involved in binding to phospholipid. This proposal is consistent with the observation that a spin label covalently bound to methionine-65 of horse heart cytochrome *c* is immobilized on binding of the protein to phospholipid or to cytochrome *c* depleted mitochondria (Azzi *et al.*, 1972; Vanderkooi *et al.*, 1973b).

The very marked decrease in exchange of the complexed cytochrome *c* on reduction of the heme iron (eq 3 *vs.* eq 1) indicates that ferrocytochrome *c* binds more firmly to mixed mitochondrial phospholipid than does ferricytochrome *c*. A differential in the strength of binding to lipid by ferri- and ferrocytochrome *c* might be anticipated in view of the marked conformational changes accompanying a change in the oxidation state of the protein (Dickerson *et al.*, 1971; Takano *et al.*, 1971; Margoliash and Schejter, 1966).

The enhanced binding strength of cytochrome *c* to mixed mitochondrial phospholipid on reduction is in contrast to the reports of Vanderkooi *et al.* (1973a,b) where oxidized cytochrome *c* was reported to bind more firmly to mixtures of cardiolipin and egg-yolk phosphatidylcholine than does ferrocytochrome *c*. This conclusion was based on studies of fluorescence quenching and midpoint potentials. Ferricytochrome *c* was similarly shown to bind more firmly to mitochondria than ferrocytochrome *c* (Vanderkooi *et al.*, 1973a). This differential binding to mitochondria may reflect gross structural changes in the mitochondrial membrane depending on the redox state of the components of the electron-transfer chain rather than alterations in the affinity of cytochrome *c* for a given site.

A difference in affinity of ferri- and ferrocytochrome *c* for phospholipid may be an integral part of the mechanism of action of cytochrome *c* in the mitochondrion and/or of the mechanism of transfer of newly synthesized cytochrome *c* from the endoplasmic reticulum to the mitochondria *in vivo* (Kadenbach, 1968).

Cytochrome *c* was reduced in the absence of added reducing agent when complexed to phospholipids and/or when solubilized by sodium deoxycholate. The inhibition of the observed nonenzymic reduction of the ultrasonically dispersed complex by superoxide dismutase (McCord and Fridovich, 1969) or by carrying out the ultrasonication under nitrogen indicates that the reduction is mediated by the superoxide radical. Superoxide radical can arise from ultrasonication (Lippitt *et al.*, 1972) and is capable of reducing ferricytochrome *c* (McCord and Fridovich, 1968, 1969). The absence of reduction of an aqueous solution of cytochrome *c* after ultrasonication under our experimental conditions implies that the phospholipid plays a leading role in the generation of superoxide radicals in the ultrasonicated complex. The deoxycholate can apparently also induce the formation of superoxide radical since the reduction of sodium deoxycholate solutions of cytochrome *c* or its phospholipid complex in the absence of added reducing agent is inhibited by superoxide dismutase (Figure 2). The reduction of cytochrome *c* in the absence of added reducing agent has been reported (Boeri, 1955; Paléus, 1952; Brady and Flatmark, 1971; Margoliash, 1954), but has been shown to result from reduction of ferricytochrome *c* by the glycine buffer in some instances (Aviram, 1972).

Determination of the activity of the ferricytochrome *c*–phospholipid complex in the NADH–cytochrome *c* reductase assay revealed that the complex and cytochrome *c* are essentially equivalent as substrates for this enzyme (Table I). The slight differences in reactivity observed in the order: KCl-disrupted complex > complex > cytochrome *c* = cytochrome *c* + KCl, probably result from activation of the reductase by lipid (Hatefi and Rieske, 1967).

The oxidation of ultrasonically dispersed ferrocytochrome *c*–phospholipid complex in the absence of added oxidant is enhanced 2- to 13-fold relative to aqueous solutions of cytochrome *c* (Table II). This enhanced rate of oxidation probably arises from lipid peroxides generated by ultrasonication of the phospholipid in the presence of oxygen. The generation of peroxide by ultrasonication of lipid has been observed (Klein, 1970; Hauser, 1971).

Ultrasonication does not appear to be essential for the generation of peroxides in the complex since the deoxycholate-solubilized complex (which has not undergone ultrasonication at any time) also exhibits an enhanced rate of oxidation relative to cytochrome *c*. Since deoxycholate does not markedly enhance the oxidation of ferrocytochrome *c* in the absence of lipid, the major source of oxidation in the deoxycholate-solubilized complex is probably, therefore, also lipid peroxide.

The observation that the type of reducing agent initially used to reduce the ferricytochrome *c* before the protein was incorporated into the ferrocytochrome *c*–phospholipid complex influences the rate of oxidation, implies that at least one of the reductants has an effect on cytochrome *c* over and above reduction of the heme iron. Biphasic oxidation reactions (Figure 3) have been reported for the autoxidation reaction of cytochrome *c* which had been previously reduced with ferrous ions (Taborsky, 1972). Treatment of the data of Taborsky with the first-order rate equation yields biphasic plots resolvable into two linear portions.

To compare the activities of cytochrome *c* and its phospholipid complex as substrates for cytochrome oxidase, one must recognize that phospholipids alone can enhance the activity of both membranous and lipid-depleted cytochrome oxidase (*e.g.*, Wharton and Griffiths, 1962; Cohen and Wainio, 1963). From our studies with membranous cytochrome oxidase it is apparent that aggregation of the complex at pH 5 renders it a poorer substrate than cytochrome *c* under the same conditions (Table II). Aggregation does not, however, completely destroy the activity of the complex at this pH as reported by Sun and Crane (1969). The observed enhanced activity of the complex at pH 6 and 7, as compared to cytochrome *c* may possibly indicate that the complex is a slightly better substrate for the oxidase under these conditions. It is possible that some lipid molecules are dissociating from the complex and binding to (and activating) the enzyme. The experiments in 0.15 M KCl

(Table II) appear to mitigate against lipid activation of the oxidase as an explanation for the enhanced activity of the complex. The marked decrease in the activity of the KCl-disrupted complex in the cytochrome oxidase assay is difficult to explain but may reflect a slight change in the effective ionic strength due to the release of lipid. Ionic strength is known to have a marked effect on the activity of cytochrome oxidase (Davies *et al.*, 1964).

In oxidation experiments performed with intact mitochondria, ferrocytochrome *c* was two- to sixfold more effective than the ferrocytochrome *c* complex as a substrate for endogenous cytochrome oxidase (Table II). The diminished effectiveness of the complex here is probably due to its limited ability to penetrate the outer mitochondrial membrane.

The ability of chemically modified cytochromes *c* to complex phospholipid (Table IV) depends on the net charge on the protein at pH 5, the pH at which the complex is prepared. The modified cytochromes *c* retaining a net positive charge at pH 5 form complexes with phospholipid. The modified cytochromes *c* with a net negative charge at this pH neither complexed to phospholipid nor exhibited activity in the cytochrome oxidase assay, even if the native conformation about the heme crevice was retained. The modified cytochromes *c* with a net negative charge may be inactive in the oxidase assay as a consequence of their lack of phospholipid-binding capacity. This interpretation is consistent with the proposal that phospholipids directly mediate the binding of cytochrome *c* to the oxidase (Tzagoloff and MacLennan, 1965). Alternatively, the lack of activity of the fully acetylated, succinylated, and trifluoroacetylated cytochromes *c* may result from a destruction of binding sites for the oxidase enzyme (Takemori *et al.*, 1962; Okunuki, 1966).

It has previously been shown that complexing of cytochrome *c* with phospholipid stabilizes the protein against denaturation by hydrophobic solvents (Ulmer, 1965; Kaminsky *et al.*, 1972b). The results presented in Figure 4 demonstrate that the heme crevice region of phospholipid complexed ferricytochrome *c* is destabilized relative to ferricytochrome *c* against denaturation by urea. These results suggest that phospholipids weaken the heme crevice of ferricytochrome *c* in the complex. The failure of the ferrocytochrome *c* complex to react with carbon monoxide indicates that reduction of the complex overcomes the lipid induced weakening of the crevice of ferricytochrome *c*. In the presence of 8 M urea, carbon monoxide interacts to a similar extent with ferrocytochrome *c* and the ferrocytochrome *c* complex (Figure 5) (Kaminsky *et al.*, 1972a). This observation suggests that there is no marked influence of the lipids on the conformation of ferrocytochrome *c* under these conditions.

In conclusion, it is apparent that apart from desolubilizing the protein, weakening the heme crevice of the oxidized protein, and the peroxide and superoxide radical induced enhancement of oxidation and reduction, the complexing of cytochrome *c* to phospholipid does not produce any major changes in its properties. If cytochrome *c* is complexed to phospholipids *in vivo*, then in the light of the minor alterations which occur on complexing of cytochrome *c*, the large number of studies of cytochrome *c* in aqueous solution are pertinent to its behavior in the mitochondrion.

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Peptide Antibiotic–Nucleotide Interactions. Nuclear Magnetic Resonance Investigations of Complex Formation between Actinomycin D and Deoxyguanosine 5′-Monophosphate in Aqueous Solution†

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ABSTRACT: The structure of the 1 : 2 complex of actinomycin D with deoxyguanosine 5′-monophosphate (d-pG) has been investigated by nuclear magnetic resonance (nmr) spectroscopy in aqueous solution. The ^{1}H, ^{13}C, and ^{31}P chemical shifts of d-pG were monitored on addition of actinomycin D and the complexation shifts analyzed in terms of ring current effects of the phenoxazone ring of actinomycin D. The ^{13}C nmr data suggest a range of stacking geometries for the purine ring of d-pG relative to the phenoxazone ring for actinomycin D in solution. A comparison of the proton chemical shifts, temperature coefficients, and line widths of the exchangeable protons of d-pG, actinomycin D, and the 1 : 2 actinomycin D–d-pG complex at superconducting fields suggests that the NH_2 proton(s) of d-pG participate in intermolecular hydrogen bonds with acceptor group(s) on the actinomycin D in the complex in solution.

The peptide antibiotic actinomycin D consists of a phenoxazone ring system to which are attached two cyclic pentapeptide lactones. Actinomycin D binds reversibly to double-helical DNA (for a review, see Reich and Goldberg, 1964) and inhibits RNA synthesis. Actinomycin D exhibits a specificity for guanosine residues (Reich and Goldberg, 1964) and its attachment to guanosine-rich regions in the DNA sequence has been attributed to base stacking between the phenoxazone and the nucleic acid bases (Sobell *et al.*, 1971; Muller and Crothers, 1968; Waring, 1970), hydrogen bonding by the amino guanosine proton with an acceptor on the actinomycin D molecule (Sobell *et al.*, 1971; Hamilton *et al.*, 1963) and hydrophobic interactions between groups on the peptide and the nucleotide (Sobell *et al.*, 1971).

The recent X-ray investigation of the 1 : 2 complex between actinomycin D and deoxyguanosine (d-G) defines the conformation in the crystal and the interactions responsible for the stability of the complex (Sobell *et al.*, 1971; Jain and Sobell, 1972; Sobell and Jain, 1972). The cyclic pentapeptide lactones are on either side of the phenoxazone ring and exhibit twofold symmetry in the crystal. The lactone rings are related to each other by two intramolecular hydrogen bonds between the D-Val N proton of one ring and the D-Val carbonyl of the other ring. The d-G ring stacks on either side of the phenoxazone ring and the complex is further stabilized by two strong intermolecular hydrogen bonds between the guanine amino group and the carbonyl of L-Thr, two weak intermolecular hydrogen bonds between the N-3 ring proton of guanine and the N proton of L-Thr, and hydrophobic interactions between the sugar ring of d-G and the side chain of L-MeVal residue.

The structure of the antibiotic has also been approached by the application of conformational calculations (DeSantis *et al.*, 1972) which take into acount the available nuclear magnetic resonance (nmr) and infrared (ir) experimental data in nonaqueous solution (Victor *et al.*, 1969; Arison and Hoogsteen, 1970).

Actinomycin D dimerizes in aqueous solution through vertical stacking of phenoxazone rings (inverted relative to each other) with a dimerization equilibrium constant of 1.4×10^{3} M^{-1} at 18° (Angerman *et al.*, 1972; Krugh and Neely, 1973).

Complex formation between actinomycin D and deoxyguanosine 5′-monophosphate (d-pG) has been investigated by proton nmr spectroscopy in D_2O solution (Arison and Hoogsteen, 1970; Danyluk and Victor, 1970; Krugh and Neely, 1973). Upfield shifts observed for proton resonances of the phenoxazone groups in the complex relative to those in the antibiotic were interpreted to support the intercalation model.

Experimental Section

d-pG was purchased from Collaborative Research, Inc., Waltham, Mass., and was passed through a Chelex column prior to use. Actinomycin D was purchased from Merck.

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