The side-chain conformations listed in Table III (in context with the basic assumption that the remainder of the protein is identical with horse-heart cytochrome *c*) may be considered to be predicitons of the tertiary structures of these eight cytochromes *c*. It will be interesting to compare the calculated side-chain conformations for tuna cytochrome *c* with the actual conformations of these residues when the X-ray structure becomes available. The results obtained in this work indicate that 17 of the 18 substitutions found in tuna cytochrome *c* can be incorporated readily into the refined structure of horse-heart cytochrome *c*, and this provides encouragement for future attempts to apply the information gained from the more accurately determined X-ray structure of tuna cytochrome *c* to the task of further refining the structure of horse-heart cytochrome *c* by conformational energy calculations.

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Cobalt–Cytochrome *c*. I. Preparation, Properties, and Enzymic Activity†

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ABSTRACT: An improved procedure for the preparation of cobalt-cytochrome *c* has been developed. Various factors influencing the cobalt insertion process are discussed. The optical spectra of cobalt-cytochrome *c* suggest a six-coordinated species. The spectral shifts occurring with oxidation-reduction are compared with those observed for deoxycobaltohemoglobin and ferrocytochrome *c* and attributed to the effect of d_{z^2} electron on stereoelectronic interactions between the axial ligands and the porphyrin π systems. Cobalt-cytochrome *c* has $E_{m,7} = -140 \pm 20$ mV as compared to an $E_{m,7}$ of +250 mV for ferrocytochrome *c*. An explanation for this negative $E_{m,7}$ is offered. Cobaltocytochrome *c* is oxidized by cytochrome oxidase at about 45% of the rate for native cytochrome *c*. On the other hand cobalticytochrome *c* was not reduced by microsomal NADH or NADPH cytochrome *c* reductase nor by mitochondrial NADH or succinate cytochrome *c* reductase. It appears that the integrity of the reductase binding site is destroyed and the oxidase binding site has been modified by cobalt substitution.

The functional properties of a metalloenzyme are a direct manifestation of its primary sequence, protein conformation, the metal ion, and the prosthetic group. Even though X-ray diffraction can provide a detailed image of an enzyme molecule, it is less useful in answering questions concerning metal ion specificities. For instance, why does nature select Fe for hemoglobin but Co for vitamin B_{12} or Zn to activate carboxypeptidase but Mg to activate enolase? Recently, there is a growing interest in the study of metal substituted enzymes to understand structure-function relationships. A case in point is that of cobalthemoglobin, an allosteric analog of hemoglobin. Comparisons of the properties of these two molecules (Hoffman and Petering, 1970;

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Chien and Dickinson, 1972; Dickinson and Chien, 1973a,b; Woodruff et al., 1974; Yonetani et al., 1974) showed that it is the motion of the proximal histidine, but not that of the metal ion, with respect to the heme plane which constitutes the necessary and sufficient condition for the triggering of the allosteric transition.

There are several physicochemical reasons to study cobalt-cytochrome *c*. Because of the additional 3d electron in Co, ^{Co}cyt-c^+ is diamagnetic and ^{Co}cyt-*c* is paramagnetic whereas ^{Fe}cyt-c^+ is paramagnetic and ^{Fe}cyt-*c* is diamagnetic.[1] Comparison of the EPR spectra of the paramagnetic molecules and ^{1}H NMR spectra of all these species could reveal structural perturbations due to metal ion substitution. ^{Co}cyt-*c* and ^{Fe}cyt-c^+ should have easily distinguishable EPR spectra and it should be possible to follow electron transfers between them with EPR. Such a study of electron transfers between Fe and Co hemoglobins has already been completed (Dickinson and Chien, 1975b). Finally, the $3d_{z^2}$ electron in Co(II) tends to repel axial ligands and its effects on coordination chemistry and redox behavior would be of interest.

There are also biochemical reasons for investigating cobalt-cytochrome *c*. Several electron-transfer mechanisms involving cyt-*c* in the respiratory chain are still being debated, such as through the heme edge of the prosthetic group (McArdle et al., 1974, and references therein), the protein aromatic channel (Takano et al., 1973), or the Tyr-67 within the crevice (Grimes et al., 1974). Study of ^{Co}cyt-*c* could yield useful information about the electron-transfer pathway. Also, EPR is capable of differentiating Co, Cu, and Fe signals and can be used to determine electronic interactions between these metal ions in a ^{Co}cyt-*c*–cytochrome oxidase complex.

In this paper we present the results on the preparation and purification of ^{Co}cyt-*c*, its electrophoretic mobility, redox potential, optical spectra, and enzymic assays. A brief account has been given previously (Dickinson and Chien, 1974). The EPR and ^{1}H NMR of cobalt cytochrome *c* are described in part II (Dickinson and Chien, 1975a); autoxidation, ligand binding, reduction, and electron-transfer properties of ^{Co}cyt-*c* will be reported elsewhere.

Experimental Section

Materials. Pure cobalt metal powder was obtained from Alfa/Ventron (50 mesh, stock No. 00089, Lot 082373, <10 ppm of copper). Horse heart ^{Fe}cyt-c^+ was purchased from Sigma Chemical Co. (Type VI, 99–99.5% pure). Hydrogen fluoride was purchased from Matheson. NADH, NADPH, sodium cholate, and Tween 80 were from Sigma Chemical Co; sodium succinate was from Mallinckrodt Chemical Co.

Methyl viologen, phenazine ethosulfate, toluylene blue, 2,6-dichlorophenol indophenol, and 2-hydroxy-1,4-naphthoquinone were supplied by K&K Laboratories. The other mediators, 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene), and phenazine methosulfate were obtained from Aldrich Chemicals, and thionine and Methylene Blue were from Allied Chemicals. Pyocyanine perchlorate was a gift from Dr. D. F. Wilson.

Cytochrome oxidase was partially purified from bovine heart mitochondria by procedure A of Tzagoloff and MacLennan (1965). It was diluted 20-fold to an activity of 4.0 μM min^{-1} with 0.29 μg ml^{-1} of ^{Fe}cyt-*c*. Bovine heart mitochondria was prepared according to the method of Green et al. (1957). Rat liver microsomes were prepared according to the method of Amar-Costesec et al. (1974). NADH cytochrome *c* reductase (pig heart crude) was obtained from Sigma Chemical Co. with an activity of 1.3 μM min^{-1} with 0.24 μg ml^{-1} of ^{Fe}cyt-c^+. Partially purified rat liver microsomal NADPH-cytochrome *c* reductase is a gift from Dr. A. Y. H. Lu prepared by the procedure of Lu et al. (1974). The solution containing 15.5 mg of protein ml^{-1} had an activity of 60.9 μmol of ^{Fe}cyt-c^+ reduced min^{-1} ml^{-1} and a specific activity of 3.9 μmol of ^{Fe}cyt-c^+ reduced min^{-1} mg^{-1}.

All buffers were prepared with reagent grade chemicals and distilled water deionized with a Milli-Q ion-exchange system.

Preparation of $Co(OAc)_2$. All glassware was prerinsed in warm HOAc to remove copper contamination from local distilled water. Excess HOAc was removed by flushing with prepurified argon with mild heating of the flask. Cobaltous acetate was prepared by refluxing 1 g of Ultra Pure cobalt metal powder in 50 ml of glacial acetic acid. The purple product was redissolved in a minimum of boiling distilled-deionized water. A very small amount of unreacted metal powder was removed by filtration. Finally, the water was evaporated to give $Co(OAc)_2$.

Preparation of p-cyt-c. Porphyrin cytochrome *c* was prepared by the method of Robinson and Kamen (1968). An all Kel-F-vacuum line was constructed for this synthesis. ^{Fe}cyt-c^+ (100 mg) was freed of moisture by evacuation at 30 Torr for 1 hr at room temperature. With the Kel-F vessel containing this ^{Fe}cyt-c^+ kept at −196°, about 2 ml of HF made anhydrous by CoF_3 which fluorinates the trace H_2O present was transferred into the vessel. The temperature was then raised with a salt–ice bath and HF was pumped off immediately after it had come into contact with the protein. Pumping was continued until the protein pulled away from the vessel.

The p-cyt-*c* was then dissolved in 0.02 *M* pH 8.0 Naphos and passed through a Sephadex G-25 column (30 × 2 cm preequilibrated with the same buffer). The desired product moved rapidly as the major purple band. A side reaction product forms a slow moving minor band faintly purple in color. p-cyt-*c* is not very stable in solution. Upon standing at 4°, the color of the p-cyt-*c* in pH 8.0 1 *M* Naphos buffer gradually changed over several days from a strikingly rich magenta to a duller, browner purple color. Spectroscopically, there are changes in both the Soret band (blue shifted from 404 to 400 nm with an increase of bandwidth) and the visible bands (appreciable broadening). Freshly prepared p-cyt-*c* is always used on the same day for cobalt insertion experiments.

Cobalt insertion. Turner (1971) has used a dialysis method to insert Cu into p-cyt-*c* with good yields. Similar procedures with a variety of anions failed to effect any Co incorporation. Flatmark and Robinson (1968) reported the insertion of Fe into p-cyt-*c* in glacial HOAc with a low yield of native product. Several variations of their procedure afforded a product which is oxygenated and does not have the native-folded structure as judged by its electrophoretic mobili-

[1] Abbreviations used in this paper are: ^{Co}cyt-*c*, cobaltocytochrome *c*; ^{Co}cyt-c^+, cobalticytochrome *c*; ^{Fe}cyt-*c*, ferrocytochrome *c*; ^{Fe}cyt-c^+, ferricytochrome *c*; p-cyt-*c*, porphyrin cytochrome *c*; ^{Co}Hb, deoxycobaltohemoglobin; ^{Co}Hb$^+$, cobaltihemoglobin; ^{Fe}Hb, deoxyhemoglobin; ^{Fe}Hb$^+$, methemoglobin; TPP, mesotetraphenylporphyrin; ^{1}H NMR, proton magnetic resonance; EPR, electron paramagnetic resonance; $E_{m,7}$, midpoint reduction potential at pH 7; Naphos, sodium phosphate buffer.

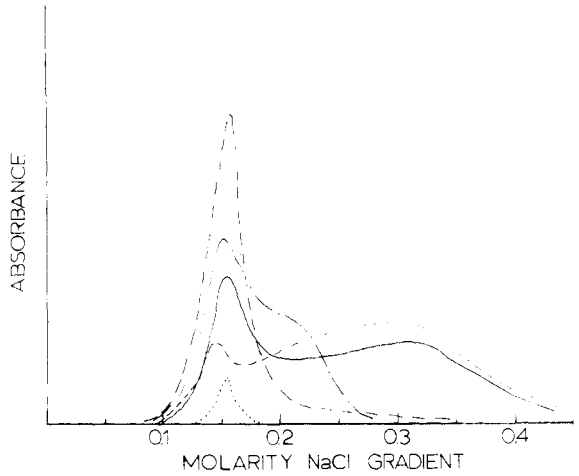

FIGURE 1: Elution pattern of various preparations of cobalt-cytochrome *c* on Amberlite CG-50 under 0–0.5 *M* NaCl gradient in 0.02 *M* pH 8.0 Naphos buffer at 4°. Reactions were run as described in the text under the following conditions: (- - -) 15% HOAc, 0.015 *M* $Co(OAc)_2$; (—) 15% HOAc, 0.03 *M* NaH_2PO_4, 0.01 *M* $Co(OAc)_2$; (- · -) 0.1 *M* NaCl, 0.05 *M* $Co(OAc)_2$; (- - -) 0.5 *M* NaCl, 0.05 *M* $Co(OAc)_2$, 0.07 *M* NaH_2PO_4, 5% HOAc; (· · ·) 0.5 *M* NaCl, 0.05 *M* $Co(OAc)_2$.

ty and EPR spectrum. Reactions under less acidic environment (15% HOAc, pH 2.15; 4 m*M* p-cyt-*c*, 40 m*M* $Co(OAc)_2$) at 65° for 20 min gave a homogeneous product, free of p-cyt-*c* but with electrophoretic mobility 70% of the native protein.

Phosphate ion was next used to help stabilize p-cyt-*c* in the native conformation. A reaction similar to the preceding one was carried out in the presence of 30 m*M* NaH_2PO_4. The product is applied onto a 1 × 3 cm column of Amberlite CG-50 (Mallinckrodt Chemicals) preequilibrated with pH 8 0.02 *M* Naphos and washed according to the procedure given by Margoliash and Walasek (1967). ^{Co}cyt-*c* was absorbed into the column and eluted by 200 ml of a linear gradient of 0–0.5 *M* NaCl in 0.02 *M* pH 8.0 Naphos buffer. The elution rate was typically 7.5 ml/hr with 2.5 ml per fraction. NaCl concentration was determined by refractive index. Two fractions were thus separated. The schematic gradient elution is shown in Figure 1. The component which was eluted (15% of total) is designated ^{Co}cyt-*c*(A); this is the same elution concentration as ^{Fe}cyt-c^+ within an experimental error of ±0.01 *M*. This protein has electrophoretic mobility identical with the native cytochrome *c*. A second major component (85% of total), designated as ^{Co}cyt-*c*(B), eluted at 0.16 *M* NaCl and has 70% of the electrophoretic mobility of the native cytochrome *c*.

Unsatisfactory though this above procedure is, it is apparently suitable for iron insertion. Anaerobic reconstitution using ferrous sulfate as above except at 80° for 15 min gave a 70% yield of ^{Fe}cyt-*c*. The unfractionated product has electrophoretic mobility which was identical with native ^{Fe}cyt-c^+. Furthermore, its reaction rate with NADH cytochrome *c* reductase is 50% of the native ^{Fe}cyt-c^+.

Over 50 variations in reaction conditions were performed to arrive at the following best compromise recipe to give the highest yield of ^{Co}cyt-*c*(A) (Figure 1). In a 25-ml flask 290 mg of NaCl (5 mmol), 70 mg of $NaH_2PO_4 \cdot H_2O$ (0.5 mmol), 125 mg of $Co(OAc)_2 \cdot 4H_2O$ (0.5 mmol), 0.5 ml of glacial HOAc (8.5 mmol), and 4.5 ml of distilled deionized water were combined. Stirring and crushing are required to get all the salts into solution. To this is added 5 ml of 15 mg/ml of p-cyt-*c* (6.5 μmol) in 0.02 *M* pH 8.0 Naphos buffer. The flask is loosely stoppered and suspended in a stirred water bath at 71 ± 1°. The reaction is generally complete after 15 min but with variation of bath temperature this reaction time may vary. The progress of the insertion reaction was monitored by injecting 5 μl of the reaction mixture into 1.0 ml of 0.1 *M* pH 7 buffer and observing the disappearance of the p-cyt-*c* Soret band at 404 nm. A small amount of precipitate generally forms within the first 2 min of heating. After completion the reaction mixture is filtered onto a 30 × 2 cm Sephadex G-25 column equilibrated with pH 8.0 0.02 *M* Naphos buffer, followed by fractionation on Amberlite CG-50. The yield of A fraction is typically 50% of the starting material; there are only small amounts of fraction B formed (Figure 1). The product is oxidized ^{Co}cyt-c^+ judged by ultraviolet (uv)–visible spectra and absence of EPR signal.

Preparation of Reduced ^{Co}cyt-c. Because of the rapid rate of autoxidation of ^{Co}cyt-*c* (approximately 0.5 μ*M*/min at room temperature in air-saturated 0.1 *M* pH 7 buffer) a special procedure was developed to prepare fully reduced ^{Co}cyt-*c* which does not contain excess reducing agent. One milliliter of approximately 3% ^{Co}cyt-c^+ was deoxygenated and treated with a tenfold excess of sodium dithionite (4.5 mg) and immediately transferred to a small dialysis bag which was placed in 250 ml of scrubbed argon sparged buffer in a 250-ml crown top bottle. After 3 hr of rapid stirring at 4°, the buffer was pumped out via pressure and needles and new sparged buffer was pumped in. The dialysis bag contents were then transferred to a 30-ml crown top bottle in a glove bag. The capped bottle was then flushed with scrubbed argon. Solutions thus stored had a half-life in the reduced state of about a day.

Redox Potential. A stainless steel cell equipped with quartz windows (1.84 cm effective path length) was constructed to measure redox potential. It is fitted with a 1-cm^2 platinum flag electrode and a saturated calomel electrode with a KCl-agar salt bridge. Voltages were measured with either a Leeds and Northrup potentiometer or a Corning Model 10 pH millivolt meter. Absorbance at 550 nm was followed by a Cary 14 spectrometer. After the cell was flushed with prepurified argon which was further scrubbed with a chromous ion solution, a sample of ^{Co}cyt-*c* containing the appropriate mediators was introduced. Microliter amounts of titrants (0.1 *M* $K_3Fe(CN)_6$ as the oxidant and 0.1 *M* dithionite or 0.08 *M* methyl viologen[2] as the reductants) were added to produce a desired voltage change with drifts of less than 0.5 mV min^{-1}. Generally titrations were begun with fully oxidized material; after complete reduction the oxidative titration was performed. The fraction oxidized was calculated from the absorption spectra in the visible region (usually at 550 nm for ^{Co}cyt-*c* and ^{Fe}cyt-*c*) using the absorbance at the extremes of oxidizing and reducing conditions.

Mediators to enhance exchange current of solute to electrode were introduced to speed equilibration of voltage measurements. Different mediator systems were used to determine $E_{m,7}$ of various samples. They are: $K_3Fe(CN)_6$

[2] Methyl viologen was prepared in the reduced form by placing 1 ml of buffer containing 15 mg of the compound in a small test tube of 1.5 ml volume fitted with a rubber septum. Also contained in the tube is 0.1 g of 5% platinum on asbestos (Matheson Coleman and Bell) held to the bottom by a small wad of glass wool. The tube was flushed with hydrogen and gently shaken until the deep purple color characteristic of a fully reduced methyl viologen solution appeared.

Table I. Extinction Coefficients for Visible and Soret Bands.

	α		β		Soret	
	λ_m (nm)	ϵ_m (mM^{-1})	λ_m (nm)	ϵ_m (mM^{-1})	λ_m (nm)	ϵ_m (mM^{-1})
^{Co}cyt-c^+	567	7.77	530	6.08	426	106.1
^{Co}cyt-c	549	11.6	520	9.1 sh	416.5	128.7
^{Fe}cyt-c^+ [a]	530	11.0			410	106.1
^{Fe}cyt-c [a]	550	29.5	520		416	129.1
^{Co}Hb$^+$	572	9.0	538	9.0	426	99
^{Co}Hb	552	17	523	sh	402	110
^{Fe}Hb$^+$ (acid) [b]	631	4.4	500	10.0	405	179
^{Fe}Hb [b]	555	12.5	523	sh	430	133

[a] Myer (1968). [b] Antonini and Brunori (1971).

(+430), diaminodurene (+420), 2,6-dichlorophenol indophenol (+217), Methylene Blue (+115), phenazine methosulfate (+80), thionine (+62), phenazine ethosulfate (+55), toluylene blue (+5), pyocyanine perchlorate (−34), and 2-hydroxy-1,4-naphthoquinone (−139), where the quantities in parentheses are the $E_{m,7}$ values in millivolts (Clark, 1960).

Electrophoresis was carried out on cellulose acetate strips (Sepraphore III, Gelman) in pH 7.0 0.05 *M* Naphos at about 200 V with 2 mA per strip. In all cases a strip with native ^{Fe}cyt-c^+ was run for comparative mobility purposes.

Uv-Visible Spectra. Uv-visible spectra were obtained on a Cary 14 spectrometer using 1-cm pathlength cuvets. Extinction coefficients were determined by both atomic absorption and by dry weight. The values agree well within experimental errors.

Assay for Enzymic Activities. Deoxygenated buffers were prepared in 250-ml crown-topped bottles fitted with self-sealing neoprene septa (Ace Glass Co.) and crimped metal cap with holes for insertion of a syringe needle. The buffer was either sparged vigorously with chromous ion scrubbed prepurified argon, or deoxygenated by triple cycles of pumping and pressurizing with scrubbed argon for 10 min for each operation. For detergent-solubilized experiments, the buffer was made 0.1% in Tween 80. Solutions of each substrate were prepared by weighing an appropriate amount into a small vial which was then sealed with a septum and rinsed with scrubbed nitrogen gas for 5 min. NADH and NADPH were about 1.0 mg ml^{-1}; sodium succinate was 9 mg ml^{-1}. ^{Fe}cyt-c^+ (Sigma Type VI) (15 m*M* in 0.1 *M* pH 7.0 Naphos) and ^{Co}cyt-*c*(A) (4 m*M*) were deoxygenated and stored in a 30-ml crown-top bottle (vide supra).

The general assay procedure for each enzyme-substrate-cyt-*c* mixture was as follows. A 2.5-ml cylindrical 1-cm path length cuvet containing a small magnetic stirring bar and capped with a tight-fitting rubber septum was rinsed with nitrogen gas for 5 min with needles for inlet and outlet. Into the cuvet was injected 50 μl of ^{Fe}cyt-c^+ or 30 μl of ^{Co}cyt-c^+ solutions. The optical spectrum was checked for stability. With the solution rapidly stirred, 2 to 4 μl of particle suspension was injected and the cuvet immediately placed in the spectrometer and the absorbance at 550 nm was monitored as a function of time. In all cases parallel runs with native cytochrome *c* were made so that relative rates could be determined.

Reductase assay was performed with four different systems. In all cases, the buffer was 0.1 *M* pH 7.5 Naphos and 1 m*M* KCN deoxygenated by sparging 2 hr with scrubbed nitrogen gas. For the detergent-solubilized experiments a buffer suspension of rat liver microsomes was treated with increments of 10% Tween 80 and 0.1 *M* pH 7.0 Naphos until the turbidity of the solution markedly lessened at about 2% Tween 80. Bovine heart mitochondria for the detergent-solubilized experiments was treated similarly with 10% sodium cholate solution. The assay with pig heart NADH cyt-*c* reductase was performed at pH 7.5, in the presence of azide, with sodium cholate solubilization according to the method of Mackler (1967).

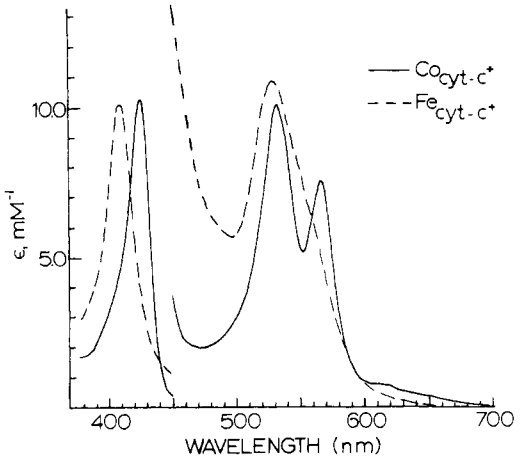

FIGURE 2: Ultraviolet-visible spectrum of ^{Co}cyt-c^+ and ^{Fe}cyt-c^+ in 0.1 *M* pH 7.0 Naphos. ε values apply from 450 to 700 nm; below 450 nm the sample is diluted tenfold.

Oxygen is needed for oxidase assay, yet ^{Co}cyt-*c* was readily autoxidized in air. It was found that when the oxygen content is reduced to 0.22%, ^{Co}cyt-*c* is relatively stable. Therefore, oxidase assays were performed in 1% air-saturated buffer.

Results

Uv-Visible Spectra. The electronic spectra of ^{Co}cyt-c^+(A) are shown in Figure 2. The visible bands are well resolved. The ratio of intensities of the β and α bands varied from 1.22 to 1.33 with the method of preparation. Early preparations all had a Soret band at 422 nm. After the preparative procedure was modified to include phosphate and chloride during Co insertion, the new ^{Co}cyt-*c* samples had a Soret band at 426 nm. The values of extinction coefficients are given in Table I.

Addition of sodium dithionite results in the spectra shown in Figure 3 and Table I. The reduction is fairly slow and pH dependent. The kinetics of chemical reduction will be given elsewhere.

Comparison of these spectra with those of cobalt-hemoglobins (Dickinson and Chien, 1973a) and indeed also any of the cobalt-porphyrin complexes (Falk, 1964) makes it clear that as prepared the cobalt is in the trivalent state and reduction with dithionite yields the Co(II) state.

Though there is appreciable similarity between the spectra of ^{Co}cyt-*c* and ^{Fe}cyt-*c*, there is one important dissimilarity, namely, the characteristic weak absorption band at 695 nm of ^{Fe}cyt-c^+ is absent in the spectra of ^{Co}cyt-c^+. There is a shallow isosbestic at 592 nm upon reduction of ^{Co}cyt-c^+, but definitive detailed search for a well-defined weak peak is hampered by the presence of the 620-nm peak from small residual amounts of p-cyt-*c*.

Half-Reduction Potentials. Because of the much more negative $E_{m,7}$ of ^{Co}cyt-*c* as compared to ^{Fe}cyt-*c*, the following mediator system was used at 30 μM each: diaminodurene, phenazine methosulfate, phenazine ethosulfate, pyrocyanine, $K_3Fe(CN)_6$, and hydroxynaphthoquinone. The first two components are unstable and were renewed by 10 μM addition about every 30 min. Titrations with and without pyocyanine gave the same $E_{m,7}$. The $E_{m,7}$ measured for ^{Co}cyt-*c*(A) was -140 ± 20 mV relative to normal hydrogen electrode with $N = 1.0 \pm 0.1$ (Figure 4). This value was reproduced on several separate preparations of ^{Co}cyt-*c* and with sodium dithionite, methyl viologen, or ferricyanide as titrant. In some cases the oxidizing and reducing titrations are separated by as much as 30 mV in the half-reduced region but averaging gives $E_{m,7} = -140$ mV.

Enzymic Activities. The rate of enzymic oxidation of ^{Co}cyt-*c* by bovine heart cytochrome oxidase (EC 1.9.3.1) was compared with the oxidation rate of ^{Fe}cyt-*c* under identical experimental conditions of pH 7.0, 25°, 0.1 *M* Naphos, 1% air-saturated buffer with 6 μM cytochrome *c* and 1 μl (about 1.3 n*M*) of cytochrome oxidase stock. Figure 5 showed that the reactions for both ^{Fe}cyt-*c* and ^{Co}cyt-*c* follow first-order kinetics with respect to cytochrome *c;* the apparent rate constants are 13.7×10^{-3} and 6.13×10^{-3} sec^{-1}, respectively. Because of the ease of autoxidation of ^{Co}cyt-*c,* blank runs were also made without cytochrome oxidase. The rate of autoxidation of ^{Co}cyt-*c* prior to oxidase injection is only 0.1 μM/min ($k_{1,app} = 2.8 \times 10^{-4}$ sec^{-1}). Thus, oxidation of ^{Co}cyt-*c* by oxidase is about 45% of the rate of the native ^{Fe}cyt-*c;* all the ^{Co}cyt-*c* was oxidized at the end of the reaction.

The data of reductase assay were converted to plots of ln $(A_\infty - A_t/A_\infty)$ vs. time where A_∞ and A_t are the absorbances at 550 nm at completion and at time *t,* respectively. The plots were all strictly linear indicating pseudo-first-order kinetics with respect to cytochrome *c* concentration. The slopes of these plots are directly converted to rate constants k_1 listed in Table II. With the three enzyme systems, the rat liver microsome, bovine heart mitochondria, and purified microsomal NADPH cytochrome *c* reductase, in no case was any reduction of ^{Co}cyt-c^+ observed.[3] Since a reaction rate less than 1% of that measured for ^{Fe}cyt-c^+ would be observable, it is certain that any reduction of ^{Co}cyt-c^+ is much less than 1% of ^{Fe}cyt-c^+.

Discussion of Results

Cobalt Insertion. There are two major considerations in the insertion of cobalt into p-cyt-*c:* the process of insertion itself and the maintenance of the native tertiary structure. A number of studies have dealt with the problem of how metal ions (M) are incorporated into porphyrin molecules (PH_2):

$$M^{2+} + PH_2 \rightleftharpoons MP + 2H^+ \qquad (1)$$

Even though the exact sequence of events is not yet clear, it is obviously necessary for the metal ion to lose much of its solvation sphere or ligands and the porphyrin to release its two central protons. The kinetics of Cu(II) incorporation into the water-soluble 2,4-disulfonated deuteroporphyrin dimethyl ester in acidic and neutral solutions followed the rate law (Weaver and Hambright, 1969):

$$d[CuP]/dt = kK[PH_2][Cu]/(K + [H^+]) \qquad (2)$$

in accord with the mechanisms:

$$PH_3^+ \rightleftharpoons PH_2 + H^+ \qquad K \qquad (3)$$

$$Cu^{2+} + PH_2 \rightarrow CuP + 2H^+ \qquad k \qquad (4)$$

The rate of metal incorporation into *meso*-tetrapyridylporphyrin in HOAc–H_2O mixture is also first order in metal–porphyrin concentrations and has inverse dependence on $[H^+]$ (Choi and Fleischer, 1963). Our observations on the rate of cobalt insertion into p-cyt-*c* are consistent with the above kinetics.

Choi and Fleischer (1963) and Stein and Plane (1969) have shown that the relative incorporation rates in aqueous solution are in the approximate order for the divalent ions Cu > Zn > Co, Fe, Mn > Mg, Ni and that the rates are very much slower for the corresponding trivalent ions. Our observation that p-cyt-*c* has much higher affinity for the Cu(II) than for the Co(II) ion is in agreement with this order of reactivity. These relative rates roughly parallel the rate of water exchange by aquo ions (Choi and Fleischer, 1963) and point to the importance for the metal ion to shed much of its solvation sphere. It is apparent that for successful preparation of ^{Co}cyt-*c,* the cobalt ion should be kept in its divalent state and that contamination of Cu(II) and Zn(II) ions should be minimized.

However, in order to conserve the native protein tertiary structure, low pH condition should be clearly avoided. Much is known about the effect of pH on the conformations of ^{Fe}cyt-*c* and ^{Fe}cyt-c^+. In the reduced state, the native cytochrome *c* is stable and remains in the native conformation from pH 4 to 12. The molecule becomes unfolded beyond this pH range. The oxidized ^{Fe}cyt-c^+ is known to have five states; the pK_a values for the four transitions are 0.42, 2.50, 9.35, and 12.76. Deferring the discussion of alkaline transitions to part II, we note that below pH 2.50 the molecule is unfolded with an open heme crevice apparently the result of ionization of His-18 which has an abnormally low pK_a of 2.5 induced by its hydrophobic environment (Babul and Stellwagen, 1972; Stellwagen and Shulman, 1973; Cohen et al., 1974). This unfolding is reversible. At pH 0.42, ^{Fe}-cyt-c^+ is completely and irreversibly denatured to a high spin form.

Not knowing whether the conformation of p-cyt-*c* varies with pH like ^{Fe}cyt-*c* or ^{Fe}cyt-c^+, one can be reasonably sure that at pH 0.9 prevailing in the glacial HOAc procedure p-cyt-*c* is denatured. In these preparations, the insertion product has abnormal electrophoretic behavior and oxygen binding ability. In the milder procedure of 15% HOAc, the media has a pH of 2.15. The insertion product is homogeneous but has only 70% of the native electrophoretic mobility. This pH is still too low to keep either the oxidized or reduced cytochrome *c* in the native conformation. It is unlikely that p-cyt-*c* would have the native tertiary structure under these conditions. However, the addition of phosphate did produce 15% of ^{Co}cyt-*c*(A) which has the native electrophoretic mobility. All the iron inserted product at pH 2.15 with $H_2PO_4^-$ present has the native mobility. This illustrates the stabilizing effect of phosphate as well as the destabilizing influence of the Co(II) ion. Cobalt has been shown to bond to the globin moiety of hemoglobin (Schulman et al., 1973).

[3] Previously we reported that unfractionated ^{Co}cyt-c^+ was reduced at about 3% of the rate of ^{Fe}cyt-c^+ by crude pig heart NADH cytochrome *c* reductase. That result is believed to be due to either impurities causing nonenzymic reduction or the reduction of ^{Co}cyt-c^+ in nonnative conformations.

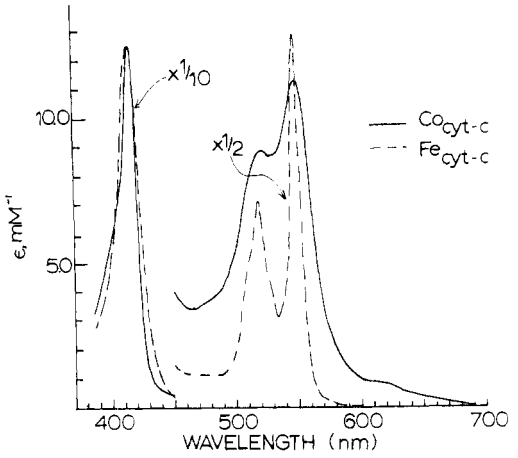

FIGURE 3: Ultraviolet-visible spectrum of ^{Co}cyt-*c* and ^{Fe}cyt-*c* in 0.1 *M* pH 7.0 Naphos. ϵ values on the ordinate apply directly to the 450–700 nm region for ^{Co}cyt-*c* where ^{Fe}cyt-*c* has been diluted twofold for scale convenience. For below 450 nm the ϵ values of the ordinate should be multiplied by ten.

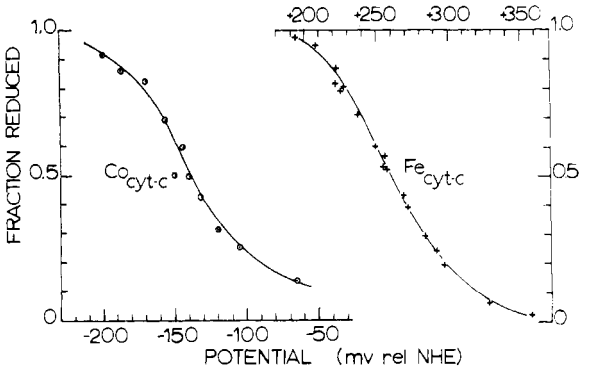

FIGURE 4: Oxidation-reduction titration of ^{Co}cyt-*c* and ^{Fe}cyt-*c* in 0.1 *M* pH 7.0 Naphos with mediators and titrants as described in the text.

With the preferred procedure which gives the highest yield of ^{Co}cyt-*c*(A), the pH of the reaction mixture at 25° is 3.6. At this pH ^{Fe}cyt-c^+ has the native conformation but ^{Fe}cyt-*c* does not. However, with the presence of PO_4^{3-} and Cl^- the native folded structure of p-cyt-*c* may well have been conserved.

Let us now summarize our observations of over 50 experiments performed to develop the best procedure of Co insertion. Such information could be valuable to biochemists who may wish to insert other metal ions into p-cyt-*c* or other enzymes.

The rate of insertion of Co(II) ion into p-cyt-*c* at 71° in 5 min for solutions, where $[Co(II)] = [H_2PO_4^-] = 42$ m*M*, decreases with increasing HOAc concentration. With a minimal amount of HOAc, the rate is about twice as fast as with 10% HOAc. At very low concentrations of HOAc, the cobalt phosphate is not completely solubilized and about two-thirds of the protein precipitates upon heating. An excess of free acetate ion is also detrimental. At 1.0 *M* NaOAc the level of precipitation is high and the elution pattern peaked at 0.13 *M* NaCl with a shoulder at 0.16 *M* NaCl. The rate of insertion can be doubled for each 0.02 *M* increase in $Co(OAc)_2$ concentration in 0.21 *M* NaH_2PO_4 and 5% HOAc. However, this acceleration is accompanied by increases in protein precipitation which reached 50% at 0.04 *M* $Co(OAc)_2$.

Even though phosphate ion apparently stabilizes the native folded conformation of p-cyt-*c*, it tends to retard Co insertion. For instance, with $Co(OAc)_2$ = 5 m*M* and 5% HOAc, the reaction at 70° is 50% complete after 5 min in the presence of 5 m*M* NaH_2PO_4; the same reaction is 75% complete in the absence of phosphate.

It has been suggested by Phillips (1960) that chloride ion enhances the rate of insertion of cobalt into porphyrin rings. This was found to be the case for p-cyt-*c* but has the drawback of a high level of precipitation and the A and B fractions were not resolved sufficiently to give useful amounts of fraction A.

The effect of variation of reaction temperature and reaction time was explored for mixtures containing 10% glacial HOAc, 0.1 *M* $Co(OAc)_2$, and 0.05 *M* NaH_2PO_4. Reacting at 81, 70, and 60° required 5, 15, and 20 min, respectively, for completion. The 81° run gave about the same amount of A fraction but there were two distinct B fractions eluted at 0.24 and 0.32 *M* NaCl. The 61° run showed a marked decrease in A fraction yield and an increase in B fraction.

In summary, it is clear that Co^{2+}, $H_2PO_4^-$, Cl^-, and OAc^- ions all have positive and negative effects on the quantity and/or quality of the product of the insertion reaction. Acetic acid lowers the amount of protein precipitated but it also slows the insertion reaction and enhances the production of the B fraction. NaH_2PO_4 increases the amount of A fraction relative to B fraction but slows the insertion of cobalt. NaCl greatly speeds the reaction rate but does not eliminate generation of significant quantities of B fraction and precipitate.

Optical Spectra. In this section we discuss the optical spectra of cobalt cytochrome *c* and compare them with the corresponding spectra of native cytochrome *c* as well as the cobalt hemoglobin spectra.

All the visible bands of ^{Co}cyt-*c* have λ_{max} values identical with those of ^{Fe}cyt-*c*. This agrees with the assignment of these bands to π–π^* transitions of porphyrin ($a_{1u}(\pi) \rightarrow e_g^*(\pi)$; $a_{2u}(\pi) \rightarrow e_g^*(\pi)$ (Zerner et al., 1966, and references cited therein). Comparison of ^{Co}cyt-*c* and $^{Co}Hb_A$, both low-spin complexes, showed that the Soret band is 780 cm^{-1} more to the red and the α band is 100 cm^{-1} to the blue for the former. These shifts could be attributed to the possible presence of an additional axial ligand in ^{Co}cyt-*c*. Extensive studies on transition metal mesoporphyrin complexes have shown that there is pronounced stereoelectronic interaction between the axial ligands with the π-electron system of the porphyrin to shift the Soret band to longer wavelengths. The visible bands are also affected but the shifts are smaller and can be in either direction energetically (Corwin et al., 1963; Baker et al., 1964; Storm et al., 1966). For similar reasons both the Soret and the α bands of $^{Co}HbO_2$ are respectively red shifted by 1180 and 600 cm^{-1} with reference to ^{Co}Hb.

The above interpretation of optical spectra suggests that ^{Co}cyt-*c* is six-coordinated. There are, however, more definitive magnetic resonance evidences regarding this point described in part II.

The presence of one electron in the d_{z^2} orbital in the reduced cobalt species probably reduces the above-mentioned ligand stereoelectronic interaction. Removal of this d_{z^2} electron should promote greater interaction with the π orbitals and cause bathochromic shifts. This expectation is realized when ^{Co}cyt-*c* is oxidized to ^{Co}cyt-c^+. All the bands are red shifted; the shifts are 620, 580, and 1050 cm^{-1} for Soret, α, and β, respectively. Similar bathochromic shifts of 1400 and 630 cm^{-1} for the Soret and α bands occur when ^{Co}Hb

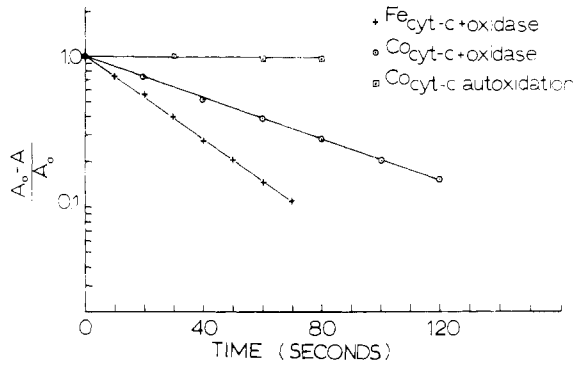

FIGURE 5: Comparative reaction of oxidase with ^{Co}cyt-c and ^{Fe}cyt-c in 0.1 M pH 7.0 Naphos saturated with 1% air. A is the absorbance at 550 nm at any time. A_0 is the absorbance at 550 nm before injection of oxidase: (+) ^{Fe}cyt-c; (○) ^{Co}cyt-c; (▣) ^{Co}cyt-c autoxidation before injection of oxidase.

is oxidized to $^{Co}Hb^+$. X-Ray results (Ibers et al., 1974; Scheidt, 1974) on model compounds also show significant structural influence of the d_{z^2} electron. The Co–axial nitrogen distance is 2.436 Å in ^{Co}TPP(piperidine)$_2$; it decreases by 15% to 2.060 Å in $^{Co}TPP^+$(piperidine)$_2$. It should be noted that a similar analysis would not be valid for hemoglobin because of differences in the π occupancy for the ferrous and ferric complexes. Since these orbitals are directly involved in the metal–porphyrin π bonding, any changes in their occupancy would have overriding effects on the electronic transitions (Zerner et al., 1966).

The 695-nm band in ^{Fe}cyt-c^+ has been considered to be strong evidence for the ligation of Met-80. The absence of a band at similar wavelength in ^{Co}cyt-c^+ and ^{Co}cyt-c was at first disconcerting. However, ^{1}H NMR results given in part II showed Met-80 to be the sixth ligand in both molecules. Therefore, the 695-nm band may or may not be characteristic for the Fe–S bond; it is certainly not characteristic for the Co–S bond.

Half-Reduction Potential. The enthalpy for the reduction of ^{Co}cyt-c^+ is the electron affinity, A, of the molecule:

$$^{Co}\text{cyt-}c^+ + e \rightarrow {}^{Co}\text{cyt-}c \qquad \Delta H^\circ = -A \tag{5}$$

The free-energy change represented by the potential of this reversible one-electron addition is:

$$\Delta G^\circ = G_{red}^\circ - G_{ox}^\circ + \Delta\Delta G_{sol}^\circ \tag{6}$$

where $\Delta\Delta G_{sol}^\circ$ is the difference in free energies of solvation which should be nearly zero. Since there are no significant entropy changes:

$$E_{m,7} = \Delta G^\circ/F = -A/F \tag{7}$$

where F is the Faraday. The electron affinity can be equated with the energy of the lowest vacant molecular orbital which is the $3d_{z^2}$ orbital (Streitwieser, 1967).

With the above theory we can understand why $E_{m,7}$ for ^{Co}cyt-c is −140 mV as compared to $E_{m,7}$ values of +100 mV for ^{Co}Hb. The difference can be attributed to the presence of an additional ligand in ^{Co}cyt-c. Whereas the energies of the $3d_{z^2}$ orbital are not available for cobalt porphyrins, Zerner et al. (1966) had calculated this energy for the 5- and 6-coordinated ferrous porphyrin. The difference between the energies of $a_{1g}(d_{z^2})$ orbitals with one and two H_2O molecules as ligands is 1.0 V with Fe in-plane and 530 mV with Fe out-of-plane.[4] A similar argument can be used to explain why ^{Fe}cyt-c has a much more positive $E_{m,7}$ of +260 mV than ^{Co}cyt-c. Reduction of the native enzyme corresponds to the introduction of an electron into the $e_g(d\pi)$ orbital. This orbital lies much lower than the $a_{1g}(d_{z^2})$ orbital (Zerner et al., 1966) by about 700 mV. Therefore, ^{Co}cyt-c should have much more negative reduction potential than ^{Fe}cyt-c.

Table II: Reduction of Cytochrome c for Various Enzymes and Substrates.

Enzyme System	Detergent	Substrate	k_1 (min^{-1}) ^{Fe}cyt-c^+	k_1 (min^{-1}) ^{Co}cyt-c^+
Rat liver microsome[a]	None	NADPH	0.20	0
	None	NADPH	4.3	0
	Tween 80	NADPH	0.17	0
	Tween 80	NADH	2.8	0
Bovine heart mitochondria	None	NADH	0.68	0
	None	Sodium succinate	0.35	0
	Sodium cholate	NADH	0.10	0
	Sodium cholate	Sodium succinate	2.5	0
Purified NADPH-cytochrome reductase	Triton	NADPH	7.2	0

[a] With antimycin A present to inhibit mitochondrial activity.

Oxidation–Reduction Mechanisms. Enzymic redox reactions are controlled both by thermodynamics and kinetics. The thermodynamics of the processes:

$$A_{ox} + B_{red} \rightleftharpoons A_{red} + B_{ox} \tag{8}$$

is expressed by the Nernst equation:

$$E_m(A) - E_m(B) = (RT/NF) \ln ([B_{ox}][A_{red}]/[B_{red}][A_{ox}]) \tag{9}$$

The oxidation of ^{Co}cyt-c by cytochrome oxidase is thermodynamically favored. The midpoint potentials for cyt-a and cyt-a_3 are +230 and +380 mV, respectively (Wilson et al., 1972). The fact that ^{Co}cyt-c is oxidized by oxidase at 45% of the rate of ^{Fe}cyt-c implies some modification of the oxidase binding site. Alteration of the electron-transfer path is eliminated by the ^{1}H NMR results (part II, Dickinson and Chien, 1975a).

^{Co}cyt-c^+ was not reduced by any of the enzyme systems used in this work. The normal electron donor for cyt-c in the mitochondrial respiratory chain is cyt-c_1. Because it has a value of +215 mV for $E_{m,7}$, cyt-c_1 is incapable of reducing ^{Co}cyt-c^+. The free-energy change is also positive for the reduction of ^{Co}cyt-c^+ by succinate cytochrome c reductase which has two cytochrome b components with $E_{m,7}$ = −25

[4] The difference may not entirely be electronic. Kassner (1972, 1973) had suggested a correlation between reduction potential and the hydrophobicity of the heme environment. Thus, a more hydrophobic environment tends to stabilize the neutral Co(II) state, whereas a more open structure and polar environment favors the Co(III) state with its formal 1+ charge. According to this hypothesis, if ^{Co}cyt-c has a more open crevice than ^{Co}Hb, the perturbing influence would tend to lower $E_{m,7}$.

and +60 mV. Much more reducing enzymes are apparently called for.

Liver microsomal cytochrome reductase readily reduces ^{Fe}cyt-*c*. Okuda et al. (1972) and Lu et al. (1974) showed that the reaction sequence involving NADH-cytochrome *c* reductase (cytochrome b_5, EC 1.6.2.2) is:

$$\text{NADH} \rightarrow f_{p_1}\text{ (flavoprotein)} \rightarrow \text{cyt-}b_5 \rightarrow \text{cyt-}c \qquad (10)$$

The more recently determined half-reduction potential for cyt-b_5 is −140 mV for particulate and +10 mV for purified nonparticulate preparations (Kawai et al., 1963). Even more reducing is NADPH cytochrome P-450 reductase which readily reduces ^{Fe}cyt-c^+ (van der Hoeven and Coon, 1974). The half-reduction potential for cyt-P-450 was reported to be −410 mV (Waterman and Mason, 1970). Dr. D. F. Wilson, in a personal communication, said that cyt-P-450 has actually three components with $E_{m,7}$ values of −410, −340, and −280 mV. Based on the reduction potentials, both microsomal reductase systems should be able to reduce ^{Co}cyt-c^+. Yet, no reduction occurred. The lack of reductase effect is therefore kinetically controlled. It will be shown that the reductase binding site is probably destroyed in ^{Co}cyt-*c* as a result of modification of a number of surface lysyl residues (part II, Dickinson and Chien, 1975a).

In conclusion, we have prepared a metal-substituted derivative of cytochrome *c* which has a slightly modified oxidase binding site but lacks the ability to bind reductase. It would be interesting to compare the X-ray structure of ^{Co}cyt-*c* with ^{Fe}cyt-*c*. Crystallization of ^{Co}cyt-*c* for this purpose is underway in our laboratories.

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Cobalt–Cytochrome *c*. II. Magnetic Resonance Spectra and Conformational Transitions†

L. Charles Dickinson and James C. W. Chien*

ABSTRACT: Between pH ~4 and 10 cobaltocytochrome *c* (^{Co}cyt-*c*) gives an electron paramagnetic resonance (EPR) spectrum with $g_{\parallel}$ = 2.035, $g_{\perp}$ = 2.223, $^{Co}A_{\parallel}$ = 61.4 G, $^{Co}A_{\perp}$ = 49.8 G, $^{N}A_{\parallel}$ = 15.3 G, and $^{N}A_{\perp}$ = 12.5 G. Comparisons with the EPR spectra of deoxycobaltomyoglobin, deoxycobaltohemoglobin, and model compounds and together with other evidence showed cobaltocytochrome *c* to have Met-80 and His-18 as its axial ligands. The protons of these ligands are seen as resonances shifted by the ring-current field of the porphyrin in the 300-MHz ^{1}H nuclear magnetic resonance (NMR) spectra of cobalticytochrome *c* (^{Co}cyt-c^{+}). The methyl and γ-methylene protons of Met-80 in this molecule occupy positions with respect to heme *c* which are somewhat different from those in ferrocytochrome *c*. The ^{1}H NMR spectra also showed that the methyl groups of Leu-32, Ile-75, Thr-63, thioether bridges, and the porphyrin ring in the cobalt protein are in the same state as in native enzyme; the same is also true for Tyr-59, His-26, and His-33 and also possibly Tyr-67, Tyr-74, and Phe-82. Above pH 11, ^{Co}cyt-*c* is converted to a five-coordinated form having $g_{\parallel}$ = 2.026, $g_{\perp}$ = 2.325, $^{Co}A_{\parallel}$ = 80 G, $^{Co}A_{\perp}$ ≈ 10 G, $^{N}A_{\parallel}$ = 17.5 G,, and $^{N}A_{\perp}$ not resolved. Below pH 1.0 the EPR spectrum of ^{Co}cyt-*c* is also five-coordinated with $g_{\parallel}$ = 2.014, $g_{\perp}$ = 2.359, $^{Co}A_{\parallel}$ = 93.8 G, and $^{Co}A_{\perp}$ = 38.8 G. The axial ligands in the alkaline and the acidic forms of ^{Co}cyt-*c* are His-18 and Met-80, respectively. New prominent proton resonance peaks are observed in cobalt–cytochrome *c* which are either absent or weak in native cytochrome *c*. These are situated at 3.0, 1.7, and 1.44 ppm, attributable, respectively, to the ε-CH_2, δ-CH_2 + β-CH_2, and γ-CH_2 of lysyl residues in random-coil peptides. From the areas of these peaks, it is estimated that one–two lysyl residues in ^{Co}cyt-*c* have been modified; four–five lysyl residues in ^{Co}cyt-c^{+} have been modified. These alterations of surface charged groups are probably responsible for the lowered reactivity of ^{Co}cyt-*c* with cytochrome oxidase and the lack of reactivity of ^{Co}cyt-c^{+} with several cytochrome reductase systems.

The preparation of cobalt–cytochrome *c* was first reported by Dickinson and Chien (1974). In the preceding paper (part I, Dickinson and Chien, 1975a) we described an improved method of preparation and gave some of the properties of cobalt–cytochrome *c* and its enzymic activities. The optical spectra and half-reduction potential of ^{Co}cyt-*c*[1] suggest that the Co atom is six-coordinated. On the other hand, the visible spectrum of ^{Co}cyt-c^{+} is devoid of absorption in the vicinity of 695 nm. The 695-nm band is a characteristic spectral feature of ^{Fe}cyt-c^{+} and has been taken by many workers to be ligand specific and to originate in the S–Fe coordination (Schechter and Saludjian, 1967; Sreenathan and Taylor, 1971; Folin et al., 1972; Lambeth et al., 1973). In contrast to this interpretation, Shejter and George (1964) and most recently Castro (1974) argued for a protein conformational origin for the 695-nm band. Because of these discordant views, it behooves us to identify the axial ligands in ^{Co}cyt-*c* by independent means such as magnetic resonance techniques.

Native ^{Fe}cyt-*c* is known to have three pH-dependent conformations in acidic, neutral, and alkaline media designated as species I, II, and III, respectively. Species I and III are said to be partially unfolded; however, their state of coordination of Fe seems to be unknown. A second objective of this study is to determine whether ^{Co}cyt-*c* also exhibits these analogous conformational transitions, and to use EPR to elucidate the state of ligation of its conformational isomers.

Finally, cobalt–cytochrome *c* has somewhat lower reactivity with cytochrome oxidase than the native enzyme and no activity whatsoever with cytochrome *c* reductase (part I). A third objective of this work is to discover the causes for the decreased enzymic activities and to learn more about the mitochondrial electron transfer processes.

Experimental Section

Materials. Sigma Type VI horse heart cytochrome *c* was used in this work. ^{Fe}cyt-*c* was prepared from it by reduction with dithionite and chromatography on a Sephadex G-25 column. The ^{Fe}cyt-*c* samples are pH 8.5; the ^{Fe}cyt-c^{+} samples are pH 6.3. Both are virtually salt free.

Unless stated otherwise, the spectra of cobalt cytochrome

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[1] Abbreviations used are: ^{Co}cyt-*c*, cobaltocytochrome *c*; ^{Co}cyt-c^{+}, cobalticytochrome *c*; ^{Fe}cyt-*c*, ferrocytochrome *c*; ^{Fe}cyt-c^{+}, ferricytochrome *c*; ^{Co}Mb, deoxycobaltomyoglobin; ^{Co}Hb, deoxycobaltohemoglobin; ^{Co}TPP, cobalto-*meso*-tetraphenylporphyrin; ^{Co}PP IX (DME), cobalt protoporphyrin IX dimethyl ester; $E_{m,7}$, half-reduction potential; EPR, electron paramagnetic resonance; ^{1}H NMR, proton magnetic resonance; DSS, sodium 2,2-dimethyl-2-silapentanesulfonic acid; shfs, superhyperfine splitting.