Cobalt Cytochrome C: Enzymic Assays

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Summary: An improved synthesis for cobalt-cytochrome c has been developed; its half reduction potential is -140 ± 20mV. Reduced ^{Co}cyt-c‡ is oxidized by bovine heart cytochrome c oxidase at a rate ~45% that of the native cytochrome c. It is not reduced by mitochondrial NADH or succinate cytochrome c reductase nor by microsomal NADH or NADPH cytochrome c reductase.

Introduction: The preparation of ^{Co}cyt-c by cobalt insertion into p-cyt-c in 15% acetic acid (1) gave low yields of fraction A which has the same electrophoretic mobility of the native species. The majority of the product, fraction B, has abnormal electrophoretic mobility. The unfractionated product was found to be a poor electron acceptor for NADH cytochrome c reductase. After more than fifty modifications of the above procedure, a recipe has been developed which gives high yield of fraction A. The central purpose of this work is to determine the ability of ^{Co}cyt-c(A) to act as a substrate in enzymic redox reactions.

Procedures: P-cyt-c was prepared as described earlier (1). Cobalt cytochrome c was synthesized as follows: 290 mg of NaCl (5m mole), 70 mg of $NaH_2PO_4 \cdot H_2O$ (0.5m mole), 125 mg of $Co(OAc)_2 \cdot 4H_2O$ (0.5 m mole), 0.5 ml of glacial acetic acid (8.5m mole) and 4.5 ml distilled deionized water were combined in a 25 ml flask. To this was added of 1.5 mg ml^{-1} p-cyt-c (6.5 μmole) in 0.02M, pH 8.0 Na phosphate buffer. The reaction was complete in 15 min. at 71°C as judged by the disappearance of the 404 nm band of p-cyt-c. Frac-

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‡Abbreviations: ^{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; Em,7 midpoint reduction potential.

tionation on Amberlite CG-50 gave typically 50% yield of ^{Co}cyt-c(A).

The rate of enzymic oxidation of ^{Co}cyt-c by bovine heart cytochrome oxidase (EC 1.0.3.1) was compared with the oxidation rate of ^{Fe}cyt-c under identical experimental conditions of pH 7.0, 25°, 0.1M Naphos, 1% air saturated buffer with 6μM cytochrome c and about 1.3 nM cytochrome oxidase. The oxidase was partially purified from bovine heart mitochondria by Procedure A of Tzagoloff and MacLennan (2); bovine heart mitochondria were prepared according to the method of Green et.al. (3)

Enzymic reduction of ^{Co}cyt-c^{+} was assayed with several systems. Partially purified rat liver microsomal NADPH-cytochrome c reductase was a gift from Dr. A.Y.H. Lu. In these assays, the substrate and the cytochrome c were dissolved in pH 7.5 0.1M Naphos and 1mM KCN buffer 0.1% in Tween 80 and sparged with prepurified N_2 scrubbed with chromous solution. For the detergent solubilized experiments Tween 80 was added to rat liver microsomes and sodium cholate was added to bovine heart mitochondria until the turbidity markedly lessened at a final concentration of 1 to 2% detergent.

Cytochrome c reductase assays were performed amaerobically. Cytochrome oxidase was assayed in buffers which were a 1% of air saturation so as to minimize the autoxidation of ^{Co}cyt-c. The enzymic reactions were followed by monitoring the absorbance at 550 nm with a Cary 14 spectrometer in 1 an pathlength curvettes at 25°C. The assays were always performed in parallel sets for the native and cobalt cytochrome c.

Results: Figure 1 shows that the reactions of cytochrome oxidase with 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 amount of autoxidation of ^{Co}cyt-c prior to oxidase injection is only 0.1μM/min. ($k_{1\ app}=2.8 \times 10^{-4}$ sec.$^{-1}$). Thus ^{Co}cyt-c is about 45% as effective as the native cytochrome c in the oxidase reaction. All of it was oxidized by cytochrome oxidase.

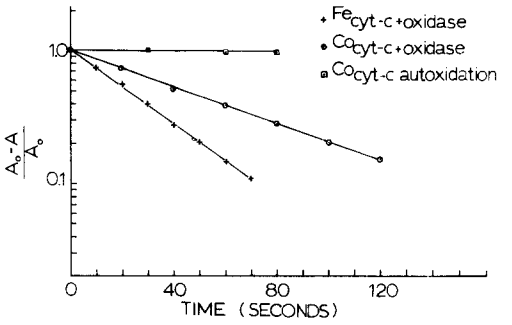

Figure 1

Table I Reduction of cytochrome c

Enzyme system	Detergent	Substrate	$k_1(min.^{-1})$ Fe_{cyt-c^+}	$k_1(min.^{-1})$ Co_{cyt-c^+}
Rat liver microsome*	none	NADPH	0.20	0
"	"	NADH	4.3	0
"	Tween 80	NADPH	0.17	0
"	"	NADH	2.8	0
Bovine heart mitochondria	none	NADH	0.68	0
"	"	Na succinate	0.35	0
"	Na Cholate	NADH	0.10	0
"	"	Na succinate	2.5	0
Purified NADPH-cytochrome c reductase	Triton	NADPH	7.2	0

*With Antimycin A to inhibit mitochondrial activity

The results on the reduction of cytochrome c are summarized in Table I. No reduction of $^{Co}cyt\text{-}c^+$ was detected with any of the enzyme systems which reduce $^{Fe}cyt\text{-}c^+$ rapidly. Any reduction of $^{Co}cyt\text{-}c$ must be $\leq 1\%$ of that measured for $^{Fe}cyt\text{-}c^+$. Previously we reported that unfractionated $^{Co}cyt\text{-}c$ prepared with the more concentrated acetic acid, phosphate-free procedure was reduced by crude pig heart NADH cytochrome c reductase at about 2% of the rate of $^{Fe}cyt\text{-}c$. That result is now believed to be due to either impurities affecting nonenzymic reduction or the reduction of $^{Co}cyt\text{-}c^+$ in non-native conformations.

Discussion: In order for enzymic redox reaction to occur the free energy change must be negative in accordance with the Nernst equation. Furthermore, the rate of the process is controlled by stereochemical factors concerning the enzyme binding sites and electron transfer pathways.

We have determined the half reduction potential of ^{Co}cyt-c to be -140 ± 20mV. The values of $E_{m,7}$ for cyt-a and cyt-a_3 are +230mV and +380mV, respectively (4). Therefore, the oxidation of ^{Co}cyt-c by cytochrome oxidase is thermodynamically favored. The fact that ^{Co}cyt-c has 45% of the oxidase activity of ^{Fe}cyt-c implies also the absence of kinetic barriers.

^{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 +215mV for $E_{m,7}$, cyt-c_1 should be incapable of reducing ^{Co}cyt-c^+. On the other hand the reductions of ^{Co}cyt-c^+ by the other systems are expected to be spontaneous on thermodynamic ground alone. The value of $E_{m,7}$ for particulate preparations of cyt-b_5 is -140mV for microsomal cytochrome b_5 (5); it is -410mV for cyt-P450 (6). Yet there was no reduction of ^{Co}cyt-c^+ with any of these systems. Therefore the redox reactions are prevented by stereochemical effects as a result of cobalt substitution. The preparative procedure is unlikely to be the major cause of structural alteration because ^{Fe}cyt-c^+ similarly prepared by the insertion of Fe(II) into p-cyt-c is reduced by NADH cytochrome c reductase with 50% of the rate of the native ^{Fe}cyt-c^+.

In conclusion, we have shown that cobalt substitution alters somewhat the oxidase binding site but totally destroys the reductase binding site. Comparison of the X-ray structures of ^{Co}cyt-c and ^{Fe}cyt-c and further chemical structural elucidation could lead to clarification of the oxidation and reduction mechanisms of cytochrome c.

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