Cobalt Cytochrome C: Preparation and Characterization

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Summary: Cobalt cytochrome c has been prepared from porphyrin cytochrome c in water/acetic acid solvent. The dominant band in the electrophoresis of the product at pH7 has the same mobility as the native protein. Dithionite changes the ultraviolet/visible spectrum markedly and generates an epr signal with cobalt hyperfine and other superhyperfine features. Nitric oxide removes part of the epr signal. Fractionation on Amberlite CG-50 under NaCl gradient at pH8.0 yields two major components distinguished by their rates of reactivity to dithionite and electrophoretic mobility. Cobalt cytochrome c is reduced by DPNH cytochrome c reductase to produce the same electron paramagnetic resonance signal as that generated by dithionite.

Introduction: The fascinating problem of understanding the range of properties of the heme group in hemoproteins as tuned by the protein environment and vice versa continues to be attacked by a variety of methods. The central role of the metal can be highlighted by changing the metal. In the case of cobalt hemoglobin the metal-replaced protein reversibly binds oxygen, has some of the allosteric properties (1,2), and very nearly the same redox potential (2) as the native protein. Cobaltohemoglobin and cobaltomyoglobin also were amenable to epr in the single crystal form to give structural detail (3,4). In this work the stable electron transfer enzyme, cytochrome c has been prepared in cobalt replaced form and a study of its physical and biochemical properties begun. We hope to contribute to an understanding of the role of the metal in determining the electron transfer mechanism and protein structure.

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Abbreviations: ^{Co}cytc, cobaltocytochrome c; ^{Co}cytc^{+}, cobalticytochrome c; ^{Fe}cytc, ferrocytochrome c; ^{Fe}cytc^{+}, ferricytochrome c; p-cytc, porphyrin-cytochrome c; epr, electron paramagnetic resonance.

Procedures: P-cytc was prepared from horse heart cytc^{+} (Sigma Chemical Co., Type VI) in 100 mg batches using liquid HF as described by previous workers (5) except that a salt/ice bath was used for vacuum removal of the HF from the reaction vessel instead of a 0°C bath. Attempts to add cobalt to porphyrin cytochrome c by the glacial acetic acid method previously used (6) to reinsert iron resulted in low yield of a protein with poor stability and very different electrophoretic properties than native cytc. In contrast to a previous report (5), we find that cobalt ions do not insert into p-cytc in aqueous solution at neutral pH.

^{Co}cytc^{+} was prepared by reacting 100 mg of p-cytc in 15% acetic acid with 20 mg of cobaltous acetate at 70°C for 15 minutes. The reaction is complete as monitored by the Soret band shift from 4040 Å to 4220 Å. After the reaction the mixture was passed through a Sephadex G-10 column. This product shows no epr signal at 77°K.

The same procedure was used to reinsert iron except ferrous sulfate was used at 80°C to compensate for the slower rate of insertion of iron (9). The reaction was carried to 70% completion in 15 minutes.

Cellulose acetate paper electrophoresis in 0.05M pH7.0 Naphos buffer at 2 ma/strip was used to analyze the products. Fractionation of products by ion exchange was accomplished on Amberlite CG-50 prepared in 0.02 pH8.0 Naphos according to the procedure of Margoliash and Walasek (7). Assay of enzymic activity was carried out by the procedure of Mackler (8) with DPNH cytochrome c reductase (Sigma Chemical Co. Type I, crude, from pig heart). Epr spectra were obtained on a Varian E-9 X-band spectrometer at 77°K.

Results: Figure 1 shows the UV/visible spectrum of unfractionated ^{Co}cytc^{+} in 0.05M pH7.0 Naphos. Extinction coefficients were determined by dry weight measurement. Also shown is the spectrum obtained after reduction with dithionite. Fractionation of the products at pH8.0 on Amberlite CG-50 with linear NaCl gradient 0-0.5M yields two major components. Electro-

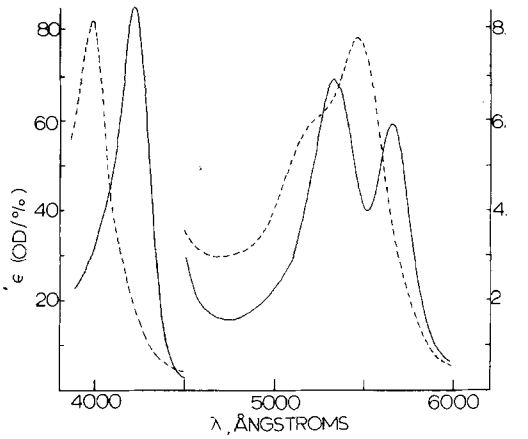

Figure 1. The UV/visible spectrum of cobalt cytochrome c in 0.02M pH8.0 Naphos as 25°C. Solid line: cobalticytochrome c. Dashed line: cobaltocytochrome c as prepared by reduction with dithionite.

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phoresis at pH7 shows that the component which elutes at 0.12 - 0.2M NaCl has identical mobility to native $^{Fe}cytc^{+}$. The other component which is eluted by 0.2 - 0.5M NaCl has 75% of the mobility of $^{Fe}cytc^{+}$.

Dithionite reduction of $^{Co}cytc^{+}$ is much slower than with native $^{Fe}cytc^{+}$. In 3 ml 0.05% of $^{Co}cytc^{+}$ solution at pH8 3 mg of sodium dithionite completely reduces the first eluted fraction in 20 minutes; the second, in 10 hours. The epr spectrum generated by dithionite reduction of unfractionated $^{Co}cytc$ is shown in Figure 2A. Exposure of the sample for 2 minutes to a nitric oxide atmosphere results in the second spectrum shown in Figure 2B. The same spectrum as in Figure 2A can be generated by cytochrome c reductase in the presence of NaN_3, DPNH in 0.2M pH7.5 Kphos buffer at 25°C in the dark. The epr intensity at 77°K was used to follow the rate of reduction of $^{Co}cytc^{+}$. The rate for unfractionated $^{Co}cytc^{+}$ is about 3% that of native $^{Fe}cytc^{+}$.

In dithionite free solutions the $^{Co}cytc$ is very rapidly oxidized to $^{Co}cytc^{+}$ with an autoxidation rate of 1μM/min in 0.02M pH8 buffer which was purged with nitrogen for 15 minutes.

The unfractionated reconstituted $^{Fe}cytc^{+}$ has 50% of the DPNH reductase

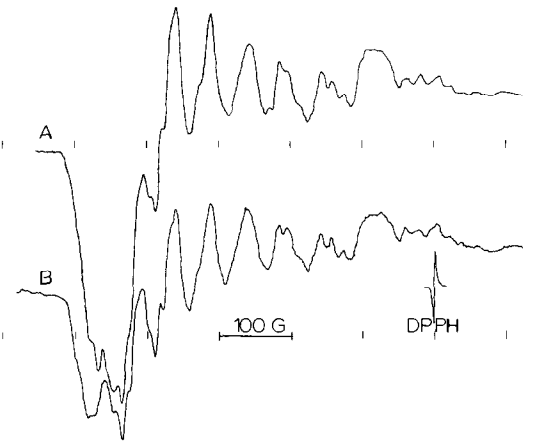

Figure 2A. EPR spectrum of 0.1% cobaltocytochrome c in 0.02M pH8.0 Naphos at 77 K as reduced by dithionite. Microwave power 20 mw, modulation amplitude 4 Gauss, microwave frequency, 9.290 GHz.

2B. EPR spectrum of the same sample after exposure to nitric oxide as described in the text. Instrument settings are the same as in Figure 2A.

activity and electrophoretic mobility identical to that of native $^{Fe}cytc^{+}$.

Discussion: The red shift of the Soret band upon oxidation of $^{Co}cytc$ contrasts with the blue shift for the $^{Fe}cytc$. Cobalt and iron hemoglobins show similar shifts. The $^{Co}cytc$ electronic spectrum resembles that of deoxycobaltohemoglobin. The cobalt proteins show no sharp intense transition comparable to the 5500 Å "spike" of $^{Fe}cytc$.

The epr spectrum of unfractionated $^{Co}cytc$ is that of porphyrin coordinated cobalt but is not of sufficient quality to be unambiguously interpreted. The more intense set of eight low field lines are taken as perpendicular features with g = 2.19 as their center and hyperfine splitting of 50 gauss. There is an incomplete (i.e. less than 8 apparent triplets) set of very weak lines apparent between 3100 and 3500 Gauss. The center of this pattern is crucial to assignment and should be clear with further purification and improved preparation. The observed g and hyperfine values do not fall clearly into either the one- or two-nitrogen ligation categories

of Walker (10). Because of the triplet splitting of the low field parallel lines there must be at least one nitrogen ligand so this is indirect evidence that the other ligand may be the Met 80 thiomethyl group.

The two species in unfractionated ^{Co}cytc may differ in their ligation. It was thought that a five-coordinated species or one with very weakly bonded sixth ligand may react with NO much more rapidly than a species with strongly bonded axial ligands. Experimental results are consistent with this hypothesis. The epr spectrum of ^{Co}cytc (Figure 2a) changed immediately to that of Figure 2b. Comparison of these two spectra showed that the rapidly reacting species has featureless epr absorption centered at g = 2.28. The other species also combines with NO but the reaction takes over two hours.

The comparison of results for ^{Co}cytc^{+} and ^{Fe}cytc^{+} for electrophoresis, Na^{+} elution concentration from Amberlite, and DPNH reductase activity are taken as strong indications that the ^{Co}cytc^{+} has a structure comparable to the native protein. Thus, further comparative studies especially in terms of the ligation of the cobalt, activity and binding to cytochrome oxidase, and possible protein conformation modifications are expected to shed new light upon the electron transfer mechanism of this protein. Such studies are underway and will be published elsewhere in an expanded article.

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