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Infrared evidence for the presence of Superoxide in a Co(III)-ATP complex used for the labeling of nucleotide in binding sites of ATPases

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Summary

The inert coordination complexes of Co(III) and ATP are used as dead end inhibitors to determine enzyme mechanisms. Conclusive infrared evidence for the presence of superoxide ion in a Co(III)-ATP complex is presented. The superoxide is displaced by monovalent anions such as cyanide. The presence of superoxide explains the biological reactivity of the inert Co(III) complex.

Introduction

Several Co(III)-nucleotides have recently been used either as reversible structural probes (1,2) or as irreversible labeling reagents (3-8) for the nucleotide binding sites of kinases and ATPases. In some labeling reactions, the Co(III)-nucleotide complexes have been formed *in situ*, i.e., by oxidation of Co(II) complexes in the presence of enzymes (6,7,8). In other cases a Co(III)-nucleotide complex was presynthesized and allowed to react with the enzyme, thereby inducing labeling, probably via exchange of labile ligands of the complex for liganding protein side chains. In order to elucidate the nature of the labeling process as well as to learn about the conformation of the binding site, it is important to determine the structure of the complex used. We have recently characterized one of these complexes "Co(III)-(phen)-ATP"[†] (9), which was found to be relatively labile for a Co(III)-complex. It has been suggested that this labilization was due to the presence of superoxide as a ligand for the cobaltic ion. This suggestion was

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[†] Abbreviation: phen-1,10-phenanthroline

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supported by the paramagnetism of the complex observed by both NMR and EPR (9). The NMR spectrum shows a significant broadening of proton and phosphorus resonances, while the EPR spectrum is characterized by the presence of a broad line centered at g = 2 and split into eight lines by the central Co(III) ion (nuclear spin 7/2). Additional evidence for the presence of superoxide of the complex is based on competition experiments with cyanide (9). In this communication, we present direct evidence, based on infrared data, for the existence of a Co(III)-O_2^- linkage in this complex (10).

Materials and Methods

"Co(III)-(phen)-ATP" was prepared either by H_2O_2 or O_2 oxidation and purified by ethanol precipitation as previously described (11). The complexes were either lyophilized or dried under nitrogen at room temperature. Complex concentrations were based on the cobalt content, as determined by atomic absorption on a Perkin-Elmer Spectrophotometer Model 303.

Phosphate determination was performed according to a modified Fiske-Subbarow method (12).

The superoxide content of the complexes were determined following their displacement by cyanide ions. To solutions of the complexes (10-250 μM), brought to pH 10 by the addition of 45 mM sodium carbonate buffer, 25 μM of nitrotetrazolium blue, and 8 mM of NaCN were added. The absorbance at 560 nm was read and the superoxide concentration estimated by using an extinction coefficient of 30,000 M^{-1} cm^{-1} (13).

Infrared Method: The infrared spectra were recorded with a Perkin-Elmer 180 infrared spectrophotometer, interfaced with a Tektronix graphic computing system, Model 4051 (14). The cells were equipped with CaF_2 windows with 0.027 mm Teflon spacer, and water-jacketed holder to maintain sample temperature at 25°C. A variable pathlength cell containing water was used as a reference cell.

Results

The inset in Figure 1 presents an IR spectrum of the "Co(III)-(phen)-ATP" complex vs water between 1170 and 1090 cm^{-1}. The broad spectrum consists of several unresolved absorptions. To obtain well-resolved single band for a specific vibration in this range, a carefully measured difference IR spectrum has been required (14). Figure 1 shows such a spectrum obtained by accumulation of 9 spectra at aqueous solutions of Co(III)-(phen)-ATP-O_2^- versus Co(III)-(phen)-ATP-CN^-. Two prominent bands are clearly distinguished. The band at 1105 cm^{-1} is typical of a $^{16}O-^{16}O$ stretch of a superoxo-Co(III)

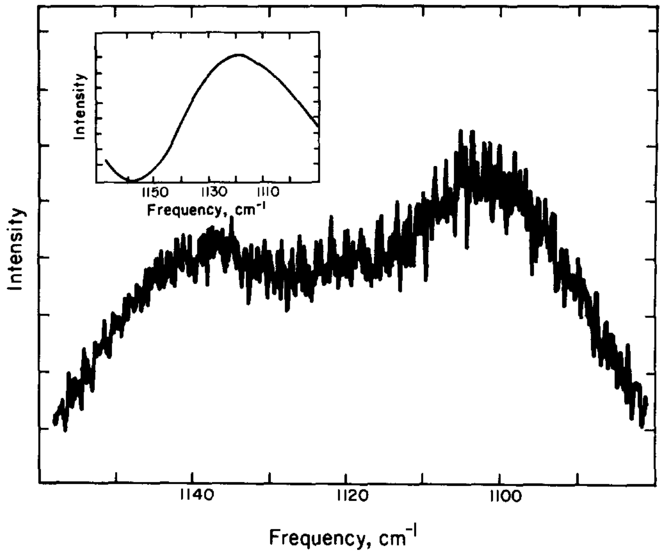

Figure 1. Infrared difference spectrum of Co(III)-(phen)-ATP-O_2^- 50 mM vs its cyanide adduct. The spectrum was obtained with a five-fold expansion of the ordinate. Inset: infrared spectrum of Co(III)-(phen)-ATP-O_2^- scanned from 1170 to 1090 cm^{-1} without scale expansion.

complex, in which the O_2^- is bound to the metal in a bent-end-on fashion $(\mathrm{M}{-}\mathrm{O}_{\searrow \mathrm{O}})$ (15,16). The origin of the band at 1140 cm^{-1} is not known. However, it may well be that the appearance of two bands is caused by Fermi resonance. Recently, two absorption bands of dioxygen at 1156 and 1107 cm^{-1} were observed in oxyhemoglobin, and it has been interpreted that they were split by Fermi resonance due to strong vibration coupling with the Fe-O overtone at 567 cm^{-1} (17).

The superoxide ion content in the "Co-(III)-(phen)-ATP" complex was found to depend critically on the method of its preparation. In the complex made by oxygen oxidation the amplitude of the 1105 cm^{-1} band was ca. ten times more intense than that observed in the complex, which has been prepared by H_2O_2 oxidation. Similarly a 10-fold difference in the O_2^- content of the "Co(III)-(phen)-ATP" complexes was found to depend on whether the oxidation

TABLE 1

Superoxide Content of Co(III)-(phen)-ATP Complexes Prepared by O_2 or H_2O_2 Oxidation

Method of oxidation	Cobalt, μM[a]	Phosphate[b]: Cobalt	O_2^- μM[c]	% of O_2^- in complex
O_2	14.4	3.1	4.1	28.3
H_2O_2	240	2.5	6.0	2.6

[a] Determined by atomic absorption.

[b] Based on phosphate determination.

[c] Determined by the nitrotetrazolium blue method, after cyanide displacement.

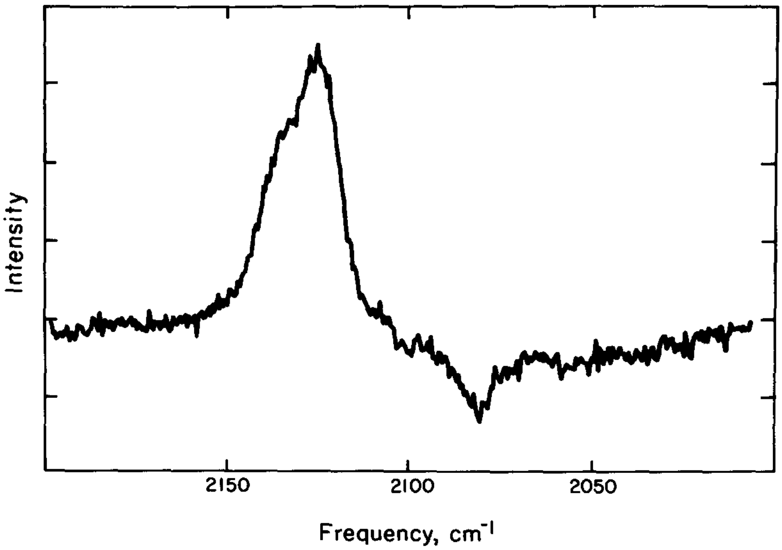

Figure 2. Infrared difference spectrum of Co(III)-(phen)-ATP-CN (50 mM) vs KCN at a similar concentration.

had been performed by O_2 or by H_2O_2 (Table 1). The agreement between these two methods supports the assignment of the 1105 cm^{-1} and 1140 cm^{-1} band to a superoxide ion.

The replacement of ligands in Co(III)-(phen)-ATP-O_2^- by cyanide was also followed by the decrease in the amplitude of the free cyanide infrared band at 2080 cm^{-1}. A titration revealed the binding of two CN^- groups to the tervalent cobalt. Concomitant with the decrease at 2080 cm^{-1} (see Figure 2), two prominent peaks, at 2125 and 2134 cm^{-1} appeared in the spectrum, clearly reflecting the stretching bands of cobalt-bound cyanide groups (18).

Discussion

Co(III)-nucleotide complexes have been extensively used for labeling the Mg^{2+} or Ca^{2+}-nucleotide binding sites in kinases and ATPases (3-8). The efficiency of the labeling depends on the nature of ligands around the cobalt ion and on their ability to exchange. A relatively labile ligand around the tervalent cobalt ion could be exchanged for a protein ligand, leading to the binding of the inert complex to the protein. Thus, elucidation the structure of Co(III)-nucleotide complexes is important for understanding their inactivation potential.

Of particular interest is the structure of the "Co(III)-(phen)-ATP" complex, a reagent that has proved to be useful in inhibitory studies of rabbit muscle myosin (3), coupling factor 1 (4) and Na^+ + K^+ ATPases (5). Previous magnetic resonance studies indicated that this complex was paramagnetic and a superoxo-cobalt(III) free radical complex has been suggested as the source of paramagnetism (9).

In this work we provide conclusive evidence to support this proposal. The O^{16}-O^{16} stretching vibration in Co(III)-O_2^- complexes has been found to occur near 1100 cm^{-1} (15). The infrared bands at 1105 and 1140 cm^{-1} in the difference spectrum of Co(III)-(phen)-ATP-O_2^- *vs* the cyanide complex clearly indicate the existence of a superoxo-cobalt(III) complex.

However, a question which remains unresolved is the low content of superoxide found in the complexes. It seems that the complex prepared by H_2O_2 oxidation contains very little of the superoxo species. It is also interesting to note in this context that the rate of inactivation of myosin and myosin subfragment-1 ATPases by this complex is 10-15 fold slower than the rate observed in the presence of the complex prepared by oxygen oxidation (M. M. Werber, unpublished results). However, the inactivation of the enzymes is probably not caused by deleterious effects of the superoxide radical, since the rate of inactivation was not affected by the presence of superoxide dismutase (8).

The less-than-stoichiometric content of superoxide found in the complex prepared by oxygen oxidation is probably due to the presence of other species, such as bridged superoxo- or peroxo-complexes. It is also possible that under the conditions used for the determination i.e., cyanide displacement, some loss of superoxide ions occur, which leads to an underestimation of its content in the complex.

It should be mentioned that the bonding of dioxygen in various 1:1 cobalt(II)-molecular oxygen adducts was shown to be consistent with the presence of the $Co(III)-O_2^-$-group (19,21). Cobalt(III) is a one electron reductant and thus typically forms superoxo-complexes with a bend-end-on configuration.

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