Nitrile Hydratase from *Rhodococcus rhodochrous* J1 Contains a Non-Corrin Cobalt Ion with Two Sulfur Ligands

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We present spectroscopic data that show that a nitrile hydratase from *Rhodococcus rhodochrous* J1 is the first reported example of a native protein that contains a non-corrin Co^{3+} ion with a mixed S and N(O) ligand field. Nitrile hydratases catalyze the addition of water to nitriles, yielding amides as the exclusive product,[3] and are used as industrial catalysts for the production of acrylamide.[4] The most thoroughly characterized nitrile hydratase is from *Rhodococcus* sp. R312[5] and is a $(\alpha\beta)_2$ tetramer that contains two low-spin non-heme ferric ions of unknown function. These metal ions exist in a tetragonally distorted octahedral ligand field of three histidine imidazoles, two cysteine thiolates, and a hydroxide.[6−8] Two cobalt-containing nitrile hydratases have been identified in *R. rhodochrous* J1.[9] We purified one of those enzymes[10,11] a multimer of $\alpha\beta$ heterodimers totaling approximately 500 000 Da and containing non-corrin Co^{3+}.[10] We measured the cobalt[12] and the protein[13] concentrations of samples of purified enzyme and found one cobalt ion per $(\alpha\beta)$. The same experiment yielded an unusually high ϵ_{280} (2.7 $(mg/mL)^{-1}$ cm^{-1}), consistent with an earlier report.[10] EPR spectra of concentrated samples (0.3 mM cobalt) showed no signals attributable to the protein from 4 to 77 K, consistent with the presence of Co^{3+}. When treated with sodium dithionite and methyl viologen, the samples developed an EPR spectrum characteristic of low-spin Co^{2+} (Figure 1; $g_{1,2,3} = 2.378, 2.206, 1.998$; $A^{Co}_{1,2,3} = 58, 11, 97$ G).[14]

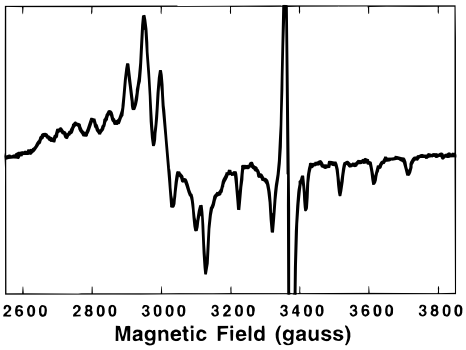

Figure 1. EPR Spectrum of *R. rhodochrous* J1 nitrile hydratase reduced with dithionite and methyl viologen. The sharp feature at 3365 G arises from reduced methyl viologen.

The cobalt K-edge X-ray absorbance spectrum of this nitrile hydratase (in the presumed Co^{3+} form) is very similar to the Fe^{3+} K-edge spectrum of the *Rhodococcus* sp. R312 enzyme[15,16] (Figure 2a,b). Using the method of Roe et al.,[17] the area (in units of eV (% edge height)) of the lowest energy pre-edge peak in the cobalt spectrum (assigned to a 1s → 3d transition) is 6.3, slightly larger than areas we obtained for six-coordinate $Co(S_2CNEt_2)_3$ (3.6), $Co(acac)_3$ (3.9), and $[Co(en)_3]Cl_3$ (4.6), but much smaller than that found for the four-coordinate $Co(im)_2Cl_2$ (16.8). The size of the pre-edge peak is consistent with six- or possibly five-coordinate cobalt in nitrile hydratase, with distortions from octahedral symmetry that increase the peak area by approximately 50% compared to those of symmetrical six-coordinate models. The pre-edge peak for the six-coordinate[7,8,16] iron in *Rhodococcus* sp. R312 nitrile hydratase is also approximately 50% larger than those of symmetrical six-coordinate Fe^{3+} model complexes.[16]

The best fits of the first sphere Fourier-filtered EXAFS are shown in Figure 2c and assume two sulfur scatterers at 2.20 Å and three ($\epsilon_\nu^2 = 1.3$)[18] or four ($\epsilon_\nu^2 = 1.5$) nitrogen scatterers at 1.95 Å. Any other integer values of n_S or n_N in two-shell fits gave $\epsilon_\nu^2 \geq 3.0$ and were rejected on the basis of the criterion that ϵ_ν^2 for a correct model is expected to be within one unit of the minimum ϵ_ν^2 obtained.[19] The value of ϵ_ν^2 increased by only 0.2−0.3 for each nitrogen replaced by an oxygen in the scattering model; therefore, one or more of the non-sulfur scatterers may be oxygen rather than nitrogen.

A search of the Cambridge Structure Database[20] revealed 76 $S_2N_nO_{4-n}$ coordination spheres in low-spin Co^{3+} complexes. Those with average bond lengths most similar to those found

(1) DuPont. Contribution 7410 from Central Research and Development.
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(3) Nagasawa, T.; Yamada, H. *Trends Biotechnol*. **1989**, *7*, 153−158.
(4) Kobayashi, M.; Nagasawa, T.; Yamada, H. *Trends Biotechnol*. **1992**, *10*, 402−408. Nagasawa, T.; Shimizu, H.; Yamada, H. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 189−195.
(5) Formerly *Brevibacterium* sp. R312. Briand, D.; Dubreucq, E.; Perrier, V.; Grimaud, J.; Galzy, P. *Microbios* **1994**, *78*, 205−214.
(6) Nelson, M. J.; Jin, H.; Turner, I. M., Jr.; Grove, G.; Scarrow, R. C.; Brennan, B. A.; Que, L., Jr. *J. Am. Chem. Soc*. **1991**, *113*, 7072−7073.
(7) Jin, H.; Turner, I. M., Jr.; Nelson, M. J.; Gurbiel, R. J.; Doan, P. E.; Hoffman, B. M. *J. Am. Chem. Soc*. **1993**, *115*, 5290−5291.
(8) Doan, P. E.; Jin, H.; Nelson, M. J.; Hoffman, B. M. *J. Am. Chem. Soc*. **1996**, *118*, 7014−7015.
(9) Kobayashi, M.; Nishiyama, M.; Nagasawa, T.; Horinouchi, S.; Beppu, T.; Yamada, H. *Biochim. Biophys. Acta* **1991**, *1129*, 23−33.
(10) Nagasawa, T.; Takeuchi, K.; Yamada, H. *Eur. J. Biochem*. **1991**, *196*, 2581−589.
(11) Nitrile hydratase was purified by a modification of ref 6. The specific activity of the purified protein was 250 U/mg, where 1 U = 1 μmol of methacrylonitrile hydrolyzed per min. This corresponds approximately to 500 U/mg by propionitrile hydration, compared to 371 U(propionitrile)/mg previously reported.[10] Samples recovered after irradiation retained 90% of their activity.
(12) Samples were added to 1 M nitric acid (Ultrapure nitric acid, J. T. Baker) and heated in closed containers at 90 °C for 24 h. The liquid was allowed to evaporate, and the samples were taken up in 0.5 mL of 1 M nitric acid. The concentration of cobalt in each sample was quantified in triplicate using a Perkin-Elmer 3300 atomic absorption spectrophotometer.
(13) Purified nitrile hydratase was hydrolyzed by treatment with 6 N HCl for 24 h at 100 °C, and the total amino acid composition was determined using a Beckman System 6300 amino acid analyzer.
(14) EPR parameters: frequency, 9.465 GHz; power, 1.0 mW; modulation amplitude, 9.7 G; sweep rate, 4.8 G/s; *T*, 20 K. The *g*- and *A*-values were determined by simulating the spectrum.
(15) A 360 mg/L sample of the enzyme in 0.1 M HEPES, 0.04 M butyrate buffer at pH 7.0 was frozen and maintained at ca. 30 K during data collection. Fluorescence-detected data collection and analysis followed procedures used for the *Rhodococcus* sp. R312 enzyme[16] except that E_0 was shifted by 598 eV and we applied a central atom phase shift that was taken as the difference between scattering phases for $Co(im)_6^{2+}$ and $Fe(im)_6^{2+}$ as calculated by FEFF 5.05 (Mustre de Leon, J.; Rehr, J. J.; Zabinsky, S. I.; Albers, R. C. *Phys. Rev., B* **1991**, *44*, 4146−4156). EXAFS data from crystalline model complexes $[Co(en)_3]Cl_3$, $Co(acac)_3$, and $Co(S_2CNEt_2)_3$ (powdered in BN) were used to verify that correct bond lengths (±0.01 Å) and numbers of coordinated atoms (±15%) are obtained from our analysis procedure.
(16) Scarrow, R. C.; Brennan, B. A.; Cummings, J. G.; Jin, H.; Duong, D. J.; Kindt, J. T.; Nelson, M. J. *Biochemistry* **1996**, *35*, 10078−10088.
(17) Roe, A. L.; Schneider, D. J.; Mayer, R. J.; Pyrz, J. W.; Widom, J.; Que, L., Jr. *J. Am. Chem. Soc*. **1984**, *106*, 1676−1681. Randall, C. R.; Shu, L.; Chiou, Y.-M.; Kagen, K. S.; Ito, M.; Kitajima, N.; Lachicotte, R. J.; Zang, Y.; Que, L., Jr. *Inorg. Chem*. **1995**, *34*, 1036−1039.
(18) ϵ_ν^2 is the least-squares-minimized weighted residual after normalization to account for the number of independent data and the number of varied parameters.[16,19]
(19) Bunker, G.; Hasnain, S.; Sayers, D., Eds. In *X-ray Absorption Fine Structure*; S. S. Hasnain, Ed.; Ellis Horwood: New York, 1991; pp 751−770.
(20) Allen, F. H.; Davies, J. E.; Galloy, J. J.; Johnson, O.; Kennard, O.; Macrae, C. F.; Mitchell, E. M.; Mitchell, G. F.; Smith, J. M.; Watson, D. G. *J. Chem. Inf. Comput. Sci*. **1991**, *31*, 187−204.

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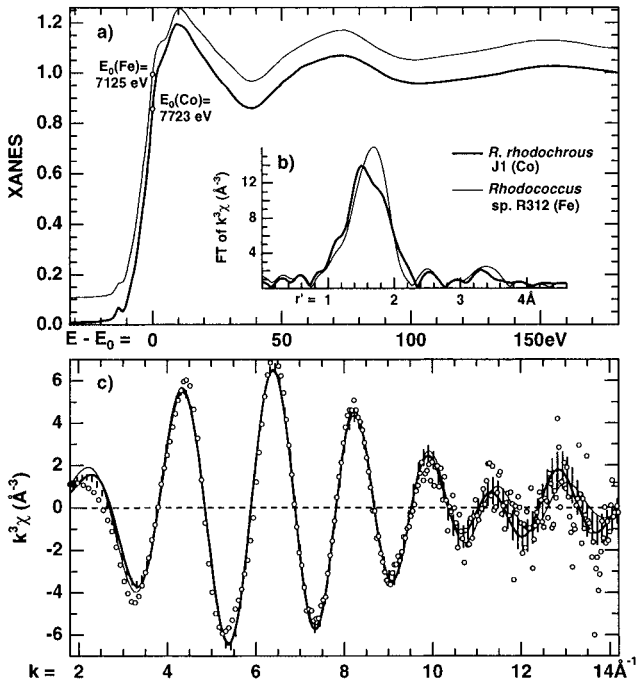

Figure 2. X-ray absorbance spectra of cobalt- and iron-containing nitrile hydratases at pH 7. (a) X-ray near-edge spectra. The Co spectrum is slightly better resolved due to the slit width employed (0.2 vs 0.5 mm with the same Si 111 monochromator). The Fe spectrum is offset by +0.1. (b) Fourier transformed EXAFS ($k = 1.0-14.3$ Å^{-1}). (c) Base-line-corrected (○) and Fourier-filtered (|) EXAFS of the cobalt enzyme and two fits (curves) to the filtered data. The filtered EXAFS is a back-transform ($r' = 1.0-2.3$ Å) of data in (b); the bars indicate the estimated uncertainty.[16] Thick line: two S and three N, $r = 2.204$-(9) and 1.946(10) Å, $\Delta\sigma^2 = -1(10)$ and $-25(14)$ pm^2. Thin line: two S and four N, $r = 2.196(11)$ and 1.955(12), $\Delta\sigma^2 = 10(12)$ and 10(17) pm^2. Values in parentheses are esd values of last digit.[16,19]

for *R. rhodochrous* J1 nitrile hydratase are three six-coordinate $CoS_2N_2O_2$ complexes: [bis(glyoxalic acid thiosemicarbizonato)cobaltate]$^-$ ($\langle r_S\rangle = 2.21$ Å, $\langle r_{N/O}\rangle = 1.93$ Å),[21] [(2,9-diamino-3,3,8,8-tetramethyl-4,7-dithiadecane-1,10-diolato)cobalt]$^+$ ($\langle r_S\rangle = 2.21$ Å, $\langle r_{N/O}\rangle = 1.95$ Å),[22] and [bis(penicillaminato)cobalt]$^-$ ($\langle r_S\rangle = 2.22$ Å, $\langle r_{N/O}\rangle = 1.95$ Å).[23] The average Co−S bond length found for *R. rhodochrous* J1 nitrile hydratase ($\langle r_S\rangle = 2.20$ Å) is relatively short, suggesting *cis* Co−S bonds because $CoS_2N_nO_{4-n}$ complexes with this arrangement tend to have shorter $\langle r_S\rangle$ than do complexes with *trans* Co−S bonds (Figure 3). This effect is probably due to an electronic *trans* influence which lengthens bonds *trans* to the Co−S bond[24] and appears to be general for square planar and pseudo-octahedral first row transition metal complexes.[16] Thus, if the cobalt is six-coordinate in *R. rhodochrous* J1 nitrile hydratase, the Co−S bond lengths argue in favor of mutually *cis* Co−S bonds.

Co−S bond lengths of 2.20 Å are also found in the five-coordinate diamagnetic Co^{2+}-nitrosyl complex of *N,N′*-ethylenebis(monothioacetylacetonimine) in which the Co−NO bond length is 1.80 Å. That observation and a previous claim of NO bound to iron in the nitrile hydratase from *Rhodococcus* sp. N771[25] prompted us to analyze the EXAFS for evidence for or against a short Co−N bond in the *R. rhodochrous* J1 nitrile hydratase. A third shell of a single Co−N bond, with *r*

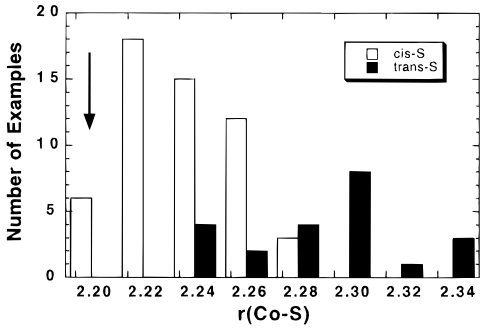

Figure 3. Histogram of the average Co−S bond lengths (±0.01 Å) for structurally characterized low-spin Co^{3+} complexes with $S_2N_nO_{4-n}$ coordination spheres. The arrow marks $\langle r_S\rangle$ determined for *R. rhodochrous* J1 nitrile hydratase.

fixed at values between 1.50 and 1.85 Å, was added to the two-shell models used to fit the EXAFS. All such three-shell refinements gave poor fits ($\epsilon_\nu^2 > 3$). On that basis we rule out a short Co−N bond and, thus, NO coordination to cobalt in the nitrile hydratase from *R. rhodochrous* J1.

The similarities of the pre-edge and EXAFS spectra (Figure 2a,b and analyses described above) suggest that the ligand environments of the metal ions in the cobalt- and the iron-containing nitrile hydratases are very similar. Of particular interest is the biologically unprecedented cobalt−sulfur coordination in the *R. rhodochrous* J1 nitrile hydratase, that, in analogy to the iron-containing enzyme,[6,26] we presume to arise from cysteine ligands. The -V-C-S-L-C-S-C-T- sequence in the α-subunit of the iron-containing nitrile hydratases that is thought to supply the thiolate ligands to the metal ion[6] is conserved as -V-C-T-L-C-S-C-Y- in the *R. rhodochrous* J1 enzyme.[9]

Low-spin six-coordinate Co^{3+} complexes show S → Co^{3+} charge transfer transitions at approximately 280 nm.[27] Although featureless in the visible region, the absorption spectrum of nitrile hydratase from *R. rhodochrous* J1 (data not shown) has a significant shoulder above 300 nm that we suggest may have S → Co ligand-to-metal charge transfer character. The absence of coenzyme B_{12} in the enzyme is shown by the lack of corrin absorbance in the visible spectrum and by the strong similarity in metal sites in the iron- and cobalt-containing nitrile hydratases revealed by EXAFS. Although there are several naturally occurring non-corrin Co^{2+} enzymes,[28] the nitrile hydratase from *R. rhodochrous* J1 is the first example, to our knowledge, of a native non-corrin Co^{3+} enzyme as well as of biological Co−S coordination.

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(21) Burstein, I. F.; Gerbeleu, N. V.; Bologa, O. A.; Verezhan, A. V.; Malinovskii, T. I. *Dokl. Akad. Nauk SSSR* **1988**, *300*, 1382−1386.
(22) Kanesaka, M.; Okamoto, K.; Nomoto, M.; Hidaka, J. *Bull. Chem. Soc. Jpn.* **1984**, *57*, 3111−3116.
(23) Helis, H. M.; de Meester, P.; Hodgson, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 3309−3312.
(24) Hambley, T. W. *Acta Crystallogr., Sect. B: Struct. Sci.* **1988**, *44*, 601−609. Hambley, T. W.; Gahan, L. R.; Searle, G. H. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1989**, *45*, 864−870.
(25) Noguchi, T.; Honda, J.; Nagamune, T.; Sasabe, H.; Inoue, Y.; Endo, I. *FEBS Lett.* **1995**, *358*, 9−12.
(26) Brennan, B. A.; Cummings, J. G.; Chase, D. B.; Turner, I. M., Jr.; Nelson, M. J. *Biochemistry* **1996**, *35*, 10068−10077.
(27) Lydon, J. D.; Elder, R. C.; Deutsch, E. *Inorg. Chem.* **1982**, *21*, 3186−3197. Elder, R. C.; Kennard, G. J.; Payne, M. D.; Deutsch, E. *Inorg. Chem.* **1978**, *17*, 1296−1303.
(28) Vogel, H. J.; Bonner, D. M. *J. Biol. Chem.* **1956**, *218*, 97−106. Petrovich, R. M.; Ruzicka, F. J.; Hemker, J.; Kleinschmidt, L.; Witzel, H. *Recl. Trav. Chim.* **1987**, *106*, 350. Reed, G. H.; Frey, P. A. *J. Biol. Chem.* **1991**, *266*, 7656−7660. Grard, T.; Saint-Pol, A.; Haeuw, J.-F.; Alonso, C.; Wieruszeski, J.-M.; Strecker, G.; Michalski, J.-C. *Eur. J. Biochem.* **1994**, *223*, 99−106. van Alebeek, G.-J.; Keuwels, M. J. J.; Keltjens, J. T.; Vogels, G. D. *Arch Microbiol.* **1994**, *161*, 514−520.