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Nitrile hydratase and related non-heme iron sulfur complexes

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Contents

Abstract

Iron-containing nitrile hydratases (NHases) are characterized by a low spin Fe(III) state and a high wavelength absorption at 700 nm. The first X-ray structure reveals a new type of $N_2S_3(O)$ iron coordination sphere, all the ligands being part of a small peptide, Cys X Y Cys Ser Cys, the two nitrogens arising from the amides of the peptide main chain, and the three thiolates from the cysteines. Several iron complexes with a mixed nitrogen sulfur coordination sphere have been synthesized and characterized by UV, EPR and electrochemistry, the two nitrogens arising from amide residues or bipyridine moiety. Iron(III) complexes, with deprotonated carboxamido nitrogen donors, are pentacoordinated. They are stable over a wide range of potential, have a $S = 3/2$ spin state and exhibit a visible absorption between

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470 and 550 nm. Iron(III) complexes, coordinated to the imine resonance form of the amide or to a bipyridine moiety, are reduced to the Fe(II) state at a potential quite similar to that of NHase (−480 mV). Moreover the amidate complexes are water soluble and stable over the pH range 3–10 as NHase. One of them yields, upon reaction with NO, a stable iron(III) nitrosyl complex, $[Fe(NO)(N_2S_2)]NEt_4$, characterized by a NO stretching at 1780 cm^{-1} instead of 1850 cm^{-1} for the [Fe(III)–NO] state of NHase.

Keywords: Nitrile hydratase; Iron sulfur complex; Amide; Imine; NO

1. Introduction

Nitrile hydratases (NHases) are bacterial enzymes that catalyze the hydration of nitriles to the corresponding amides and that contain non-heme iron or non corrinoid cobalt at their active site. The best known so far are the iron-containing enzymes. Two of them, NHase from *Chlororaphis* B23 and NHase from *Rhodococcus rhodochrous* J1 are commonly used in Japan for the industrial production of acrylamide and nicotinamide [1]. These enzymes consist of two kinds of subunits α and β and contain two moles of iron per $\alpha_2\beta_2$ tetramer. A consensus sequence Cys X Y Cys Ser Cys, conserved among all the α subunits has been very early assigned to the iron-binding site [2]. On the basis of spectroscopic studies including EPR [3], UV [4], ENDOR [5,6] and EXAFS [2,7], the iron coordination sphere was first proposed to be N_3S_2O with the two thiolates in *cis* position [8]. Actually, iron NHases are the first non-heme enzyme with a low spin Fe(III) state. They exhibit in UV-vis spectroscopy a protein absorption at 280 nm with a shoulder at 400 nm and a clearly less intense band around 700 nm tentatively assigned to a thiolate→ iron charge transfer band. Indeed, the iron enzymes are characterized by two states depending on the presence of their stabilizing agent or on pH [8,9].

2. The two states of the enzyme with and without butyric acid

NHases are purified and stored in an inactive state with a very high concentration of butyric acid, used as a stabilizing agent, in Hepes buffer at pH 7.2. Upon dilution of the stock solution in Hepes buffer, the activity is recovered. The butyric acid containing enzyme from *Rhodococcus* R312 and the dialyzed enzyme display two sets of EPR signals with one at 2.278, 2.136, 1.965 and another one at 2.202, 2.12, 1.98, respectively, the latter being more isotropic with a *g* value splitting of 0.222 instead of 0.313 [9].

The charge transfer band is also shifted from 710 to 680 nm upon dialysis. Increasing the pH of the butyric acid containing enzyme solution has the same propensity to reduce the *g* value splitting and to induce a hypsochromic shift of the UV absorption [8,9]. Hence, it is the acid form of butyric acid that interacts with the active site. This acid is a competitive inhibitor of nitrile hydration with a K_i value of 0.9 (±0.1) mM ($K_i' = 4$ μM relative to the acid form at pH 7.2) [9].

Another important feature of NHase is its particularly high affinity for NO, in the range of the one known for methemoglobin, a Fe(III) hemeprotein.

3. Interaction of the iron active site with NO

When purified in the dark, NHase from *Rhodococcus* N771 (a NHase identical to that isolated from *Rhodococcus* R312) is inactive, its activity being restored by photoirradiation [10]. The inactive state is EPR silent and its UV-vis spectrum shows the two absorptions at 280 and 370 nm but the broad band at 700 nm is lacking [11], indicating that the presence of this band is directly correlated to the enzyme activity. Fourier transform Infrared difference spectrum of NHase before and after photoirradiation shows a large negative band around 1850 cm^{-1} attributed to NO stretching [12]. This was confirmed by ^{15}N labeling experiments. Furthermore, resonance Raman studies showed that NO was directly interacting with the iron in the inactive state but was released upon photoirradiation [13].

This affinity of *Rhodococcus* N771 NHase iron for NO seems likely to be a general property of iron-containing NHases, since NHase from *Comamonas testeroni* NI1 [14], which has 50% sequence homology with N771, and which is not reported to be photosensitive, shares the same property, namely inactivation with NO in vitro and photoactivation [15]. NI1 NHase is inactivated by stoichiometric addition of NO (relative to the iron content), either in anaerobic solution or physiologically produced in vitro by a rat brain NO synthase. This result strongly supports the hypothesis that in *Rhodococcus* N771 NHase, NO could be produced by a NO synthase present in the strain [12].

All these properties were known before the publication of the first structure.

4. Structure of the active enzyme

One year ago, the X-ray structure of *Rhodococcus* R312 NHase, an enzyme which has 100% sequence homology with *Rhodococcus* N771 NHase, has been solved to 2.65 Å resolution and has revealed an unexpected coordination sphere for the iron [16], the ligand donor set being not N_3S_2O, as previously postulated, but $N_2S_3(O)$. The existence and the nature of the sixth ligand, H_2O or HO^-, could not be determined at this resolution. All the iron ligands belong to the consensus sequence in the α subunit Cys_{110} X Y Cys_{113} Ser_{114} Cys_{115} and the iron lies at the interface between the α and β subunits. The iron is coordinated to the three thiolates of the three cysteines and, more strikingly, to two nitrogen amides of the peptide main chain at the level of Cys_{115} and Ser_{114}. Such a coordination of amide to iron in biological systems has only been reported recently in nitrogenase P-cluster [17]. Moreover the integrity of the active site within the enzyme seems likely to be related to the presence of arginine residues in the β chain in the close proximity of Cys_{113} and Cys_{115}.

Such an iron coordination sphere raises some questions:

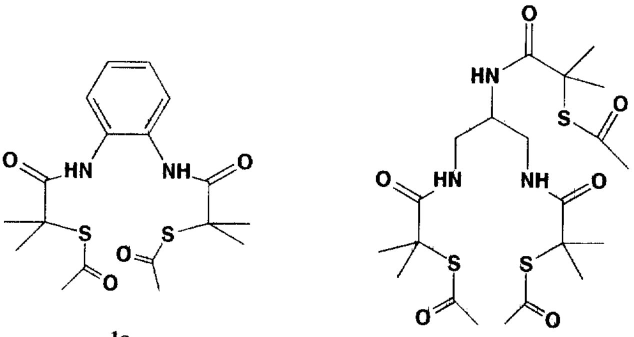
1. *What is the charge of the active site*?Non deprotonated amide are not good ligands for metals, however such a hexacoordinated complex with a $N_2S_2S_2^*$ donor set (S = thiolate and S* = thioether) has been reported on the basis of microanalysis [18]. In contrast, deprotonated amides are strong σ donor ligands and numerous complexes are described, either with N_4, or with N_2O_2 donor set [19]. With these ligands the iron is always pentacoordinated and its EPR spectrum is typical of $S = 3/2$ intermediate spin state [20]. If the amides are deprotonated, the charge of the NHase active site should be -2 or -3 whether H_2O or HO^- is the sixth ligand of the iron. There is not any example of such a negatively charged active site in biological systems, which is usually neutral. A convenient way to decrease the charge is to postulate the occurrence of the tautomeric form of the amide, the neutral imine, which could be a strong π donor ligand. With imines, the formal charge of this site becomes neutral with H_2O as sixth ligand or only one negatively charged with HO^-.
2. *Do complexes, the iron of which is coordinated to three thiolates and two amides, either in their deprotonated form or in their imine form, reproduce the low spin state and the high wavelength absorption of the enzyme*?

In the aim to check the abovementioned hypotheses, complexes with a mixed nitrogen sulfur coordination sphere were built, the nitrogen atoms arising from amide moieties.

5. Related iron(III) complexes with a mixed nitrogen sulfur coordination sphere

5.1. Iron (III) complexes with deprotonated carboxamido N and thiolato S donors

Two amide containing proligands, shown in Fig. 1, were prepared, $N_2(SCOCH_3)_2$, **1a** and $N_2(SCOCH_3)_3$, **1b**, in which the thiolates were protected as thioacetates. Cu(II) and Cu(III) complexes of the first tetradentate ligand, N_2S_2, derived from **1a**, were previously described [21]. The second ligand, N_2S_3, derived



1a **1b**

Fig. 1. Amide nitrogen containing proligands with two or three protected thiolates.

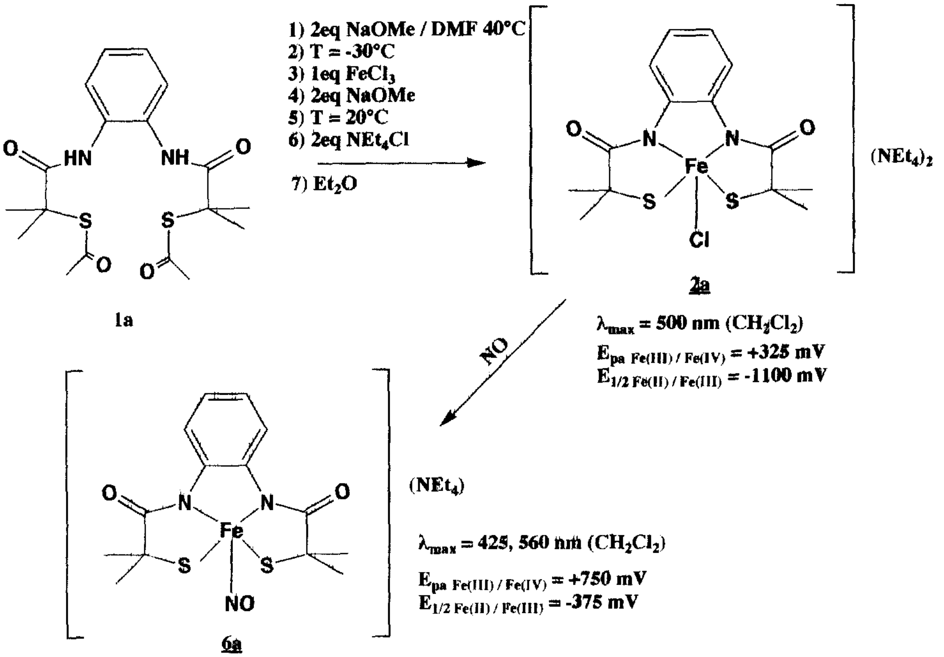


Fig. 2. Nitrosyl complex **6a** derived from complex **2c**.

from **1b**, is the closest to the NHase active site, with a 11 atoms chelate as in the protein [16].

After deprotection of the thiolates under basic conditions, and insertion of iron(III) with anhydrous $FeCl_3$ or $Fe(DMF)_6(ClO_4)_3$ in DMF, two complexes were prepared and characterized [22], $[Fe(N_2S_2)Cl](NEt_4)_2$, **2a**, derived from **1a**, (Fig. 2), and $[Fe(N_2S_3)](NEt_4)_2$, **2b** from **1b**, which both are orange colored. Single crystals of complex **2a** suitable for X-ray analysis (Fig. 3) were obtained by slow evaporation of an acetonitrile solution. Both complexes display EPR spectra, in frozen DMF solution, typical of $S = 3/2$ intermediate spin state, with a large absorption centered at $g = 5$, a broad shoulder at $g = 3.5$ and a low component at $g = 2$. The patterns of these spectra are very similar to those of bis(dithiocarbamato)-iron(III) complexes described by Chaps et al. [23]. None of them exhibit in UV-vis spectroscopy the broad absorption of NHase at 700 nm, but two well defined band around 380 and 470 nm. In cyclic voltammetry, all complexes are reversibly reduced to the Fe(II) state at a glassy carbon electrode around -1100 mV (vs. SCE) and irreversibly oxidized to the Fe(IV) state around $+470$ mV. These values are the typical ones previously found for iron(III) complexes with a N_4 donor set, N coordinated to four deprotonated amides [20]. This stabilization of the Fe(III) state over the range, -1000 to $+400$ mV, could be in agreement with a Lewis acid character of NHase iron, if the nitrile is effectively interacting with the iron during the catalytic cycle. Such an interaction could not be pointed out with our com-

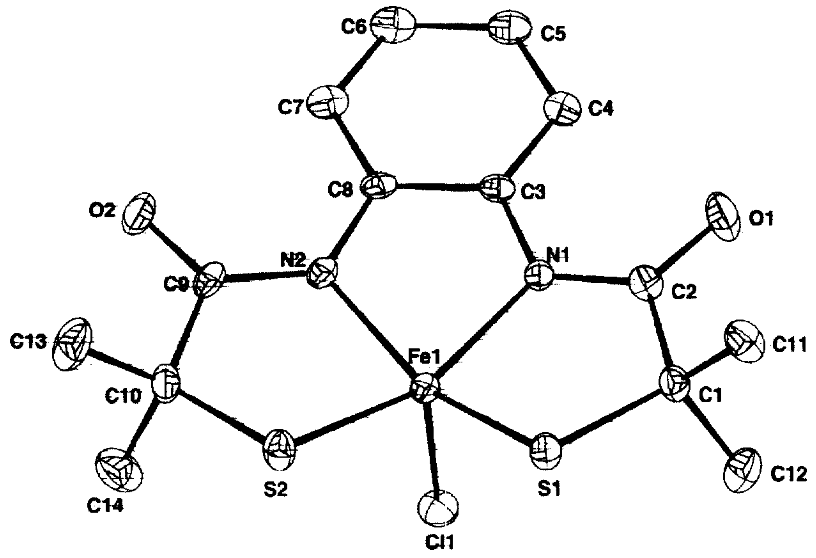


Fig. 3. Crystal structure of complex **2a** with atom labeling. Selected bond distances: Fe–N1, 1.926; N1–C3, 1.432; N1–C2, 1.303; C2–O1, 1.278; Fe–N2, 1.943; N2–C8, 1.410; N2–C9, 1.34; C9–O2, 1.256; Fe–S1, 2.206; Fe–S2 2.197 Å.

plexes, which are not active toward nitrile hydration, even though they are water soluble in the presence of a low percentage of DMF or MeOH and stable over the pH range 3–10, as NHase.

5.2. Imine state and related complexes with π nitrogen donor ligand

Insertion of iron (III) to the thiolate sodium salt of ligand derived from **1b** ($N_2S_3Na_3$) in DMF at −40°C leads to the immediate appearance of a dark blue color. Complex **3b** (Fig. 4), isolated as a blue powder from ether precipitation, is not stable even at very low temperature and it was only characterized when formed in situ in DMF at −40°C [22], by UV-vis spectroscopy, electrochemistry and EPR. Its EPR spectrum displays a fine signal at $g = 4.3$ and its UV-vis spectrum a well defined absorption at 540 nm, which is slowly displaced with two additional base equivalents to the 470 nm absorbing species characteristic of complex **2b**. Complex **3b** is reduced at a potential of −500 mV (vs. SCE), which is very close to that determined for NHase from *Rhodococcus* R312 in the presence of butyric acid ($E_{1/2\,\mathrm{Fe(III)/Fe(II)}} = -480$ mV vs. SCE) [24].

Finally, the spectroscopic data of this imine complex, **3b**, are also very similar to those of complexes $Fe(N_2S_2)Cl$, **4** [25], and $Fe(N_2S_2S')$ (S'=SCH_2COOEt), **5**, shown in Fig. 5, in which the nitrogen donors arise from a bipyridine moiety. Complex **4**, has been crystallized and its structure determined by X-ray analysis [25]. Its chloro iron bond is very long and is easily exchanged with thioacetate, leading to the formation of complex **5**. Both complexes have a $S = 3/2$ spin state and are

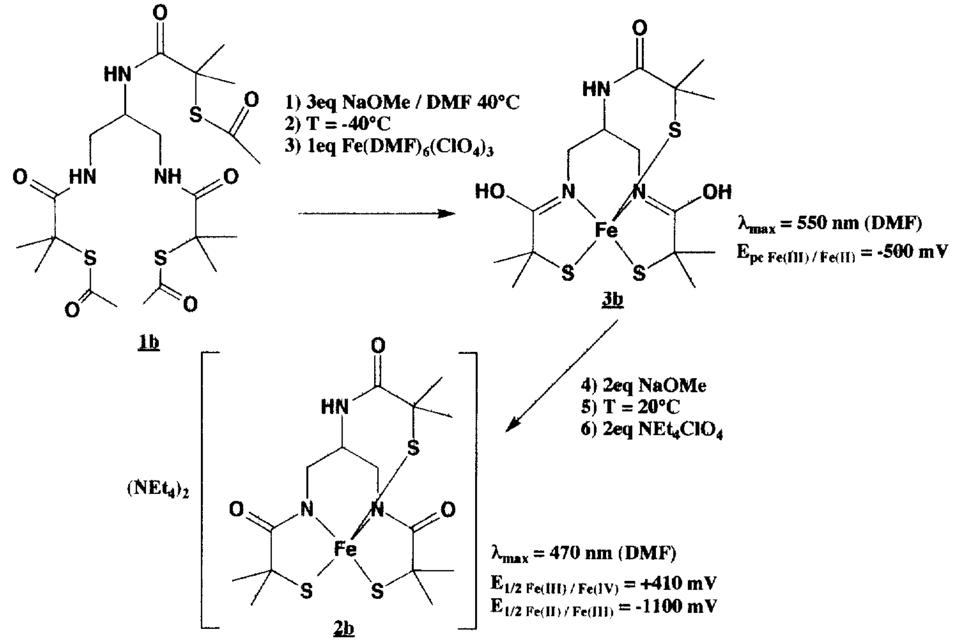


Fig. 4. Imine nitrogen and deprotonated amide nitrogen donors complexes derived from proligand **1c**.

characterized by a UV-vis absorption at 490 nm, with only a shoulder at 600 nm for **5**, and by a Fe(III)/Fe(II) reduction potential of −415 and −530 mV (vs. SCE) for **4** and **5**, respectively.

5.3. Reaction of a tetradentate $[Fe(N_2S_2)Cl]^{2-}$ *complex with NO*

The two complexes containing deprotonated nitrogen amides and thiolates, **2a** and **2b** react with exogenous NO, but **2a** is the only one to yield a stable complex

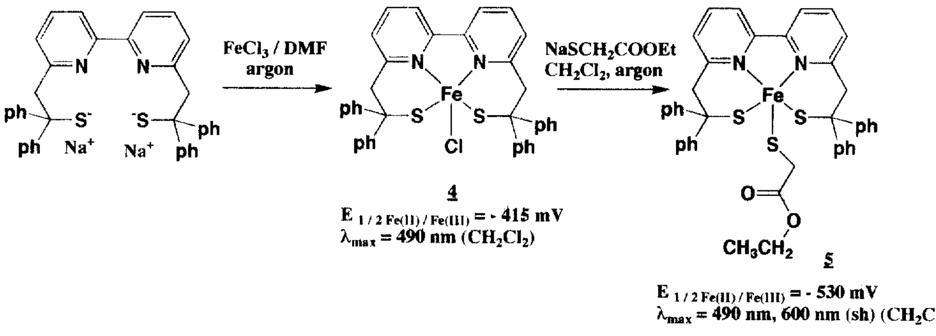


Fig. 5. Iron(III) complexes with a N_2S_2 and N_2S_2S' coordination sphere, nitrogen donors arising from a bipyridine moiety.

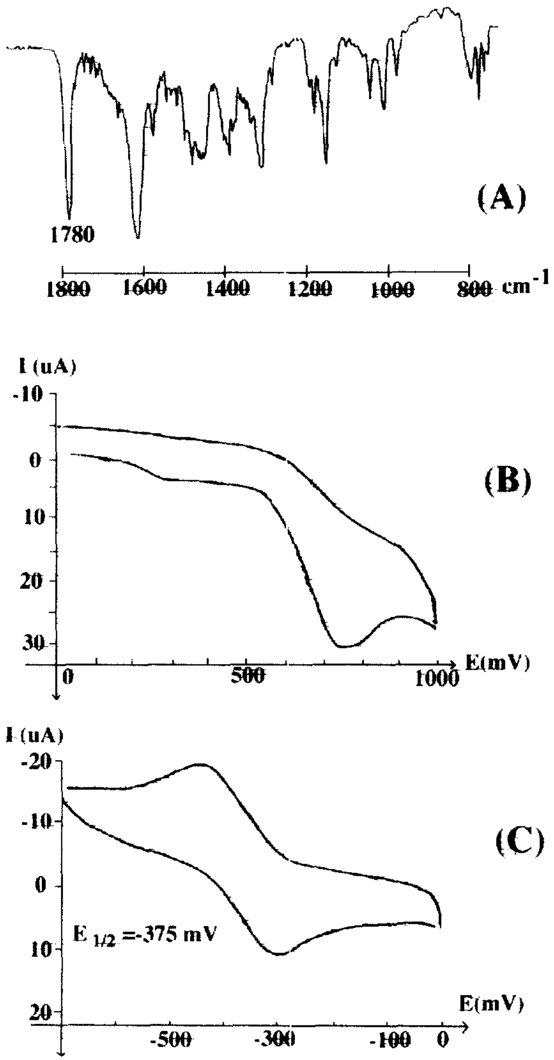


Fig. 6. Spectroscopic characterization of the nitrosyl complex **6a**: IR spectrum in KBr pastille (A). Cyclic voltammogram in deaerated CH_2Cl_2, with 0.1 M NBu_4BF_4 as supporting electrode, at a glassy carbon electrode (potential vs. SCE) and 50 mV s^{-1} sweep rate, in oxidation (B) and reduction (C).

6a [22]. The formation of **6a** (Fig. 2) could be monitored in a 90/10CH_2Cl_2\\CH_3CN mixture in UV-vis spectroscopy by the appearance of two new bands at 425 and 560 nm. The complex isolated from ether precipitation seems likely to derive from the Fe(III) state, since its cyclic voltammetry shows a quasi-reversible wave centered at − 375 mV (vs. SCE) which could correspond to the Fe(III) → Fe(II) reduction and an irreversible one at + 750 mV (vs. SCE) (Fig. 6) tentatively assigned to the Fe(III) → Fe(IV) oxidation. It is a new example of $\{FeNO\}^6$ complex among the

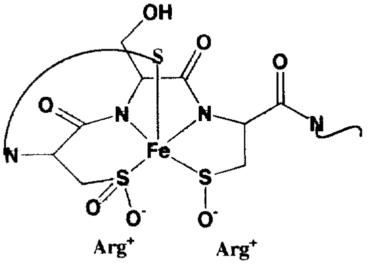


Fig. 7. Possible structure of the active enzyme centre on the basis of the NO inactivated state structure published in Ref. [27].

very few ones described in the literature, except those of the [bis-(dithiolene)FeNO]$^-$ family [26]. NO stretching of **6a** appeared at 1780 cm^{-1} (Fig. 6A) instead of 1867 and 1850 cm^{-1}, for the [Fe(C_4CN_4)NO]$^-$ bis-dithiolene complex [26] and NO inactivated NHase [11], respectively. Furthermore NO is not released upon photoirradiation, complex **6a** being stable in the presence of light.

6. Conclusion and future prospects

As far as chemical models can exactly mimic the active site of an enzyme, we are very far to reproduce the spectroscopic and chemical properties of NHase with complexes, the iron of which is coordinated to three thiolates and two imine nitrogen or two deprotonated amide nitrogen donors. The presence of three thiolates is presently a subject of discussion since, very recently, a new structure of NHase under its NO inactivated state has been published at 1.7 Å resolution. The two cysteine residues, Cys_{115} and Cys_{113}, were found to be modified into Cys-sulfenic and Cys-sulfinic acid, respectively [27]. The reversibility of NO inactivation suggests strongly that this structure is retained in the active form. This could explain our inability to mimic the electronic properties and the reactivity of the enzyme centre with the previously described iron complexes. On the basis of the last structure, the iron should be coordinated, as depicted in Fig. 7, in its first coordination sphere to three σ donor ligands, two deprotonated amides and one thiolate, and two sulfur groups with a π donor character, an environment which neutralizes the positive charge of the iron. The presence of several arginines in close proximity of the sulfinic and sulfenic moieties supports the hypothesis, that these two acids are deprotonated, and, that the negative charge is localized on the oxygen atoms. Such iron complexes are not described so far in the literature [28] The question now is to know if the aforementioned amidate complexes could be the precursors of complexes in which the thiolates of the mean plane could be selectively oxidized into sulfenic and sulfinic acids.

References

[1] (a) H. Yamada, M. Kobayashi, Biosc. Biotech. 60 (1996) 1391. (b) T. Nagazawa, H. Yamada, Trends Biotechnol. 7 (1989) 153.

[2] M.J. Nelson, H.Y. Jin, I.M. Turner, G. Grove, R.C. Scarrow, B.A. Brennan, L. Que, J. Am. Chem. Soc. 113 (1991) 7072.

[3] Y. Sugiura, J. Kuwahara, T. Nagasawa, H. Yamada, J. Am. Chem. Soc. 109 (1987) 5848.

[4] T. Nagasawa, K. Ryuno, H. Yamada, Biochem. Biophys. Res. Commun. 139 (1986) 1305.

[5] H.V. Jin, I.M. Turner, M.J. Nelson, R.J. Gurbiel, P.E. Doan, B.M. Hoffman, J. Am. Chem. Soc. 115 (1993) 5290.

[6] P.E. Doan, M.J. Nelson, H. Jin, B.M. Hoffman, J. Am. Chem. Soc. 118 (1996) 7014.

[7] R.C. Scarrow, B.A. Brennan, J.G. Cummings, H. Jin, D.J. Duong, J.T. Kindt, M.J. Nelson, Biochemistry 35 (1996) 10078.

[8] B.A. Brennan, J.G. Cummings, D.B. Chase, I.M. Turner Jr., M.J. Nelson, Biochemistry 35 (1996) 10068.

[9] M.A. Kopf, D. Bonnet, I. Artaud, D. Petre, D. Mansuy, Eur. J. Biochem. 240 (1996) 239.

[10] T. Nagamune, H. Kurata, H. Makoto, J. Honda, H. Korke, M. Ikeuchi, Y. Inoue, A. Hirata, I. Endo, Biochem. Biophys. Res. Commun. 168 (1990) 437.

[11] M. Odaka, K. Fujii, M. Hoshino, T. Noguchi, M. Tsujirama, S. Nagashima, M. Yohda, T. Nagamune, Y. Inoue, I. Endo, J. Am. Chem. Soc. 119 (1997) 3785.

[12] T. Noguchi, J. Honda, T. Nagamune, H. Sasabe, I. Inoue, I. Endo, FEBS Lett. 358 (1995) 9t.

[13] T. Nogushi, M. Hoshino, M. Tsujimura, M. Odaka, Y. Inoue, I. Endo, Biochemistry 35 (1996) 16777.

[14] E. Cerbelaud, S. Levy-Schil, D. Petre, F. Soubrier. Rhône–Poulenc Chimie, PCT Int. Appl. (1995) WO 9504828 A1 950216.

[15] D. Bonnet, I. Artaud, C. Maoli, D. Petre, D. Mansuy, FEBS Lett. 409 (1997) 216.

[16] W. Huang, J. Jia, J. Cummings, M. Nelson, G. Schneider, Y. Lindquist, Structure 5 (1997) 691.

[17] J.W. Peters, M.H.B. Stowell, M.S. Soltis, M.G. Finnegan, M.K. Johnson, D.K. Rees, Biochemistry 36 (1997) 1181.

[18] R. Shukla, P.K. Bharadwaj, Polyhedron 16 (1993) 1983.

[19] J.T. Collins, Acc. Chem. Res. 27 (1994) 279.

[20] K.L. Kostla, B.G. Fox, M.P. Hendrich, T.J. Collins, C.E.F. Rickard, E. Münck, J. Am. Chem. Soc. 115 (1993) 6746.

[21] J. Hans, H.J. Krüger, Angew. Chem. 35 (1996) 2827.

[22] S. Chatel, A.S. Chauvin, P. Leduc, J.C. Chottard, D. Mansuy, I. Artaud, unpublished results.

[23] G.E. Chapps, S.W. McCann, H.H. Wickman, R.C. Sherwood, J. Chem. Phys. 60 (1974) 990.

[24] D. Bonnet, D. Lexa, I. Artaud, D. Mansuy, unpublished results.

[25] M.A. Kopf, D. Varech, J.P. Tuchagues, D. Mansuy, I. Artaud, J. Chem. Soc. Dalton Trans. (1998) 991.

[26] J.A. McCleverty, N.M. Atherton, L. Locke, E.J. Wharton, C.J. Wiscon, J. Am. Soc. 89 (1967) 6082.

[27] S. Nagashima, M. Nakasako, N. Dhomae, M. Tsujimura, K. Takio, M. Odaka, M. Yohda, N. Kamiya, I. Endo, Nat. Struct. Biol. 5 (1998) 347.

[28] C.A. Grapperhaus, M. Darensbourg, Acc. Chem. Res. 31 (1998) 451.