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Reaction of nitric oxide with the iron(III) complex of *N*-(dithiocarboxy)sarcosine: a new type of reductive nitrosylation involving iron(IV) as an intermediate †

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Reaction of NO with the iron complex of *N*-(dithiocarboxy)sarcosine (H_2DTCS) was investigated by UV/VIS absorption spectroscopy, HPLC, LC-ESI MS and pulse radiolysis. The results of HPLC and LC-ESI MS clearly showed that the reaction products of NO with $[Fe^{III}(DTCS)_3]^{3-}$ are $[NO–Fe^{II}(DTCS)_2]^{2-}$ and a dimer of DTCS in which two DTCS bind through a disulfide bond. On pulse radiolysis of a deaerated aqueous solution of $[Fe^{III}(DTCS)_3]^{3-}$ in the presence of sodium nitrite the absorption changes consisted of three phases, as judged from kinetic difference spectra. In the faster phase, radiolytically generated NO reacted with $[Fe^{III}(DTCS)_3]^{3-}$ to form a transient intermediate I with a second-order rate constant of $(4.8 \pm 0.9) \times 10^8$ dm^3 mol^{-1} s^{-1}. Subsequently, I reacted with unchanged $[Fe^{III}(DTCS)_3]^{3-}$ to give an intermediate II with an absorption maximum at 460 nm. In the third step II was found to decay on a timescale of seconds. The final product in the sequence of the reactions was $[NO–Fe^{II}(DTCS)_2]^{2-}$. From the spectra obtained, the intermediate II is discussed in relation to the iron(IV) complex of *N*,*N*-diethyldithiocarbamate. These results suggest that the proposed reaction mechanism is a new type of reductive nitrosylation involving iron(IV) as an intermediate.

Since the discovery of the physiological roles of nitric oxide (NO) in 1987, the chemistry and biology of NO have become of growing interest.[1] It is now well established that NO is produced enzymatically by nitric oxide synthases (NOS) and has numerous important functions in mammalian tissues as a paracrine cellular mediator.[2,3]

In understanding such diverse physiological roles of NO the detection of endogenous NO is of crucial importance. The spin-trapping approach combined with EPR spectroscopy is a technique of choice for both *in vivo* and *in vitro* NO detection by virtue of its facility and wide applicability. Iron complexes with dithiocarbamate derivatives (Fe–DTC) are noted among the spin-trapping agents for NO, because NO has a high affinity for iron complexes, and the resultant nitrosyl complexes exhibit characteristic EPR spectra, both at room and low temperature. To date, NO produced from all kinds of NOS isoforms can be detected by using this technique.[4–25]

Despite this method's wide application, some of its basic chemistry is not well understood. In most literature[4–7,11–24] it is noted *a priori* that the trapping agent, $Fe^{II}(DTC)_2$ prepared by mixing iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and an excess of DTC, reacts with NO to form stable $NO–Fe^{II}(DTC)_2$ as in eqn. (1). In a previous report we have shown that $Fe^{III}(DTC)_3$

$$NO + Fe^{II}(DTC)_2 \longrightarrow NO–Fe^{II}(DTC)_2 \qquad (1)$$

complexes also readily react with NO to form $NO–Fe^{II}(DTC)_2$ in aqueous solution,[25] although the detailed mechanism is unclear. In this study, therefore, the reaction of NO with the Fe^{II}/Fe^{III} complex with *N*-(dithiocarboxy)sarcosine (DTCS; *N*-methylglycine dithiocarbamate) in aqueous solution was investigated by UV/VIS absorption spectroscopy, HPLC, LC-ESI MS, and pulse radiolysis. Here we present observations that NO directly reacts with $[Fe^{III}(DTCS)_3]^{3-}$ to produce $[NO–Fe^{II}(DTCS)_2]^{2-}$ by a new type of reductive nitrosylation.

Experimental

Materials

N-(Dithiocarboxy)sarcosine disodium salt dihydrate Na_2-(DTCS)·$2H_2O$ was purchased from Dojindo Laboratories and *N*,*N*-diethyldithiocarbamic acid sodium salt trihydrate Na_2-(DETC)·$3H_2O$ from Wako Pure Chemicals. Solvents used in HPLC analysis were special grade for HPLC. Water used in this study, except in HPLC, was purified with the Milli-Q water purification system. NO gas (99.7% minimum), purchased from Sumitomo-seika chemicals, was purified according to the literature[26] prior to use. All other chemicals and solvents were commercially available reagent grade and used without further purification.

Sample preparations

Although many versions of the preparation method of water-soluble Fe–DTC complexes as NO trapping agents have been reported,[6–10,15–23] the essential part of the preparation in the literature is as follows: $FeSO_4 \cdot 7H_2O$ (sometimes with five equivalents of sodium citrate) and DTC are dissolved in a nitrogen/argon-bubbled medium (at least 20 min; water, saline, or buffers) separately and then mixed in ratios of 1 : 3 to 1 : 10 (Fe to DTC) under a nitrogen atmosphere. In this study we prepared the complex by mixing aqueous solutions of $FeSO_4 \cdot 7H_2O/FeCl_3$ and DTCS in ratios of 1 : 2 or 1 : 3 ([Fe] = 5.0×10^{-4} mol dm^{-3}) with or without 30 min deoxygenation with nitrogen (procedure I). Sodium citrate was not used. In addition to this procedure, we prepared the NO trapping reagents in a more carefully deoxygenated medium for measurement of absorption spectra as follows (procedure II):

† Electronic supplementary information (ESI) available: LS-ESI MS chromatograms, LC-ESI mass spectra and pulse radiolysis data. See http://www.rsc.org/suppdata/dt/b0/b003942j/

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an iron salt ($FeSO_4 \cdot 7H_2O$ or $FeCl_3$), DTCS, and water were put separately in glassware which has four branches and one quartz cell. The solvent was first flushed with argon, degassed by repeated freezing, pumping, and thawing, and then introduced to DTCS and iron salt and stirred vigorously. The solution was frozen again, the parts of it and the empty quartz UV cell were isolated from the body of the glassware by a burner, and then the solution was transferred to the UV cell.

Reactions with NO were carried out in Thunberg-type cells at 25 °C. An excess of NO gas was introduced to a carefully deoxygenated aqueous solution of Fe^{II}/Fe^{III}–DTCS complex, then stirred and purged with argon.

Samples for pulse radiolysis were prepared as follows. Solutions containing $(1.2–4.0) \times 10^{-4}$ mol dm^{-3} $[Fe^{III}(DTCS)_3]^{3-}$, $(1.0–20) \times 10^{-3}$ mol dm^{-3} sodium nitrite ($NaNO_2$; for NO source),[27] and 0.1 mol dm^{-3} *tert*-butyl alcohol (for scavenging $^{\bullet}OH$ radicals) in 1.0×10^{-2} mol dm^{-3} phosphate buffer (pH 7.4) were deoxygenated in sealed cells by repeated evacuation and flushing with argon.

NO–$Fe(DTCS \cdot Na)_2 \cdot 2H_2O$ was synthesized from $Na_2(DTCS) \cdot 2H_2O$ (490 mg, 2 mmol) and $FeSO_4 \cdot 7H_2O$ (278 mg, 1 mmol) according to a modified method of Ileperuma and Feltham.[28] In this procedure, NO was introduced to a deoxygenated aqueous solution of $FeSO_4 \cdot 7H_2O$ and then a deoxygenated solution of DTCS was added dropwise. Unlike NO complexes of dialkyldithiocarbamates, NO-$Fe(DTCS \cdot Na)_2$ was water-soluble and did not precipitate. Therefore, a powder sample (316 mg, 64%) was obtained by the addition of acetone to a solution containing the NO complex under a N_2 atmosphere[25] and used as a standard for HPLC analysis (Found: C, 19.35; H, 2.91; N, 8.44. $C_8H_{10}FeN_3Na_2O_5S_4 \cdot 2H_2O$ requires C, 19.43; H, 2.85; N, 8.50%).

The complex $[Fe(DETC)_3][ClO_4]$ was synthesized according to the published procedure as follows:[29] $Fe(ClO_4)_2 \cdot H_2O$ (0.14 g, 0.5 mmol, Aldrich), dissolved in the minimum volume of ethanol, was added dropwise over a period of 20 min to a vigorously stirred benzene solution (30 cm^3) of $Fe(DETC)_3$ (0.5 g, 1 mmol). The solution was stirred for 1 h, after which time the finally divided solid (80 mg, 13%) was collected and recrystallized from CH_2Cl_2 by slow addition of benzene (Found: C, 29.96; H, 5.10; N, 6.95. $C_{15}H_{30}ClFeN_3O_4S_6$ requires C, 30.02; H, 5.04; N, 7.00%).

Elemental analyses were performed in the Chemical Analysis Centre, University of Tsukuba.

CAUTION: perchlorate salts of metal complexes with organic ligands are potentially explosive.

Physical measurements

UV/VIS absorption spectra were recorded using a Shimadzu Multispec-1500 or a Hitachi U-3000 spectrophotometer.

Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research of Osaka University.[27,30,31] The pulse width was 8 ns. The light source was a 150 W halogen lamp or a 1 kW xenon lamp. The dose was in the range of 15–100 Gy. After passing through an optical path, the transmitted light intensities were analysed by a fast spectrophotometric system composed of a Nikon monochromator, an R-928 photomultiplier, and a Unisoku data analysing system.

HPLC analyses were carried out with a reversed-phase column (Inertsil C-4, 5 μm, 4.5 × 150 mm; GL Sciences Inc., Tokyo, Japan) at a flow rate of 1.0 cm^3 min^{-1} and 40 °C using a JASCO (Tokyo, Japan) model PU-980 pump. Products of the reaction of NO with Fe–DTC complexes ($[Fe] = 5.0 \times 10^{-5}$ mol dm^{-3}; 25 °C) were detected with a water–CH_3CN 60:40 (v/v) mixture as the eluent and UV detection at 240 nm with a JASCO UV-970 detector. Tetra-*n*-butylammonium phosphate solution was added to the eluent as an ion pair reagent to give a final concentration of 5×10^{-3} mol dm^{-3}.

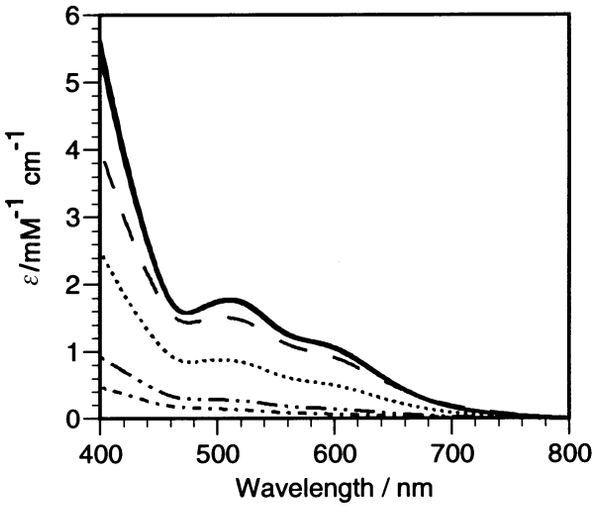


Fig. 1 Electronic absorption spectra of Fe–DTCS complexes in water at room temperature ($[Fe] = 5 \times 10^{-4}$ mol dm^{-3}). The complexes were prepared under normal experimental conditions (N_2-bubbled: procedure I) and thoroughly degassed conditions (procedure II): $[Fe^{III}(DTCS)_3]^{3-}$ complex prepared from $FeCl_3$ and DTCS with both procedures (solid lines; overlapped); Fe–DTCS complexes prepared from $FeSO_4 \cdot 7H_2O$ and DTCS with procedure I (Fe^{II}:DTCS = 1:2, dotted; 1:3, dashed) and with II (Fe^{II}:DTCS = 1:2, dash-dotted; 1:3, dash-double dotted).

LC-ESI MS analysis of the reaction products of NO with Fe-DTC complexes ($[Fe] = 1.0 \times 10^{-2}$ mol dm^{-3}; 25 °C) was also carried out using a Micromass (Manchester, UK) ZabSpec-Q mass spectrometer equipped with an ESI interface and HPLC system described above. The same apparatus and conditions were employed as in HPLC, but only 5–10% of the flow eluted from the column was introduced into the electrospray source to avoid choking up the electrospray unit. The ionization voltage was 4 kV. The analyses were performed in the negative mode.

Results and discussion

Oxidation state of NO trapping iron complexes

Fig. 1 shows absorption spectra of Fe–DTCS complexes prepared in nitrogen-bubbled medium (procedure I) and by the deoxygenation procedure with freeze–pump–thaw cycles (procedure II). In the case of iron(III) complexes, the same spectra, which are characteristic of $Fe^{III}(DTC)_3$ complexes [λ_{max}/nm = 510 (ε/dm^3 mol^{-1} cm^{-1} 1800) and 600 (sh)],[32] were obtained for both preparations (two solid lines are superimposed). In contrast, different spectra were observed for iron(II) complexes. Absorption spectra of the complex prepared by procedure I were similar to those of iron(III) complexes (Fe^{II}:DTCS = 1:3, dashed; 1:2, dotted). The decrease in absorbance of the 1:2 complex might be due to the deficiency of the ligand to form an iron(III) complex. On the other hand, when the complex was prepared by procedure II the spectra of a trace of iron(III) complexes, which is presumably formed by oxidation with a small amount of residual oxygen, was observed (Fe^{II}:DTCS = 1:2, dash-dotted; 1:3, dash-double dotted).

These observations clearly indicate that the iron(II) complexes prepared by procedure I are oxidized to Fe^{III}; that is, the brown color of the spin trap solution is an indication of oxidation. In practice, the solution of the complex prepared under a nitrogen atmosphere is added to the cultured cell, and the solution readily turns brown. Accordingly, it can be said that NO reacts with $Fe^{III}(DTC)_3$ complexes even if the complex is prepared from an iron(II) salt under a nitrogen atmosphere.[7a,b]

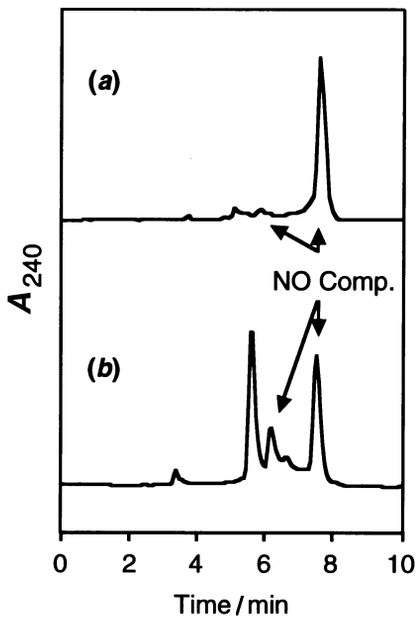


Fig. 2 HPLC profiles of (*a*) the reaction mixture of NO and $[Fe^{II}(DTCS)_2]^{2-}$, (*b*) the reaction mixture of NO and $[Fe^{III}(DTCS)_3]^{3-}$. UV absorption at 240 nm was measured. Conditions: [Fe] = 5.0×10^{-5} mol dm^{-3} in water–CH_3CN 60:40 (v/v), 40 °C. Tetra-*n*-butylammonium phosphate solution was added to the eluent as an ion pair reagent to give a final concentration of 5×10^{-3} mol dm^{-3}.

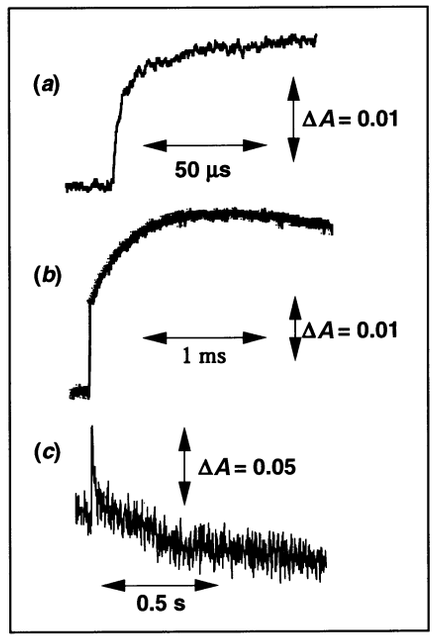


Fig. 3 Absorption changes after pulse radiolysis of $[Fe^{III}(DTCS)_3]^{3-}$ in the presence of $NaNO_2$ at pH 7.4 measured at 520 nm. The reaction mixture contained 2.5×10^{-4} mol dm^{-3} $[Fe^{III}(DTCS)_3]^{3-}$, 1.0×10^{-2} mol dm^{-3} $NaNO_2$, 0.1 mol dm^{-3} *t*-butyl alcohol, and 1.0×10^{-2} mol dm^{-3} potassium phosphate buffer (pH 7.4). The initial part of (*c*) is depicted in (*a*) and (*b*) on different time and absorbance scales.

Products of the reaction of NO with Fe^{II}/Fe^{III}–DTCS complexes

To obtain more detailed information about the reaction of NO with $Fe^{III}(DTC)_3$ we performed product analyses by HPLC. The reversed phase HPLC profiles clearly showed that the products in the reaction mixture of NO and $[Fe^{III}(DTCS)_3]^{3-}$ are quite different from those of $[Fe^{II}(DTCS)_2]^{2-}$. In the latter case only the peak of $[NO–Fe^{II}(DTCS)_2]^{2-}$ was detected [Fig. 2(*a*)], indicating that a simple addition reaction occurs [eqn. (1)]. In contrast, an additional peak at 5.5 min was observed with $[Fe^{III}(DTCS)_3]^{3-}$ [Fig. 2(*b*)]. This peak corresponds to neither DTCS ligand nor $[Fe^{III}(DTCS)_3]^{3-}$ whose elution profile in this eluent was similar to that of DTCS ligand. No extra peaks except for DTCS were detected in the reaction mixture of NO and DTCS ligand (data not shown). These facts suggest that the peak at 5.5 min is associated with a by-product of the reaction of NO and $[Fe^{III}(DTCS)_3]^{3-}$.

To identify the by-product, we then performed the product analysis by LC-ESI MS. A solution containing 1.0×10^{-2} mol dm^{-3} of $[Fe^{III}(DTCS)_3]^{3-}$ and an excess of NO was injected into the LC system, with settings the same as in normal HPLC analyses. In a chromatogram of the total ion content obtained by LC-ESI MS in the negative mode three components corresponding to HPLC profiles were found (see ESI supplementary material Fig. S1). As expected from the HPLC results, the components at 8 min 12 s whose mass numbers are 624 and 654 could be assigned to NO elimination $\{[Fe^{II}(DTCS)_2](n\text{-}Bu)_4N\}^-$ and $\{[NO–Fe^{II}(DTCS)_2](n\text{-}Bu)_4N\}^-$, respectively (Fig. S2). The elimination of NO seems to have occurred upon ionization because the two chromatograms are identical (Fig. S1). The ESI mass spectrum of the 5 min 31 s peak is well simulated if the product is a dimer of DTCS in which two DTCS bind through a disulfide bond (DTCS disulfide; Fig. S3). Thus, we conclude that the reaction products of NO with $[Fe^{III}(DTCS)_3]^{3-}$ are $[NO–Fe^{II}(DTCS)_2]^{2-}$ and DTCS disulfide.

In the reaction of NO with $[Fe^{II}(DTCS)_2]^{2-}$ only the components corresponding to NO and NO-eliminated complexes were detected in LC-ESI mass spectra.

Reaction of NO with $[Fe^{III}(DTCS)_3]^{3-}$

In a previous report, we have shown that NO is produced by the reaction of hydrated electrons (e_{aq}^-) with NO_2^- under deaerated conditions by the use of pulse radiolysis.[27] We followed the absorption change after pulse radiolysis of $[Fe^{III}(DTCS)_3]^{3-}$ in the presence of NO_2^-. In the absence of NO_2^-, e_{aq}^- reacted with $[Fe^{III}(DTCS)_3]^{3-}$ to form $[Fe^{II}(DTCS)_2]^{2-}$. This is apparent from the decrease in the absorbance in the range 340–600 nm (data not shown). In the presence of NO_2^-, e_{aq}^- reacted with both $[Fe^{III}(DTCS)_3]^{3-}$ and NO_2^-. Above 5.0×10^{-3} mol dm^{-3} NO_2^-, however, all the e_{aq}^- generated by pulse radiolysis reacted with NO_2^- to give NO, which was supported by the lack of an absorbance decrease due to the formation of $[Fe^{II}(DTCS)_2]^{2-}$.

After pulse radiolysis of 2.5×10^{-4} mol dm^{-3} $[Fe^{III}(DTCS)_3]^{3-}$ in the presence of 1.0×10^{-2} mol dm^{-3} $NaNO_2$ the absorption at 520 nm consisted of a faster [Fig. 3(*a*)] and a slower increase [Fig. 3(*b*)]. Subsequently, the decrease in absorption was observed on a timescale of seconds [Fig. 3(*c*)]. The possibility that these processes are due to reaction of the ligand DTCS can be excluded, since such absorption changes were not seen after pulse radiolysis of DTCS in the presence of 1.0×10^{-2} mol dm^{-3} $NaNO_2$ as a control experiment.

The kinetic difference spectra at 100 μs, 2 ms, and 2 s after pulse radiolysis of $[Fe^{III}(DTCS)_3]^{3-}$ and the static difference spectrum of $[NO–Fe^{II}(DTCS)_2]^{2-}$ minus $[Fe^{III}(DTCS)_3]^{3-}$ are shown in Fig. 4. The similarity between the 2 s spectrum and the static spectrum indicates that $[NO–Fe^{II}(DTCS)_2]^{2-}$ was formed as a final product after the pulse. The formation of $[NO–Fe^{II}(DTCS)_2]^{2-}$ was also confirmed by the EPR spectrum of the final product (data not shown). From these findings it can be concluded that NO reacted with $[Fe^{III}(DTCS)_3]^{3-}$ to form $[NO–Fe^{II}(DTCS)_2]^{2-}$ *via* intermediate species I and II, which correspond to the spectra at 100 μs and 2 ms, respectively. Assuming that NO quantitatively reacts with $[Fe^{III}(DTCS)_3]^{3-}$ to give the final product $[NO–Fe^{II}(DTCS)_2]^{2-}$, the concentration of NO is calculated to be 2.0×10^{-5} mol dm^{-3} from the change in absorbance at 520 nm and the absorption coefficient ($\Delta\varepsilon_{520}$/dm^3 mol^{-1} cm^{-1} 1500) between $[Fe^{III}(DTCS)_3]^{3-}$ and $[NO–Fe^{II}(DTCS)_2]^{2-}$.

In the initial process the formation of the intermediate I, with a broad absorption from 400 to 500 nm, obeyed

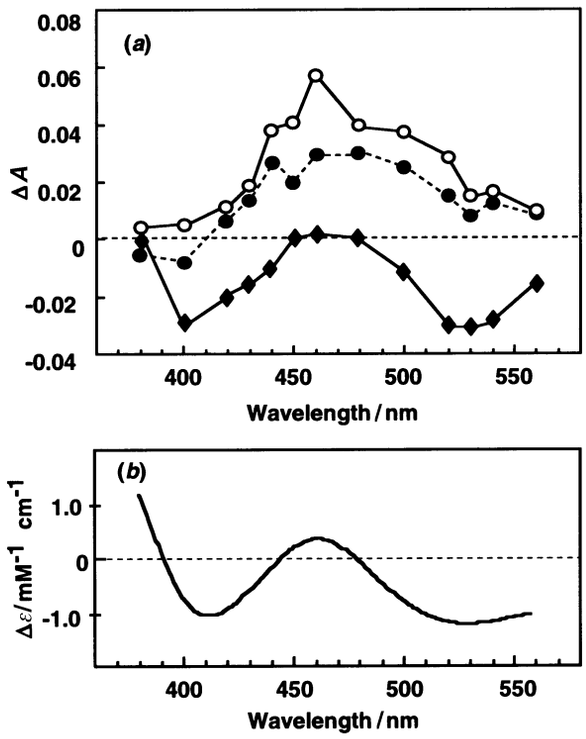


Fig. 4 (*a*) Difference spectra at 100 μs (●), 2 ms (○), and 2 s (◆) after pulse radiolysis of $[Fe^{III}(DTCS)_3]^{3-}$ in the presence of $NaNO_2$ at pH 7.4 in phosphate buffer solution. The concentrations of $[Fe^{III}(DTCS)_3]^{3-}$ and $NaNO_2$ were 2.5×10^{-4} and 1.0×10^{-2} mol dm^{-3}, respectively. (*b*) Static difference spectra of $[NO–Fe^{II}(DTCS)_2]^{2-}$ minus $[Fe^{III}(DTCS)_3]^{3-}$.

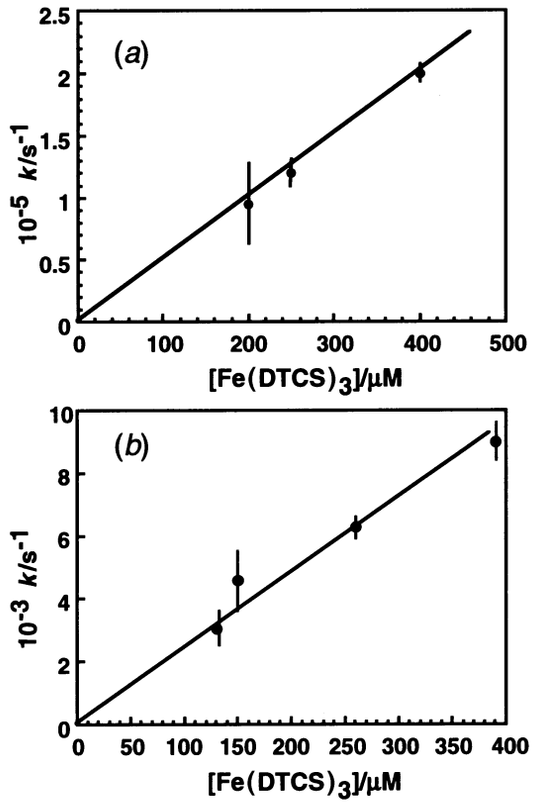


Fig. 5 Dependence on $[Fe^{III}(DTCS)_3]^{3-}$ concentration of (*a*) the pseudo-first-order rate constant for the reaction of NO with $[Fe^{III}(DTCS)_3]^{3-}$, and (*b*) the first-order rate constant for the reaction of $[NO–Fe^{III}(DTCS)_3]^{3-}$ with $[Fe^{III}(DTCS)_3]^{3-}$ [1.0×10^{-2} mol dm^{-3} $NaNO_2$, 0.1 mol dm^{-3} *t*-butyl alcohol, and 1.0×10^{-2} mol dm^{-3} potassium phosphate buffer (pH 7.4)].

pseudo-first-order kinetics and its rate constant increased with the concentration of $[Fe^{III}(DTCS)_3]^{3-}$ [Fig. 5(*a*)]. For these experiments, the concentration of NO is approximately (1.0–2.0) $\times 10^{-5}$ mol dm^{-3} and the $[Fe^{III}(DTCS)_3]^{3-}$ concentration was varied in the range (1.5–4.0) $\times 10^{-4}$ mol dm^{-3}. This indicates that the faster phase is a consequence of bimolecular reaction of NO with $[Fe^{III}(DTCS)_3]^{3-}$, eqn. (2). From the slope of

$$[Fe^{III}(DTCS)_3]^{3-} + NO \longrightarrow [NO–Fe^{III}(DTCS)_3]^{3-} \quad (2)$$

Fig. 5(*a*), the second-order rate constant of the reaction is calculated to be $(4.8 \pm 0.9) \times 10^8$ dm^3 mol^{-1} s^{-1}. This is comparable to the k_1 value, $(1.1 \pm 0.3) \times 10^8$ dm^3 mol^{-1} s^{-1}, of the reaction of NO with $[Fe^{II}(ProDTC)_2]^{2-}$ (ProDTC = L-proline dithiocarbamate).[16] This indicates that Fe–DTC systems are by no means inferior to that of NO with haemoglobin (3.7×10^7 dm^3 mol^{-1} s^{-1}),[33] which is a potent NO trap in living systems.

In the second phase the intermediate II with an absorption maximum at 460 nm [Fig. 4(*a*)] appeared in the time range of milliseconds. The rate constant of this process was affected by the concentration of $[Fe^{III}(DTCS)_3]^{3-}$ [Fig. 5(*b*)], not that of the intermediate I generated initially (data not shown). This suggests that the formation of intermediate II can be attributed to the reaction of I with unchanged $[Fe^{III}(DTCS)_3]^{3-}$. The second-order rate constant of this process is estimated to be $(2.5 \pm 0.3) \times 10^7$ dm^3 mol^{-1} s^{-1}.

Subsequently, very slow absorption changes were observed in the time range of seconds (Figs. 3(*c*) and S4). This process was affected by neither the concentration of $[Fe^{III}(DTCS)_3]^{3-}$ nor that of the intermediate I (data not shown). It is particularly interesting that the rate of this process was remarkably accelerated in the presence of additional DTCS (5×10^{-5} mol dm^{-3}) (Fig. S4), though the intermediates observed after the pulse and the rate constants of the formation of intermediates I and II were not affected. This suggests that intermediate II reacts with free DTCS. The second-order rate constant of the reaction is estimated to be $(2.6 \pm 0.5) \times 10^6$ dm^3 mol^{-1} s^{-1}.

Identification of intermediate II

The most characteristic feature of intermediate II is its absorption maximum at 460 nm, and a quite similar spectrum was seen for the iron(IV) complex with diisopropyldithiocarbamate [(*i*-Pr)$_2$DTC] in acetone.[29b] Moreover, it has been reported that $Fe^{IV}(DTC)_3$ complexes react with dithiocarbamate to form $Fe^{III}(DTC)_3$ and DTC disulfide.[29b] Thus, we investigated the reaction of iron(IV) dithiocarbamate with dithiocarbamate. Owing to the instability of iron(IV) species in water, we could not prepare $[Fe(DTCS{\cdot}Na)_3]ClO_4$ in water by a modified method of Chant *et al.*[29b] or others. DETC and acetone were used instead of DTCS and water, where the solvent and the substituent effects on the absorption spectra of Fe–DTC complexes are small. The complex $[Fe^{IV}(DETC)_3]^+$ was fairly stable in acetone without additional DETC and only a little degradation was observed within 2 h. Addition of free DETC immediately caused spectral changes of the $[Fe^{IV}(DETC)_3]^+$, and finally gave a spectrum of $Fe^{III}(DETC)_3$ [Fig. 6(*a*)]. The reaction was very fast when an acetone solution of DETC ligand was added; therefore, we added DETC as a solid and obtained the spectra at 20 s intervals. The difference spectra with a peak at 465 nm and an isosbestic point at 422 nm [Fig. 6(*b*)] are very similar to those obtained by pulse radiolysis [Fig. 4(*a*)]. The spectral similarity and the decay acceleration of the intermediate II by free DTCS strongly suggest that II is an iron(IV) species.

Postulated mechanisms of reductive nitrosylation

Taking the product analyses and pulse-radiolysis data into consideration, we can propose a mechanism for reductive nitrosylation of this system (Scheme 1). If an iron(IV) species is involved as the intermediate II in the reaction, $[Fe^{III}(DTCS)_3]^{3-}$ donates an electron to the $NO–Fe^{III}$ complex (intermediate I) to

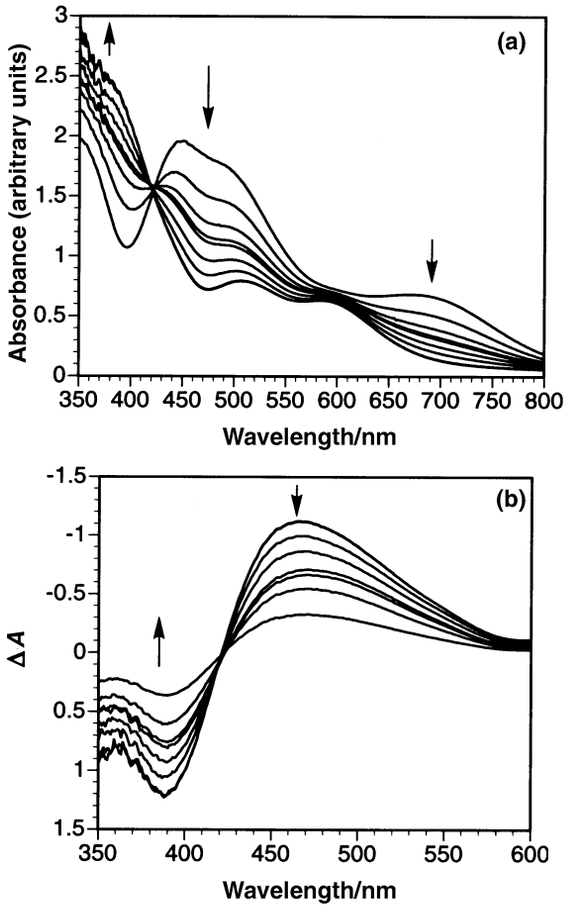
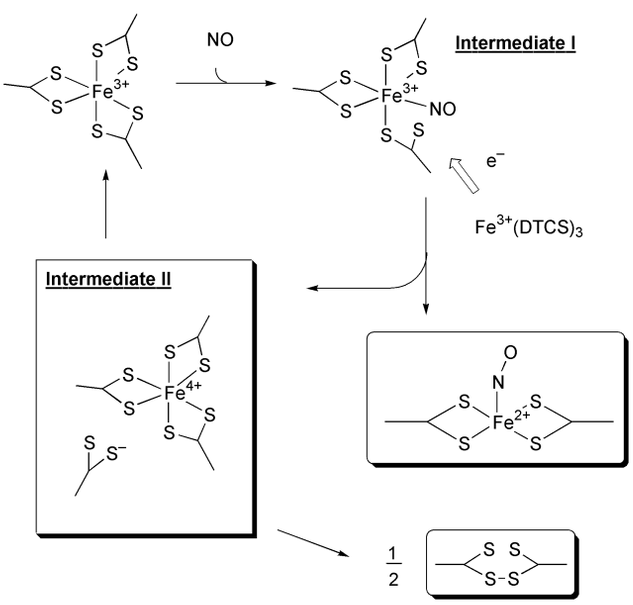


Fig. 6 (*a*) Absorption changes after addition of DETC ligand to $[Fe^{IV}(DETC)_3]^+$ in acetone solution (2.5×10^{-4} mol dm^{-3}). Successive scans are 20 s apart. (*b*) Changes in difference spectra after addition of DETC minus $[Fe^{IV}(DETC)_3]^+$. Successive scans are 20 s apart. Note that the sign of the vertical axis (ΔA) is inverse to that in Fig. 4(*a*), because the observed reaction is the inverse process of that of Fig. 4(*a*).



Scheme 1 Possible mechanism for reductive nitrosylation in Fe^{III}-$(DTC)_3$ complexes.

form $[NO–Fe^{II}(DTCS)_2]^{2-}$ and $[Fe^{IV}(DTCS)_3]^{2-}$ [eqn. (3)], respectively, where DTCS indicates free $DTCS^{2-}$.

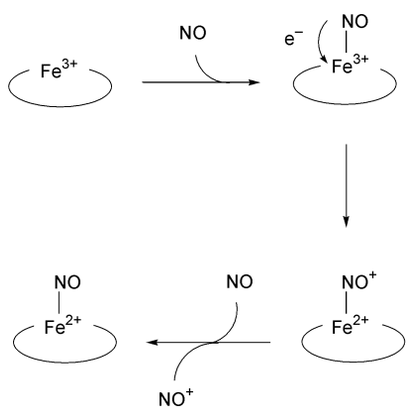
$$[NO–Fe^{III}(DTCS)_3]^{3-} + [Fe^{III}(DTCS)_3]^{3-} \longrightarrow [NO–Fe^{II}(DTCS)_2]^{2-} + [Fe^{IV}(DTCS)_3]^{2-} + DTCS \quad (3)$$

We assume that this electron transfer is induced by a positive shift of the reduction potential of $[Fe^{III}(DTCS)_3]^{3-}$ with NO binding. Compared with $[Fe^{II}(DTCS)_2]^{2-}$ relative to $[Fe^{III}(DTCS)_3]^{3-}$ in the absence of NO ligand, the NO bound form of $[Fe^{II}(DTCS)_2]^{2-}$ is expected to be more stable than the Fe^{III}–NO species. In our preliminary electrochemical study, $[Fe^{III}(DTCS)_3]^{3-}$ exhibited a quasi-reversible cyclic voltammetric wave in water with $E_{1/2} = 0.29$ V *vs.* Ag–AgCl ($\Delta E_p = 59$ mV; data not shown). The data suggest that the high-valent species of $Fe(DTC)_3$ is easily generated under fairly mild conditions.

In the third step the complex $[Fe^{IV}(DTCS)_3]^{2-}$ reacts with free DTCS to form $[Fe^{III}(DTCS)_3]^{3-}$ and DTCS disulfide.

A homolytic scission of DTCS ligand from both $[NO–Fe^{III}(DTCS)_3]^{3-}$ and unchanged $[Fe^{III}(DTCS)_3]^{3-}$ complexes may be an alternative reductive nitrosylation mechanism for this system, because two DTCS$^{\bullet}$ give a DTCS disulfide, and the $NO–Fe^{III}$ complex is reduced to $[NO–Fe^{II}(DTCS)_2]^{2-}$. This type of reaction in which a metal center is reduced by a ligand elimination was reported by Yordanov *et al.* with copper(II) dithiophosphinates, where the reaction is triggered by light.[34] However, this homolytic scission mechanism can be excluded because of the following two reasons. (1) Spectroscopic data clearly show the concentration dependence of the decay process of intermediate II on free DTCS (Fig. S4). However, free DTCS is not involved in the mechanism. (2) Although $[Fe^{II}(DTCS)_2]^{2-}$ is an only candidate for intermediate II in the mechanism, it has no intense absorption band in the visible region (Fig. 1 and the results of pulse radiolysis).

Reductive nitrosylation is thus far known in haem proteins and iron porphyrin chemistry.[35–39] The detailed mechanism has not been established yet, but the outline of the reaction is considered to be as in Scheme 2. In brief, one Fe^{III}–L (L = protein



Scheme 2 Proposed mechanism for reductive nitrosylation in haem/porphyrin complexes.

or porphyrin) reacts with two NO molecules to form an NO–Fe^{II}–L and an NO^+. However, the reductive nitrosylation of $[Fe^{III}(DTCS)_3]^{3-}$ cannot be explained by this mechanism. The concentration of NO generated by the pulse is low, $(1.0–2.0) \times 10^{-5}$ mol dm^{-3}, in $(1.5–4.0) \times 10^{-4}$ mol dm^{-3} $[Fe^{III}(DTCS)_3]^{3-}$, so that there is no excess of NO in the system. Secondly, unchanged $[Fe^{III}(DTCS)_3]^{3-}$ is likely to be involved in the reductive nitrosylation process. Thirdly, products like DTCS disulfide are not produced by the mechanism of Scheme 2. In the original meaning of the term 'reductive nitrosylation', the reducing agent is NO and a metal ion reacts with two equivalents of NO [eqn. (4)].[35] The proposed mechanism is not

$$Fe^{III}\text{–enzyme} + 2NO \longrightarrow NO–Fe^{II}\text{–enzyme} + NO^+ \quad (4)$$

strictly reductive nitrosylation in that sense. In this system, however, Fe^{III} is actually reduced by the action of NO [eqn. (5)]

$$2Fe^{III}(DTC)_3 + NO \longrightarrow NO\text{–}Fe^{II}(DTC)_2 + Fe^{III}(DTC)_3 + \tfrac{1}{2}\text{DTC disulfide} \quad (5)$$

in which NO seems to play a role of trigger for the intercomplex electron transfer by modulating redox potentials. We, therefore, propose this type of the reaction as a novel category of reductive nitrosylation.

Conclusion

This work reports the novel reaction mechanism of reductive nitrosylation realized in iron complexes with dithiocarbamate derivatives. Although an endogenous NO detection method by Fe–DTC complexes is widely used, the reaction mechanism has been unclear. We have shown here the first spectroscopic evidence for the reductive nitrosylation reaction, which enables us to detect endogenous NO easily. In the light of the results obtained by UV/VIS absorption, HPLC, LC-ESI MS, and pulse radiolysis, we postulate a mechanism involving Fe^{IV} as a by-product (Scheme 1). To our knowledge, this is a new type of reductive nitrosylation observed in non-haem iron complexes.

In this experiment the reductant is $[Fe^{III}(DTCS)_3]^{3-}$. This is probably because no other compounds are involved in this simple system. In practical use, there are many candidates for reductants in biological system; hence, other compounds may also reduce the transient $[NO\text{–}Fe^{III}(DTCS)_3]^{3-}$ species (intermediate I). In that case, intermediate II (iron(IV) species) is not produced; however, the reduction is certainly triggered by NO binding to the iron centre.

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References

1 P. L. Feldman, O. W. Griffith and D. J. Stuehr, *Chem. Eng. News*, 1993, **71**, 26.
2 J. F. Kerwin, Jr., J. R. Lancaster, Jr. and P. L. Feldman, *J. Med. Chem.*, 1995, **38**, 4343.
3 For example, *Nitric Oxide: Principles and Actions*, ed. J. Lancaster, Jr., Academic Press, San Diego, CA, 1996, chs. 3–7, pp. 111–257.
4 P. Mordvintcev, A. Mülsch, R. Busse and A. Vanin, *Anal. Biochem.*, 1991, **199**, 142.
5 A. Mülsch, A. Vanin, P. Mordvintcev, S. Hauschildt and R. Busse, *Biochem. J.*, 1992, **288**, 597; L. N. Kubrina, W. S. Caldwell, P. I. Mordvintcev, I. V. Malenkova and A. F. Vanin, *Biochim. Biophys. Acta*, 1992, **1099**, 233; A. F. Vanin, P. I. Mordvintcev, S. Hauschildt and A. Mülsch, *Biochim. Biophys. Acta*, 1993, **1177**, 37; V. D. Mikoyan, N. V. Voevodskaya, L. N. Kubrina, I. V. Malenkova and A. F. Vanin, *Biochim. Biophys. Acta*, 1995, **1269**, 19; V. D. Mikoyan, L. N. Kubrina, V. A. Serezhenkov, R. A. Stukan and A. F. Vanin, *Biochim. Biophys. Acta*, 1997, **1336**, 225.
6 A. Komarov, D. Mattson, M. M. Jones, P. K. Singh and C.-S. Lai, *Biochem. Biophys. Res. Commun.*, 1993, **195**, 1191; C.-S. Lai and A. M. Komarov, *FEBS Lett.*, 1994, **345**, 120; A. M. Komarov and C.-S. Lai, *Biochim. Biophys. Acta*, 1995, **1272**, 29; G. M. Pieper and C.-S. Lai, *Biochem. Biophys. Res. Commun.*, 1996, **219**, 584.
7 (*a*) Y. Kotake, *Methods Enzymol.*, 1996, **268**, 222; (*b*) Y. Kotake, T. Tanigawa, M. Tanigawa, I. Ueno, D. R. Allen and C.-S. Lai, *Biochim. Biophys. Acta*, 1996, **1289**, 362; (*c*) T. Miyajima and Y. Kotake, *Biochem. Biophys. Res. Commun.*, 1995, **215**, 114; (*d*) T. Tabatabaie, C. Stewart, Q. Pye, Y. Kotake and R. A. Floyd, *Biochem. Biophys. Res. Commun.*, 1996, **221**, 386; (*e*) L. A. Reinke, D. R. Moore and Y. Kotake, *Anal. Biochem.*, 1996, **243**, 8; (*f*) T. Miyajima and Y. Kotake, *Free Rad. Biol. Med.*, 1997, **22**, 463.
8 T. Yoshimura, S. Fujii, H. Yokoyama and H. Kamada, *Chem. Lett.*, 1995, 309.
9 T. Yoshimura, H. Yokoyama and S. Fujii, *J. Magn. Reson. Anal.*, 1997, **3**, 125 and refs. therein.
10 T. Yoshimura, H. Yokoyama, S. Fujii, F. Takayama, K. Oikawa and H. Kamada, *Nat. Biotechnol.*, 1996, **14**, 992; S. Fujii, Y. Suzuki, T. Yoshimura and H. Kamada, *Am. J. Physiol.*, 1998, **274**, G857.
11 S. Sato, T. Tominaga, T. Ohnishi and S. T. Ohnishi, *Biochim. Biophys. Acta*, 1993, **1181**, 195; S. Sato, T. Tominaga, T. Ohnishi and S. T. Ohnishi, *Brain Res.*, 1994, **647**, 91; T. Tominaga, S. Sato, T. Ohnishi and S. T. Ohnishi, *J. Cereb. Blood Flow Metab.*, 1994, **14**, 715; S. Sato, T. Tominaga, T. Ohnishi and S. T. Ohnishi, in *Central Nervous System Trauma: Research Techniques*, eds. S. T. Ohnishi and T. Ohnishi, CRC Press, Boca Raton, FL, 1995, pp. 455–470; S. T. Ohnishi, in *Nitric Oxide Protocols*, ed. M. A. Titheragde, Humana Press, Totawa, NJ, 1998, pp. 129–153; P. Kuppusamy, S. T. Ohnishi, Y. Numagami, T. Ohnishi and J. L. Zweier, *J. Cereb. Blood Flow Metab.*, 1995, **15**, 899.
12 G. Wallis, D. Brackett, M. Lerner, Y. Kotake, R. Bolli and P. B. McCay, *Shock*, 1996, **6**, 274.
13 V. Quaresima, H. Takehara, K. Tsushima, M. Ferrari and H. Utsumi, *Biochem. Biophys. Res. Commun.*, 1996, **221**, 729.
14 K. Tsuchiya, M. Takasugi, K. Minakuchi and K. Fukuzawa, *Free Rad. Biol. Med.*, 1996, **21**, 733.
15 J. L. Zweier, P. Wang and P. Kuppusamy, *J. Biol. Chem.*, 1995, **270**, 304; J. L. Zweier, P. Wang, A. Samouilov and P. Kuppusamy, *Nat. Med.*, 1995, **1**, 804; P. Kuppusamy, P. Wang, A. Samouilov and J. L. Zweier, *Magn. Reson. Med.*, 1996, **36**, 212.
16 S. V. Paschenko, V. V. Khramtsov, M. P. Skatchkov, V. F. Plyusnin and E. Bassenge, *Biochem. Biophys. Res. Commun.*, 1996, **225**, 577.
17 H. Fujii, J. Koscielniak and L. J. Berliner, *Magn. Reson. Med.*, 1997, **38**, 565; H. Fujii and L. J. Berliner, *Phys. Med. Biol.*, 1998, **43**, 1949.
18 A. Mülsch, P. Mordvintcev, E. Bassenge, F. Jung, B. Clement and R. Busse, *Circulation*, 1995, **92**, 1876.
19 S. W. Norby, J. A. Weyhenmeyer and R. B. Clarkson, *Free Rad. Biol. Med.*, 1997, **22**, 1.
20 A. M. Komarov, J. H. Kramer, I. T. Mak and W. B. Weglicki, *Mol. Cell. Biochem.*, 1997, **175**, 91.
21 H. Nakagawa, N. Ikota, T. Ozawa, T. Masumizu and M. Kohno, *Biochem. Mol. Biol. Int.*, 1998, **45**, 1129.
22 S. Lecour, V. Maupoil, O. Siri, A. Tabard and L. Rochette, *J. Cardiovasc. Pharmacol.*, 1999, **33**, 78.
23 V. Misik and P. Riesz, *J. Phys. Chem.*, 1996, **100**, 17986.
24 S. Pou, H. J. Halpern, P. Tsai and G. M. Rosen, *Acc. Chem. Res.*, 1999, **32**, 155.
25 S. Fujii, T. Yoshimura and H. Kamada, *Chem. Lett.*, 1996, 785.
26 J. S. Beckman, D. A. Wink and J. P. Crow, in *Methods in Nitric Oxide Research*, eds. M. Feelisch and J. S. Stamler, John Wiley & Sons, Chichester, 1996, pp. 61–70.
27 K. Kobayashi, M. Miki and S. Tagawa, *J. Chem. Soc.*, *Dalton Trans.*, 1995, 2885.
28 O. A. Ileperuma and R. D. Feltham, *Inorg. Synth.*, 1976, **16**, 5.
29 A. R. Hendrickson, R. L. Martin and N. M. Rohde, *Inorg. Chem.*, 1974, **13**, 1933; R. Chant, A. R. Hendrickson, R. L. Martin and N. M. Rohde, *Inorg. Chem.*, 1975, **14**, 1894.
30 K. Kobayashi, A. Koppenhöfer, S. J. Ferguson and S. Tagawa, *Biochemistry*, 1997, **36**, 13611.
31 K. Kobayashi, M. Tsubaki and S. Tagawa, *J. Biol. Chem.*, 1998, **273**, 16038.
32 A. H. White, R. Roper, E. Kokot, H. Waterman and R. L. Martin, *Aust. J. Chem.*, 1964, **17**, 294; A. H. Ewald, R. L. Martin, E. Sinn and A. H. White, *Inorg. Chem.*, 1969, **8**, 1837.
33 M. P. Doyle and J. W. Hoekstra, *J. Inorg. Biochem.*, 1981, **14**, 351.
34 N. D. Yordanov, V. Alexiev, C. Malakova and A. Shishkov, *Transition Met. Chem.*, 1983, **8**, 210.
35 K. G. Caulton, *Coord. Chem. Rev.*, 1975, **14**, 317.
36 J. C. W. Chien, *J. Am. Chem. Soc.* 1969, **91**, 2166.
37 B. B. Wayland and L. W. Olson, *J. Am. Chem. Soc.*, 1974, **96**, 6037.
38 T. Yoshimura, S. Suzuki, A. Nakahara, H. Iwasaki, M. Masuko and T. Matsubara, *Biochemistry*, 1986, **25**, 2436; T. Yoshimura and S. Suzuki, *Inorg. Chim. Acta*, 1988, **152**, 241.
39 M. Hoshino, M. Maeda, R. Konishi, H. Seki and P. C. Ford, *J. Am. Chem. Soc.*, 1996, **118**, 5702.