Low-temperature oxidation of [4Fe-4S] analogues: generation of a Fe/S cluster spectroscopically similar to the 3-Fe clusters in the 3-Fe ferredoxins

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magnetic susceptibilities indicative of spin states significantly higher than 1/2\(^2\) (Table III). The room temperature magnetic susceptibility and the chemical shifts of the protons of the (4Fe-4Se)\(^+\) clusters of reduced Se-substituted Cp Fd are larger than those of native reduced Cp Fd (Figure 2, Table III). These observations may be correlated with the occurrence of high-multiplicity spin states at low temperature.

At cryogenic temperatures, the reduced Se-substituted clostridial ferredoxins display three spin states, namely \( S = 1/2, S = 3/2 \), and \( S = 5/2 \), and one may wonder whether a similar spin-state heterogeneity occurs at functionally relevant temperatures. The \(^1\)H NMR spectra of the reduced Fd from Bacillus polymyxa\(^4\) and Bacillus stearothermophilus,\(^1,3\) which contain a single (4Fe-4S)\(^+\) cluster, display eight to ten manthetically shifted proton resonances, out of the 0–10 ppm range. The spectra of the reduced clostridial ferredoxins involve ca. twice as many (17–20) shifted proton resonances as those of the ferredoxins containing a single cluster\(^6\) (Table II). It may therefore be inferred that the two clusters in clostridial Fd differ from each other with respect to the chemical shifts of their neighboring protons. The reduced Se-substituted Cp Fd displays no more shifted proton resonances than its native counterpart (Table II), which suggests the presence of only two different types of (4Fe-4Se)\(^+\) clusters in the former protein; the occurrence of three sets of spin states would imply that ca. eight to ten proton resonances have escaped observation. This seems unlikely, since no additional proton resonances have been observed in the 170–330 ppm range. Proton resonances occurring at lower field would probably be difficult to detect, due to line broadening. They would however be associated with clusters having high magnetic susceptibility, and therefore the presence of very low field resonances would result in a bulk magnetic susceptibility value even higher than the measured one (6.4 mT; see Table III). The \(^1\)H NMR data thus show that two different spin-state ladders occur at room temperature. The relationship between these two spin ladders and the three low-temperature spin states remains to be established. In any case, the presence, in the spectra of [2(4Fe-4Se)\(^+\)] ferredoxins, of two sets of proton resonances differing strongly by their chemical shifts and by the temperature dependencies of these shifts indicate that the (4Fe-4Se)\(^+\) clusters bound to clostridial Fd polypeptide chains possess unusual magnetic properties not only at low temperature but also at room temperature. The evidence that high-spin states of (4Fe-4X)\(^+\) clusters are not simply a freezing artifact, at least in the presently investigated case, clearly point to the functional relevance of the high-spin states found in some native (4Fe-4S)\(^+\) proteins.\(^24\)

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**Low-Temperature Oxidation of [4Fe-4S] Analogues. Generation of an Fe/S Cluster Spectroscopically Similar to the 3-Fe Clusters in the 3-Fe Ferredoxins**

J. P. Weterings, T. A. Kent, and R. Prins*

Received March 3, 1986

The oxidation of the cubane cluster compound \([Fe_5S_4(SR)_4]^{2-}\) can be directed to yield a 3-Fe cluster (i.e. a Fe/S cluster spectroscopically similar to the 3-Fe centers in the 3-Fe ferredoxins) by the choice of DMF/water as reaction medium, \(K_2[Fe(CN)_6]\) as oxidant, and a low reaction temperature. The resulting compound at 4.2 and 40 K yields Mössbauer spectra that are typical for a 3-Fe cluster. At 40 K a quadrupole splitting of 0.58 mm/s and a isomer shift of 0.31 mm/s are observed. Its X-band ESR signal at \( g = 2.01 \) has a width of about 3 mT and displays the same shape as that of 3-Fe proteins, including the remarkably broad wing at the high-field side of the spectrum. The influences of the reaction medium, the reagents, and the initial concentrations on the chemistry of reaction have been investigated.

**Introduction**

Since 1980 it has been well established that *Azotobacter vinelandii* Fd (ferredoxin) \(1\) crystals contain a 3-Fe cluster\(^4\) with an Fe\(_S\)S\(_4\)L\(_4\) ring structure\(^4\) (where \( L = RS\) or RO\(^-\); Figure 1a). A second type of 3-Fe cluster with an Fe\(_S\)S\(_4\)L\(_3\) cap structure (Figure 1b) has been postulated for Aconitase on the basis of EXAFS measurements.\(^5\) This has been supported by X-ray diffraction measurements.\(^6\) Another type of 3-Fe cluster has recently been found in denatured Aconitase and proved to have a linear structure\(^7\) (Figure 1c). Of these three structures the cap type seems to be the most common.\(^3\) It probably also is the structure of the 3-Fe cluster in *A. vinelandii* Fd 1 solutions.\(^3,10\) The native 3-Fe structures typically display axial \( S = 3/2 \) ESR spectra slightly above \( g = 2 \) with a width of about 3 mT. When first observed, these resonances were confused with the HP (high-potential protein) signal. The linear 3-Fe cluster is readily distinguished by its \( S = 1/2 \) ESR signal around \( g = 4.3 \) and \( g = 9.6 \). Until recently it was thought that 3-Fe clusters might only occur in native 3-Fe proteins, including the remarkably broad wing at the high-field side of the spectrum. The influences of the reaction medium, the reagents, and the initial concentrations on the chemistry of reaction have been investigated.

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(1) Eindhoven University of Technology.
(2) Gray Freshwater Biological Institute.
be degradation products, induced during protein isolation, with no biological relevance. However, in the case of succinate dehydrogenase their necessity for enzymatic activity has now been proved.13

Adequate model compounds for the native 3-Fe clusters have not been isolated as yet. The few compounds reported to have a cap structure12,13 contain organic sulfur in a number of places where an analogue would have inorganic sulfur. Compounds having the ring structure either display the same property14 or have dimerized to a prismane structure.15 Only for the linear 3-Fe cluster has a fully satisfactory analogue been reported.14

Biochemically there are two reactions leading to a 3-Fe cluster. The first is self-assembly from iron, sulfide, and apoprotein.16 We have not been able to observe this reaction with synthetic tri-cysteiny1 peptides.17 The second type of reaction is oxidation of the cubane clusters, [Fe4S4L4I2-]. Apparently the 3-Fe cluster of all known 3-Fe proteins can be formed by this reaction. Yet oxidation of cubanes does not necessarily produce a 3-Fe cluster. One-electron oxidation results in the formation of a HP4-like analogue if bulky ligands are employed18 or if a special solvent prevents further reactions.19 If halide (X-) is present, [Fe4S4(SR)4]2-20 can be oxidized by X2 or another oxidant to yield [Fe4S4X4]2- and disulfide.20 Further oxidation in a polar medium results in the [Fe4S4X4]2- dianion.21 Finally, it has been reported that in nonpolar solvents cubane can be converted into [Fe4S4L4]+ clusters (prismanes) before breaking up into the dianionic species.22 It has been pointed out that even counterions like Et4N+ affect the course of this reaction, probably by stabilizing intermediates instead of through selective crystallization.23

Clearly, the result of a cubane oxidation is strongly dependent on the exact reaction circumstances. We report here on low-temperature oxidation generating the 3-Fe cluster in solution and its characterization by ESR and Mössbauer spectroscoopy.

Experimental Section

Cubane iron-sulfur clusters24 and [Fe(C5H7)2](BF4)25 were synthesized according to the literature. High-potential protein solutions of Chromatium strain D and Thioctpsa roseopersicina were kindly supplied by Dr. K. K. Rao. If not stated otherwise “buffer” signifies a 50 mM Tris-HCl solution of pH 7.5. 25Fe of 95% isotopic purity was bought (as a metal) from Intersales-Holland B.V. (Hengelo, The Netherlands) and converted to FeCl3 by heating (350 °C in a Cl2 flow. All other chemicals and solvents were of reagent grade, purchased from Aldrich Chemie and used without further purification.

The Mössbauer equipment has been described before.27 Isomer shifts are quoted relative to the centroid of the spectrum of iron metal with the 57Co source and the iron foil at room temperature. X-Band EPR spectra were recorded with a Varian E-15 spectrometer equipped with an Oxford Instruments ESR-9 continuous-flow cryostat. All ESR spectra were recorded at a microwave frequency of 9.7 GHz, a modulation amplitude of 0.5 mT, and a modulation frequency of 100 kHz. Copper(II) chloride was used as a quantitative reference (Merck A.A.S. standard). A BBC microcomputer was interfaced to the spectrometer for integration according to the method of moments.28 The same computer was used for kinetic calculations.

Aerobic handling of solutions was effected by the usual vacuum-line/syringe methods. A glass kit consisting of a sample holder and a reaction vessel connected by a siphon was immersed as a whole in an ethanol-filled Dewar. The bath was cooled by a thermostated flow of cold nitrogen gas. The standard temperature of the bath was –40 °C. Separately, nitrogen gas was flushed through a metal cooling loop (–40 °C) via the sample holder into the reaction mixture to provide mingling during the reaction. By reversal of the flow direction in the kit, a sample of the mixture could be transferred to a Mössbauer cell or to an ESR tube. The reaction in the sample was then stopped by quickly immersing it in liquid nitrogen. Once frozen, the top of the kit was opened and the sample at the bottom of the kit remained at low temperature. In a typical experiment 6 mL of a 4 mM solution of (NMMe)2[Fe4S4(S-t-Bu)4]

Figure 1. Structures of the ring (a), cap (b), and linear (c) 3-Fe cluster.

Figure 2. First-derivative ESR spectra of [Fe4S4(S-t-Bu)4]2-/DMF, 4 mM, oxidized at –40 °C by 1 equiv of [Fe(CN)6]3-/buffer: (a) spectrum sampled at –40 °C, gain 200; (b) spectrum after 2 min of incubation at room temperature, gain 1000; (c) spectrum after 4 min of incubation at room temperature. Spectra were measured at 4.2 K, with a microwave power of 0.5 mW.
of water was reduced to 1%. Again the ESR spectrum showed unreacted ferricyanide. The reaction was also run at -25 °C when cyanide was replaced by \([\text{Fe(C5H5)}_2](\text{BF}_4)\) or when DMF was used to keep the potassium ferricyanide in solution as the amount of two-thirds of its intensity. The elaborate sample preparation procedure that we tried were thus seen to be unfavorable. Isolation of the 3-Fe cluster will therefore be difficult. Furthermore, the lines of the [4Fe-4S] cluster are broad and non-Lorentzian. To approximate better the line shape, we fitted these lines with two nested Lorentzian pairs of equal area. The results were \(\Delta E_x(1) = 1.32 \text{ mm/s, } \Delta E_y(2) = 1.05 \text{ mm/s, } \delta_x(1) = 0.82 \) mm/s, and \( \Gamma(1) = 0.25 \text{ mm/s.} \) The average values are quoted above. An analogous fit to the 4.2 K data was done. To the first order, subtraction of the 4.2 K signal of \(\text{D. gigas } \text{Fd II (28\%) total }^{57}\text{Fe}\) from the spectrum of Figure 5b cancels the signal of the 3-Fe model compound. A fit to the resulting difference spectrum yielded for the [4Fe-4S] cluster \(\Delta E_x(1) = 1.34 \text{ mm/s, } \Delta E_y(2) = 1.08 \text{ mm/s, } \delta_x(1) = 0.45 \text{ mm/s, } \delta_y(2) = 0.26 \text{ mm/s, and } \Gamma(1) = 0.28 \text{ mm/s.}\)

in DMF (N,N-dimethylformamide) was cooled to -40 °C. One equivalent of \(\text{K}_3[\text{Fe(CN)}_6]\) dissolved in 1.5 mL of buffer at room temperature was injected in one squirt to avoid freezing in the needle. Mixing water and DMF is an exothermic process. The temperature profile was measured by a thermocouple in the reactor. It showed a jump from -40 °C to -22 °C upon addition of the water. After 1 min the temperature was -33 °C, and after 4 min the mixture reached -40 °C.

Results and Discussion

Stability. A sample made by the standard procedure (described under Experimental Section) and measured at 4.2 K showed the typical 3-Fe ESR spectrum depicted in the upper trace of Figure 2. A longer reaction time, up to 120 min, at -40 °C proved to be of no influence. The reaction is finished within a few minutes, and the product is stable at this temperature. Measurement at higher temperatures (>30 K) showed the presence of low concentrations of other radicals, indicating that the conversion of the cubane to the 3-Fe cluster was not complete. The fact that these species are observed at relatively high temperatures (at least up to 60 K) characterizes them as probably of organic nature. A standard sample kept at room temperature for 40 s showed a loss of two-thirds of its intensity. The elaborate sample preparation is thus justified. Incubation for 2 and 4 min at room temperature caused an almost complete disappearance of the 3-Fe signal and the dominance of the ESR spectrum by resonances from unidentified radicals (Figure 2b,c). These resonances are roughly similar to those found in excessively oxidized \(\text{A. vinelandii Fd I}\). They are different from reduced 2-Fe cluster signals, which usually show resonances up to g values of 1.89.

Variations of Reagents and Solvents. In a number of experiments the components of the standard procedure were modified one at a time. Use of (NBu)_3[Fe(S)_2(SPh)_2] resulted in quick precipitation of a fine black powder upon the injection of the aqueous ferricyanide solution. The low-temperature ESR spectrum of this suspension showed that the oxidant was largely unreacted. After it was checked that 18-crown-6-ether is of no influence on the generation of the 3-Fe cluster, 3 equiv of water was used to keep the potassium ferricyanide in solution as the amount of water used was reduced to 1%. Again the ESR spectrum showed unreacted ferricyanide. The reaction was also run at -25 °C to compensate for the absence of heat of mixture. The result was unchanged. This could be explained by the strongly diminished potential of ferricyanide in nonprotic solvents. When ferricyanide was replaced by Fe(C_(12)H_(20))(BF_4) or when DMF was replaced by acetonitrile, no ESR signal was observed. Reportedly, ferricyanide takes a special place among oxidants when iron-sulfur proteins are involved. All deviations from our standard pro-

Table I. Spectral Components Observed\(^*\) for the Mössbauer Sample at 40 K

<table>
<thead>
<tr>
<th>spectral component</th>
<th>rel. area, % of total (^{57}\text{Fe} )</th>
<th>( \Delta E_x, \text{ mm/s} )</th>
<th>( \delta, \text{ mm/s} )</th>
<th>( \Gamma(\text{fwhm}), \text{ mm/s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Fe</td>
<td>28 (3)</td>
<td>0.58 (2)</td>
<td>0.31 (2)</td>
<td>0.32</td>
</tr>
<tr>
<td>4-Fe</td>
<td>59 (3)</td>
<td>1.18 (2)</td>
<td>0.45 (2)</td>
<td>( b )</td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>13 (3)</td>
<td>2.4 (1)</td>
<td>1.2 (1)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\(^*\)Results of a least-squares fit of symmetric Lorentzian pairs to the data of Figure 5a. If we assume the recoil-free fractions of all \(^{57}\text{Fe}\) sites are the same, then the relative area of a given spectral component is directly proportional to the concentration of the associated \(^{57}\text{Fe}\).

Figure 3. Mössbauer spectra of \(\text{[Fe}_{3}\text{S}_4\text{(S-}t\text{-Bu)}_2\text{]}^{2-}\) DMF, 1 mM, oxidized at -40 °C by 1 equiv of \(\text{[Fe(CN)}_6\text{l}^{3-}\) buffer. The sample was 9 mm thick when the data of spectrum e were collected. For spectra a–d, the sample was transferred while in Ar atmosphere to a cell with 2.5 times the cross sectional area. The thinner sample was needed to avoid saturation effects. The absorption scales are uncorrected for Compton background. (a) Spectrum of the sample measured at 40 K with a 60 mT magnetic field applied parallel to the \(\gamma\) beam. The solid line is a least-squares fit described in Table I. Three quadrupole doublets are indicated by the labeled brackets. (b) Spectrum of the same sample measured at 4.2 K with 60 mT parallel field. (c) Difference spectrum generated by subtracting the 40 K data (spectrum a) from the 4.2 K data (spectrum b). The area and base line of the 40 K data were normalized to those of the 4.2 K data for the subtraction. (d) Magnetic hyperfine pattern (dots) of the synthetic 3-Fe cluster. This pattern was generated by adding a simulation of the 40 K 3-Fe signal (as described in Table I) to the spectrum c. The 4.2 K, 60 mT parallel spectrum of \(\text{D. gigas Fd II}\) is also plotted (hatchmarks, area normalized, data from ref 34). The sharp feature at +1 mm/s in the model compound data arises from the change of the 4-Fe cluster signal between 4.2 and 40 K. The solid line indicates the difference spectrum calculated for the 4-Fe cluster from the parameters quoted in Table I. (e) Field direction dependence of the 4.2 K spectra of the Mössbauer sample (dots). This difference spectrum was generated by subtracting the area-normalized 60 mT perpendicular applied field spectrum from the 60 mT parallel field spectrum. An analogous difference spectrum for \(\text{D. gigas Fd II}\) (hatchmarks) is plotted with normalized height. This Fd II spectrum and Figure 2 of ref 34 are from the same data set.

(32) Johnson, M. K., personal communication.
line in Figure 3a indicates that 13% ± 3% total 57Fe is high-spin ferrous iron that is not associated with either 4-Fe or 3-Fe clusters. The third doublet with $\Delta E = 0.58$ mm/s and $\delta = 0.31$ mm/s is characteristic of tetrahedral sulfur-coordinated ferric iron and accounts for 28% ± 3% total 57Fe. As shown below this doublet is the signal of a 3-Fe cluster. At 4.2 K (Figure 3b) the signals of the [4Fe-4S] cluster and the ferrous iron have changed little from those of the 40 K data. The [4Fe-4S] cluster line at +1 mm/s is slightly shifted (see Table I). The ferric doublet, however, has been replaced by a broad magnetic pattern. This transition from fast to slow relaxation between 40 and 4.2 K would be very unusual for monoferric iron. Figure 3c shows the difference spectrum generated by subtracting the 40 K spectrum from the 4.2 K data. In the difference spectrum, the signals of the [4Fe-4S] cluster and the ferrous iron essentially cancel and the magnetic pattern is clearly illustrated. Our best representation (Figure 3d) of the magnetic hyperfine pattern was generated by adding a theoretical simulation of the 3-Fe cluster 40 K doublet to the spectrum of Figure 3c. This pattern is very similar to those of known 3-Fe clusters. Direct comparison with the spectrum of Desulfovibrio gigas Fd II shows the two spectra are virtually identical.

Further evidence for the presence of a 3-Fe cluster is provided by the field direction dependence of the 4.2 K spectra. The data of Figure 3e is the difference between a spectrum recorded with the 60 mT applied field parallel to the gamma beam and one with the field perpendicular. With constant temperature and weak applied field, the signals of both the [4Fe-4S] cluster and the Fe2+ magnetic pattern are, within the noise, identical. The disappearance of the shoulder predicted by the model fitting the 1-equiv curve signal can be distinguished: an almost isotropic form having $g_{||} < g_{\perp}$ and a form in which an extra shoulder can be distinguished on the $g_{||}$ peak. The high-field part of the spectrum is often quite broad. Higher molecular weight 3-Fe proteins, for instance acomitase (66–89 kDa), Chromatium vinosum hydrogenase (62 kDa), and most probably Vibrio succinogenes fumarate reductase (79 + 31 + 2 × 25 kDa), also display ESR signals slightly above $g = 2$, but their shapes are different. Figure 2a shows the spectrum of our 3-Fe preparation. It is close to those of the Fds.

The relaxation of the synthetic 3-Fe center was analyzed and macroheterogeneity was convincingly demonstrated. This macroheterogeneity and the flatness of the low-field top of the spectrum suggests at least two $g$ tensors are required to fit the spectrum (as is the case for HP). A reliable explanation of the 3-Fe ESR spectrum cannot come from computer fits only; it requires more knowledge of the electronic and structural properties of the cluster.

Three-iron Fds have been reported to show an almost isotropic spectrum or one with a shoulder. Apparently the spectrum of our model is of the isotropic type. The disappearance of the shoulder from the protein spectra could be a matter of broadening (overall or in the low-field region only). The spectra of $D. \text{gigas} \text{ Fd II}$ seem to be almost isotropic for the $57\text{Fe}$-reconstituted protein whereas a shoulder can be discerned for the $56\text{Fe}$ protein.

Since the $g = 2.01$ signal has often been called HP-like, we examined the ESR signal of HP under a variety of circumstances. Different protein sources and different pH values caused only minor changes in the well-known spectrum. Denaturation at room temperature results in a very broad spectrum. However, a shoulder is discerned at $g = 2.01$ for HP from Thermus aquaticus, Chromatium vinosum hydrogenase, and Chromatium vinosum hydrogenase.

temperature by urea also had little effect on the spectral shape though the intensity of the signal diminished. The spectrum of Chromatium HP denatured in dimethyl sulfoxide has been reported to be similar to the reduced 4-Fe signal.\(^{50}\) Whether this spectrum is due to destruction products or to the unconstrained [Fe₄S₄]⁺⁺ core is uncertain. However, this does not affect our conclusion: an [Fe₄S₄]⁺⁺ cluster has, to the best of our knowledge, not yet been shown to yield an ESR spectrum similar to those of known 3-Fe clusters.

**Kinetics.** The product distribution determined by Mössbauer spectroscopy gives an important clue to the kinetics of 3-Fe formation. The data for a reaction starting with 1 mM cubane and 1 equiv of oxidant are shown in Table I. If a 4-Fe cluster yields one 3-Fe cluster and one Fe(II) ion, then 28% iron in 3-Fe cluster demands 9.3% Fe(II). Of the 13% Fe(II) found, a small part thus seems to originate from complete destruction of cubane. Only complete oxidation to disulfide and sulfur, at the same time producing Fe(II), allows for the consumption of all oxidant and the observed large amounts of residual cubane. Actually, in order to balance the oxidant and 28% Fe in 3-Fe clusters, one would expect to find 12% Fe(II) and 60% Fe in 4-Fe clusters.

A second source of information on the kinetics is the dependence of 3-Fe yield on the initial concentrations of cubane and oxidant (always 1 equiv). This was monitored by ESR. The Mössbauer and ESR data are presented in Figure 4 together with calculated curves based on the following model. Oxidation (by Ox) of cubane (Cub) first yields a HPOX analogue (Hip). The observed concentration dependence is then explained by taking into consideration two consecutive reactions of different order. First, the reaction of Ox with Hip is assumed to give a labile species that immediately falls apart. Upon reduction of Fe(III) by released ligands, 8 equiv (S²⁻ and RS⁻, denoted Lig) remain to be oxidized. Second, Hip itself could excrete one Fe(II) and one ligand thus yielding the trinuclear species (Tri). Finally, free ligands are oxidized.

\[
\begin{align*}
\text{Cub} + \text{Ox} & \rightarrow \text{Hip} \\
\text{Hip} + \text{Ox} & \rightarrow 8\text{Lig} \\
\text{Hip} & \rightarrow \text{Tri} + \text{Lig} \\
\text{Lig} + \text{Ox} & \rightarrow S, \text{RSSR}
\end{align*}
\]

At high concentrations, the HP-analogue is mainly converted via the bimolecular reaction 2 and the yield of 3-Fe cluster is vanishingly low. At low concentrations, reaction 2 is of no importance and the course of reactions is essentially

\[
\begin{align*}
\text{Cub} + \text{Ox} & \rightarrow \text{Tri} + \text{Lig} \\
\text{Lig} + \text{Ox} & \rightarrow S, \text{RSSR}
\end{align*}
\]

Assuming the latter reaction to be fast relative to the former it can be seen that the yield of 3-Fe cluster is limited to 50%. (Note that always 1 equiv was used.) The steepness of the curves in Figure 4 and the midpoint concentration are governed by the reaction rate constants \(k_i\). Denoting the reaction rates by

\[
A = k_1[\text{Cub}][\text{Ox}] \\
B = k_2[\text{Hip}][\text{Ox}] \\
C = k_4[\text{Hip}] \\
D = k_5[\text{Lig}][\text{Ox}]
\]

the differential equations describing the kinetics take the following form

\[
\begin{align*}
d[\text{Cub}] / dt & = -A \\
d[\text{Ox}] / dt & = -A - B - D \\
d[\text{Tri}] / dt & = C \\
d[\text{Hip}] / dt & = A - B - C \\
d[\text{Lig}] / dt & = 8B + C - D
\end{align*}
\]

The course of the concentrations was computed by numerical integration. The reaction was considered to be ended when \([\text{Ox}] < 0.1\%\) and \([\text{Hip}] < 0.1\%, or when \([\text{Cub}] < 0.1\%\) and \([\text{Hip}] < 0.1\%. The ratio of \(k\) values fitting the experimental results best was: \(k_1:k_2:k_3:k_4 = 1:60:250\) mM:100. The unit mM remains in the ratio because of the difference in reaction order; the unit of time is arbitrary.

According to our model, addition of several equivalents of oxidant improves the 3-Fe yield. Curves for 1, 2, and 3 equiv are shown in Figure 4. The improvement depends on the concentration of cubane, reaching almost complete conversion at extremely low concentrations and being unchanged in the high-concentration limit. Figure 5 shows the 3-Fe yield as a function of the number of equivalents of oxidant at a constant initial cubane concentration of 0.5 mM. At low numbers of equivalents, the oxidant is completely consumed and cubane is left over. At the top of the curve, at about 3/4 equiv both are consumed. At high equivalent numbers, oxidant is left over. Since it is to be expected that this amount of redundant oxidant will attack 3-Fe cluster in the long run, the model was extended with the reaction

\[
\text{Tri} + \text{Ox} \rightarrow 7\text{Lig}
\]

This reaction must be very slow with respect to the sequence (1)–(4). Therefore the effect of (7) was included by subtracting one-eighth of the amount of redundant oxidant from the 3-Fe yield (dashed curve).

Recognizing the fact that reaction 3 is the only one leading to a 3-Fe cluster, we tried to separately carry out this reaction. HP was oxidized with somewhat less than 1 equiv of ferricyanide. The solution was then injection in DMF at -40 °C, and the ice formed dissolved at the lowest temperature possible. Neither a HPO² nor a 3-Fe cluster ESR signal was found. When the protein was first denatured in cold DMF and then oxidized the result was the same. Since both 4-Fe and 3-Fe clusters are now known to be stable under these circumstances, we conclude that the impetuous unfolding of the protein causes the rupture of clusters. Dissolving the HP-analogue in DMF/water (4:1) at -40 °C could be a more successful approach if the bulky ligands do not prevent the separation of just one iron-ligand pair.

**Conclusions**

Oxidation of [Fe₄S₄(S-t-Bu)₄]⁺⁺ by [Fe(CN)₄]³⁻ in DMF/buffer (4:1) at -40 °C yields a new cluster that is spectroscopically similar to the [3Fe-xS] cluster. This 3-Fe complex is stable at -40 °C and unstable at room temperature. It could not be obtained in a different medium or by using other oxidants. An attempt to prepare it by denaturation of oxidized HP also failed. This preparation is the first and (as yet) only one yielding a manageable and relatively highly concentrated solution of a synthetic 3-Fe cluster.

Both ESR and Mössbauer spectroscopic data of the model compound are consistent with assignment to a 3-Fe structure. Moreover the kinetics demand an intermediate [Fe₄S₄]⁺⁺ cluster,
thereby ruling out the possibility of a HP analogue being the endproduct. Furthermore, HP under several denaturing circumstances did not show an ESR spectrum similar to those of 3-Fe proteins. This strongly indicates that the new compound described in this work is indeed a 3-Fe cluster. It also implies that ESR spectra of iron-sulfur clusters are not easily changed by the surroundings as is often assumed. We therefore believe that the first ESR spectrum reported for a synthetic 3-Fe cluster was the one published by Cammack et al.,51 whereas the spectra shown by Christou et al.52 most probably were neither of a HP nor those of a 3-Fe cluster.

The kinetics justify the expectation that even higher conversions of 4-Fe to 3-Fe could be attained. Alternatively the 3-Fe cluster might be produced from a HP analogue in a controlled-degradation reaction. Isolation and elucidation of the structure then come within reach.

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Registry No. (NMFe)2[Fe2S2(S-t-Bu)4], 52678-92-9; K3[Fe(CN)6], 13746-66-2; (NBu3)2[Fe2S2(SPh)4], 52586-83-1.

Notes

Contribution from the Department of Chemistry, Manipur University, Imphal 795 003, India

Studies on the Alkylation of Vitamin B12 and Related Systems Revisited: Novel Features of Oxidative-Addition Reactions

Dipankar Datta* and G. Tomba Sharma

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Vitamin B12 is a Co(III) complex of the corrin moiety. It is the only vitamin known to contain a metal center. Its chemistry1a,2 is so fascinating and challenging that there seems to be unabated constant interest in this vitamin and its derivatives to workers of various disciplines of chemistry since its isolation in 1948 (for a comprehensive study, see ref 1; a few selected aspects are covered in ref 2). This vitamin can exist in three different oxidation states—the Co(I) variety is known as B12a. Alkylation of vitamin B12a (schematically represented by reaction 1) gives rise to a

\[
\text{Co}(I)^+ + \text{RX} \rightarrow \text{Co} + X^-
\]

(1)

Co(III) species with a metal–carbon bond through an oxidative reaction mechanism.3a It may be mentioned in this connection that coenzyme B12, which has such a metal–carbon bond, and methylcobalamin (R = CH3 in 1 of eq 1), which is a substrate in the methionine biosynthesis are nature's only organometallic compounds known to date.3a

Extensive studies carried out by Schrauzer and co-workers4 led to the earlier conclusion that reaction 1, which is reversible,5 proceeds through an S2,2 mechanism (eq 2). Later observation

\[
\text{Co}(I)^+ + \text{RX} \rightarrow \text{Co} \rightarrow \text{Co}^+ + X^-
\]

(2)

of inversion of configuration at the reacting carbon center by Jensen et al.5 supported this view. However, they seemed to have missed the electron-transfer component of such reactions, and "attempts to demonstrate the expected inversion of configuration at carbon resulting from these oxidative additions led to"7 erroneous conclusions.7a 7b Herein we reanalyze the data of

An FIP fellow.

Table I. Variation of the Rate Constants of the Alkylation of Tributylphosphine–Cobaloxime, with Alkyl Halides (RX)*

<table>
<thead>
<tr>
<th>R group</th>
<th>σ*</th>
<th>k</th>
<th>Cl</th>
<th>Br</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH3</td>
<td>0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>-CH2CH3</td>
<td>-0.100</td>
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<td>0.204</td>
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<tr>
<td>-CH2CH2CH3</td>
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<td>-2.201</td>
<td>0.174</td>
<td>0.174</td>
<td>0.174</td>
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<tr>
<td>-CH2CH2CH2CH3</td>
<td>-0.130</td>
<td>-3.495</td>
<td>0.505</td>
<td>0.505</td>
<td>0.505</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH3</td>
<td>-0.210</td>
<td>-2.137</td>
<td>0.959</td>
<td>0.959</td>
<td>0.959</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH3</td>
<td>-0.125</td>
<td>-3.110</td>
<td>0.924</td>
<td>0.924</td>
<td>0.924</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH2CH3</td>
<td>0.215</td>
<td>2.640</td>
<td>4.280</td>
<td>4.280</td>
<td>4.280</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH2CH2CH3</td>
<td>0.110</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH2CH2CH2CH3</td>
<td>0.405</td>
<td>3.204</td>
<td>3.204</td>
<td>3.204</td>
<td>3.204</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH2CH2CH2CH2CH3</td>
<td>1.300</td>
<td>3.204</td>
<td>3.204</td>
<td>3.204</td>
<td>3.204</td>
</tr>
<tr>
<td>-CH2CH2CH2CH2CH2CH2CH2CH2CH2CH2CH3</td>
<td>0.600</td>
<td>1.146</td>
<td>1.146</td>
<td>1.146</td>
<td>1.146</td>
</tr>
</tbody>
</table>

*Meanings of the symbols used are same as in the text. Rate data are taken from ref 4. 4 In case of CH2CONH2* has been calculated from the σ data (0.27) given in Table 16 of: Charton, M. Prog. Phys. Org. Chem. 1981, 13, 119. The formula σ(X) = 0.45σ2(CHX) of Taft (Taft, R. W.; Lewis, I. C. J. Am. Chem. Soc. 1958, 80, 2436) was used. Others are taken from ref 13.

Schrauzer and co-workers4 to obtain certain interesting features of the alkylation reaction and the oxidative additions, in general.