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# Catalytic Intermediates in the Reaction of Deuteroferriheme with *m*-Chloroperoxybenzoic Acid

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Deuteroferriheme (DFH) reacts with *m*-chloroperoxybenzoic acid (*m*-CPBA) to produce a reaction intermediate whose optical spectrum resembles that of horseradish peroxidase (HRP) compound 0. To exclude problems related to aggregation, the fraction of dimers was maintained below 2%. Under these conditions, the reaction of DFH with *m*-CPB in 10 mM phosphate containing 1% *v/v* 1,2-dimethoxyethane ($I$ = 0.1M NaCl) at pH 5.5 at 1 °C was shown to produce an intermediate that has a distinctive Soret band near 350 nm, in agreement with earlier observations in the literature. The EPR spectrum of this intermediate indicates that it contains half ferric iron and cannot be a ferryl dimer. Its structure is more consistent with that of a ferric, ferryl dimer and a scheme supporting its formation has been proposed.

## INTRODUCTION

Protein-free hemes have been widely used as models of heme-containing enzymes such as the catalases and peroxidases that catalyze the oxidation of selected substrates. A focus of these investigations has been the elucidation of the structures of the catalytic intermediate species which play a central role in the activity of the heme. Such intermediates are presumed to be oxidized forms of the heme. The current state of knowledge of the per-

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oxidase and catalase intermediates referred to as compounds I and II is quite mature and there is no disagreement concerning their structures. However, there is currently no information available regarding the structure of compound 0 observed for HRP and Ac-MP-8. The mechanism of formation of compound 0 is

$$\mathrm{HRP} + \mathrm{H_2O_2} \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} \mathrm{HRP\text{–}H_2O_2} \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} \mathrm{O} \xrightarrow{k_3} \mathrm{I}$$

where HRP-$H_2O_2$ is a precursor complex, compound 0 is an oxidized form of HRP, and step 3 is the rate determining step. The studies described below on the reaction of ferri-DFH with *m*-CPB were carried out to address this question.

Kelly and associates[8,9,12,16,17] have studied the reaction of ferri-DFH with a variety of oxidants including NaOCl and *m*-CPB. These studies have produced a result distinct from that reported by any other workers that have studied the interaction of ferric hemes with oxidants in that they reported the formation of a catalytic intermediate with an optical spectrum (reconstructed from a series of single wavelength measurements) that resembled that reported by Baek and Van Wart[2] for HRP compound 0. Kelly[6] proposed the scheme shown in Figure 1 to account for their results. Here, resting ferri-DFH is denoted (Po)Fe(III) and exists in a pH-dependent equilibrium with the μ-oxo dimer (Po)Fe(III)-O-Fe(III)(Po). On reaction with *m*-CPB, ferri-DFH undergoes two-electron oxidation to form what they referred to as an »intermediate species« that was suggested to be the compound I analog (Po)Fe(IV)-O-Fe(IV)(Po), a ferryl μ-oxo dimer. This is the species with the

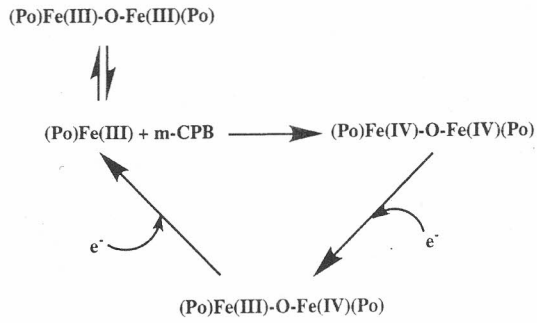


Figure 1. Scheme proposed by Kelly and associates to describe the steps in the reaction of ferri-DFH with *m*-CPB.

optical spectrum that resembles HRP compound 0 in that it exhibits a Soret band with a maximum at 350 nm. This species was reported to decay first to (Po)Fe(III)-O-Fe(IV)(Po), considered to be a compound II analog, and then back to the resting (Po)Fe(III) state. The interest in the »intermediate species« with the unusual compound 0-like optical spectrum is due to the fact that it is relatively long-lived for DFH. Thus, while HRP compound 0 has a halflife of 20 ms at –20 °C and can only be observed in rapid-scan low-temperature stopped-flow experiments, the species observed by Kelly and associates for DFH has a halflife of seconds at 23 °C. In this study, the »intermediate species« with the unusual optical spectrum has been freeze-quenched and studied by EPR spectroscopy to provide information about its structure.

## MATERIALS AND METHODS

Triply recrystallized hemin was obtained from Nutritional Biochemicals Corp. and subjected to resorcinol melt treatment for the preparation of DFH. Extraction and crystallization procedures were as described by Falk[4] and and the DFH was characterized as its pyridine hemochrome derivative, which was found to exceed 99% spectral purity ($\varepsilon = 2.4 \times 10^4$ $M^{-1}$ $cm^{-1}$, $\lambda_{max} = 544$ nm).[4] *m*-Chloroperoxymenzoic acid (*m*-CPB) was obtained from Pfalz and Bauer. Sephadex G-50 (superfine) and sephadex G-10 were the products of Pharmacia Biotechnology. All buffer solutions were prepared in reagent grade water with a resistivity of 18 MΩ/cm prepared with a millipore MILL-Q system.

The apparent protonic activity in the presence of organic cosolvents, pH*, was measured with a glass electrode and an Orion Research Model 611 pH meter.

*Low-temperature stopped-flow* experiments were carried out with a modified form of an instrument described elsewhere[3]. The instrument contains an observation cell with a path lenghth of 2 cm. For single wavelength measurements, more than 200 data points were used in each rate constant determination. The reported rate constants were the average of at least three trials. Rapid-scan experiments were performed by focusing light from an Oriel 75-W xenon lamp attenuated with a neutral density filter through the quartz observation cell of the stopped-flow instrument and into a Princeton Applied Research Model 1226 spectrograph. The light is dispersed and focused into a home-built diode array detector constructed from a Hamamatsu Model S-2304-512Q linear silicon photodiode array sensor interfaced to a Micro-Way A2D-160 12-bit 166 kHz analog-to-digital converter.[5] The current from the phototube is amplified and converted to a voltage signal that is transmitted to an IBM-XT computer using a Data Translation Model 2801 an A/D converter and stored on a floppy disk. All stopped-flow measurements were performed under pseudo-first-order conditions.

The cryosolvents used in all of the low-temperature experiments were 50% *v/v* methanol/buffer or 60 % *v/v* DMSO/buffer, where the buffer was 10 mM phosphate. A minimum of 1 hr was allowed for temperature equilibrium to occur before kinetic runs were initiated. The temperature of the the stopped-flow system was monitored throughout all experiments with an Omega copper/constantan grounded thermocouple and an Omega Model 2176 A-T digital thermometer.

*The optical spectra* were measured with a Hewlett Packard diode array spectrophotometer equipped with a Hewlett Packard diode array spectrophotometer equipped with a RMS 6 Lauda Brinkmann Circulator to control the temperature of the sample.

*Electron Paramagnetic Resonance (EPR) Spectroscopy.* EPR spectra were recorded at liquid helium temperature using a Bruker ER 220-D-SRC spectrometer with a Lake Shore Model DTC-500 cryotronic temperature controller.

## RESULTS AND DISCUSSION

The equilibrium between ferri-DFH and its μ-oxo dimer is pH and concentration dependent. In order to study the reaction between ferri-DFH and *m*-CPB under the most defined conditions, a DFH concentration and pH were selected at which all of the catalyst was monomeric. The variation in the observed extinction coefficient of DFH at 384 nm, $\varepsilon_{384}$, at several pH values and DFH concentrations is shown in Table I. The DFH dimerization reaction, which lowers the value of $\varepsilon_{384}$, may be expressed as

$$2M \longleftrightarrow D + H^+ \tag{1}$$

$$K_{eq} = [D]\,[H^+] / [M]^2 \tag{2}$$

where M and D denote the monomer and dimer, respectively. Equation (3) shows the relationship between $K_{eq}$, $\alpha$ (the fraction of DFH existing as the monomer) and the hydrogen ion and heme concentrations:

$$K_{eq} = [H^+]\,(1 - \alpha) / (2\,\alpha^2\,[DFH]_T) \tag{3}$$

From spectral measurments at 23 °C, pH 5.5, $K_{eq} = 7.6 \times 10^{-2}$, $\varepsilon_D = 3.7 \times 10^4$ $M^{-1}$, $cm^{-1}$, and $\varepsilon_M = 3.03 \times 10^5$ $M^{-1}$, $cm^{-1}$. From these parameters, the precentage of dimer at pH 5.5 at DFH concentration of 5 μM can be shown to be less than 2.5%.

*Single-Wavelength Low-Temperature Stopped-Flow Examination of the Reaction of Ferri-DFH with* m-*CPB.* The low-temperature stopped-flow technique has been applied here to study the reactions of 1.5 and 5 mM DFH

TABLE I

Effect of Dimerization of Ferri-DFH on its Soret Absorbance (384 nm)

| [DFH]* | pH | | | | | |
|---|---|---|---|---|---|---|
| | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 8.5 |
| **20.00** | 1.115 | 1.449 | 1.430 | 1.309 | 0.252 | 1.369 |
| **10.00** | 0.569 | 0.759 | 0.814 | 0.685 | 0.670 | 0.738 |
| **5.00** | 0.302 | 0.450 | 0.443 | 0.384 | 0.347 | 0.402 |
| **2.50** | 0.192 | 0.202 | 0.264 | 0.223 | 0.193 | 0.211 |
| **1.00** | 0.117 | 0.120 | 0.137 | 0.107 | 0.117 | 0.098 |
| **0.50** | 0.075 | 0.088 | 0.092 | 0.067 | 0.069 | 0.059 |
| **0.25** | 0.053 | 0.064 | 0.063 | 0.043 | 0.046 | 0.036 |
| **0.10** | 0.060 | 0.049 | 0.044 | 0.032 | 0.023 | 0.026 |

* micro M

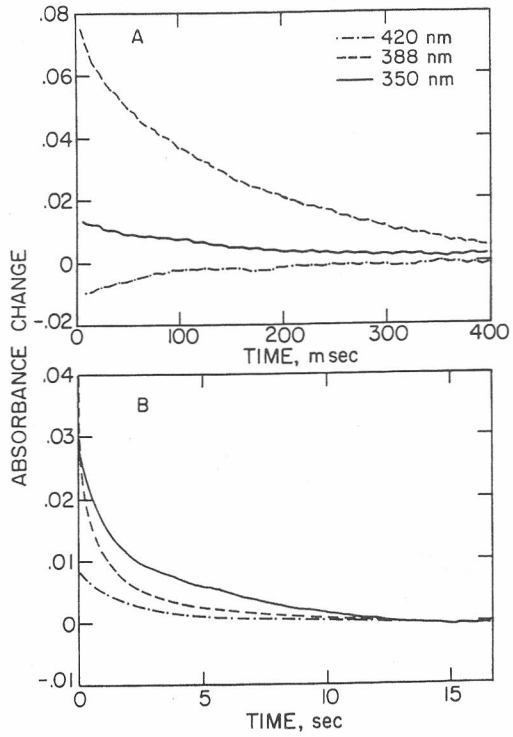


Figure 2. Low-temperature stopped-flow investigation of the reaction of 1.5 μM ferri-DFH with 3.2 μM *m*-CPB in 10 mM phosphate containing 1% *v/v* 1,2-dimethoxyethane ($I$ = 0.1 M NaCl) at pH 5.5 at 1 °C monitored at (– · – · –) 420, (– – –) 388 and (———) 350 nm on (A) faster and (B) slower time scales.

with 3.2 and 10 μM *m*-CPB, respectively, in 10 μM phosphate containing 1% *v/v* 1,2-dimethoxyethane (*I* = 0.1 M NaCl) at pH 5.5 at 1 °C, and is an effective way of detecting intermediates.

The reactions have been monitored at three wavelengths on two different time scales (Figure 2A and B). During the first 400 ms of the reaction, ferri-DFH is converted to an intermediate whose absorbance is much lower at 388 nm, slightly lower at 350 nm, and slightly higher at 420 nm (Figure 2A). Next, the first intermediate is converted to a second intermediate over the next 15 s that has a slightly lower absorbance at all three wavelengths (Figure 2B). This second intermediate then slowly decays back to ferri-DFH over the next 15 min with regeneration of its prominent Soret band at 388 nm (data not shown).

*Rapid-Scan Low-Temperature Stopped-Flow Examination of the Reaction of Ferri-DFH with* m-*CPB.* In order to observe the entire optical spectrum of the intermediates formed during the reaction described above, rapid-scan studies have been carried out. The spectrum of resting ferri-DFH with its distinctive Soret band near 388 nm is shown in Figure 3 (curve 1). Curves 2 and 3 are 1 sec scans acquired 5 s and 5 min after the start of reaction. The 5 s scan shows that an intermediate has been formed with a Soret maximum

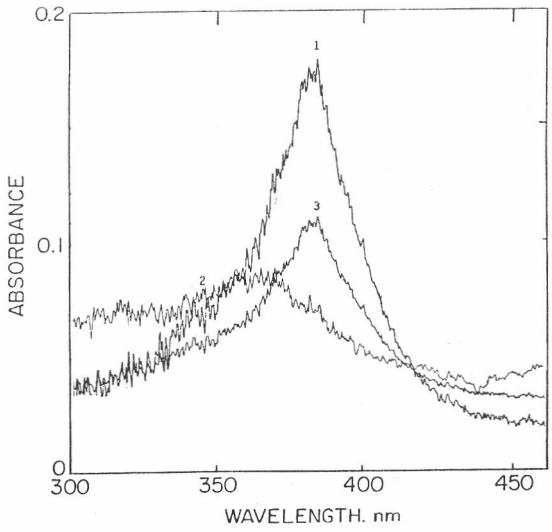


Figure 3. Rapid-scan low-temperature stopped-flow study of the reaction of 1.5 μM ferri-DFH with 3.2 mM *m*-CPB in 10 mM phosphate containing 1% *v/v* 1,2-dimethoxyethane (*I* = 0.1 M NaCl) at pH 5.5 at 0 °C. Curves 1–3 are spectra of ferri-DFH and the intermediates present 5 s and 5 min after the start the reaction, respectively.

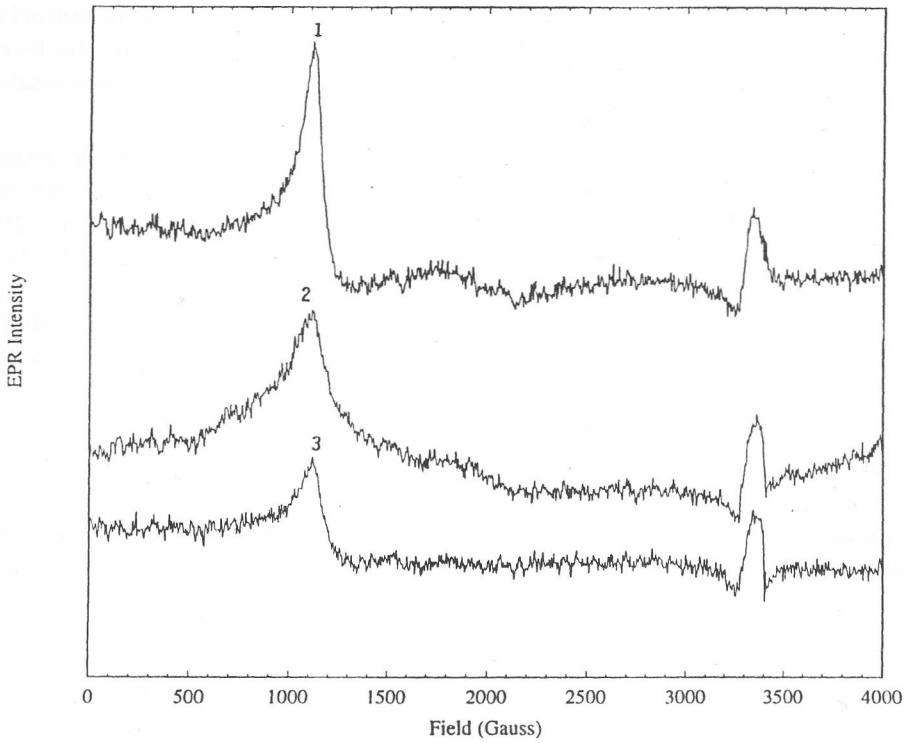


Figure 4. EPR spectra of 25 μM ferri-DFH (1) before and (2) 1 s and (3) 10 s after reaction with 50 μM *m*-CPB in 10 mM phosphate at pH 5.5. Samples were freeze quenched in liquid nitrogen and examined at 2 K.

near 350 nm. This is the second intermediate referred to above and is the »intermediate species« observed earlier by Kelly and associates. This intermediate then decays back to ferri-DFH with regeneration of a Soret band at 388 nm.

*EPR Spectrum of 350 nm Intermediate.* In order to provide some information on the identity of the intermediate showing the Soret maximum at 350 nm, its EPR spectrum has been acquired. For this experiment, 25 μM ferri-DFH was reacted with 50 μM *m*-CPB in 10 mM phosphate containing 1% *v/v* 1,2-dimethoxyethane ($I$ = 0.1 M NaCl) at pH 5.5 and the reaction freeze quenched after 1 and 10 sec in liquid nitrogen. Optical spectra showed that both of these samples contained predominantly (> 90%) the 350 nm intermediate. The EPR spectrum of the starting ferri-DFH and these two samples were then acquired at 2 K (curves 1–3, Figure 3). The spectrum shown in curve 1 for ferri-DFH is a typical high spin ferric iron spectrum with a major signal near $g$ = 6. Curves 2 and 3 are also both high-spin ferric

iron spectra, except with one-half of the amplitude of the starting material. This indicates that half of the iron in this intermediate is still in the ferric state. The loss in half of the signal compared to ferri-DFH is presumably due to partial oxidation to the EPR silent Fe(IV) state.

*Mechanism of Reaction of Ferri-DFH with* m-*CPB.*The results described above confirm the earlier report by Kelly and associates[7,16] that a 350 nm intermediate is produced during the reaction of ferri-DFH with *m*-CPB. However, the stopped-flow and EPR data are not consistent with their mechanism shown in Figure 1 for two reasons. First, the production of this intermediate is biphasic and is preceded by another earlier intermediate. Second, the 350 nm intermediate *cannot* have the structure (Po)Fe(IV)-O-Fe(IV)(Po), since the EPR data establish that approximately half of the iron present is in the ferric state.

As an alternative to the Kelly mechanism, we propose the scheme shown in Figure 5. Here, the first step in the reaction is the oxidation of resting monomeric ferri-DFH, denoted (Po)Fe(III), to the monomeric compound I analog ($Po^{\bullet+}$)Fe(IV) = O. This is almost certainly the first intermediate observed in the 400 msec trace shown in Figure 2A and is the expected product of the first step of such a reaction. The next step is the reaction of ($Po^{\bullet+}$)Fe(IV) = O with remaining unoxidized reactant, (Po)Fe(III), to give ($Po^{\bullet+}$)Fe(IV)-O-Fe(III)(Po). This species contains half ferric iron and half ferryl iron and has a structure consistent with the EPR spectrum shown for

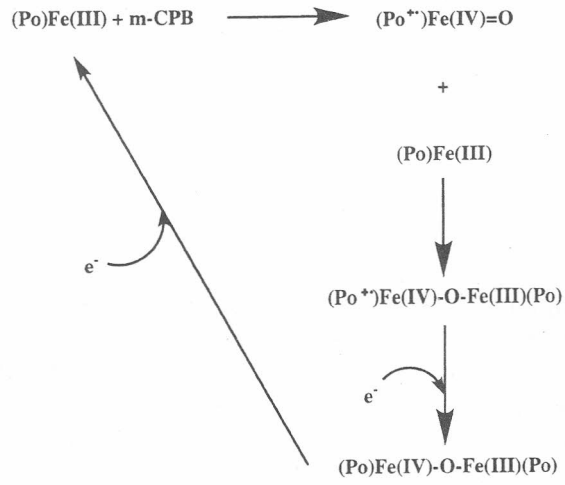


Figure 5. Scheme proposed here for the reaction of ferri-DFH and *m*-CPB that accounts for the stopped-flow and EPR data. Here, either ($Po^{\bullet+}$)Fe(IV)-O-Fe(III)(Po) or (Po)Fe(IV)-O-Fe(III)(Po) is the species with the 350 nm Soret band.

the 350-nm intermediate in Figure 4. This type of optical spectrum is characteristic of oxo-bridged dimeric hemes. Another possibility is that this species is rapidly reduced to (Po)Fe(IV)-O-Fe(III)(Po), which could also be the 350 nm intermediate. We cannot distinguish between these possibilities. Finally, the 350 nm intermediate decays back to ferri-DFH by one-electron reduction. In conclusion, the 350 nm band observed during the oxidation of ferri-DFH is due to a dimeric species such as ($Po^{\bullet+}$)Fe(IV)-O-Fe(III)(Po) or (Po)Fe(IV)-O-Fe(III)(Po). Since it is not possible to form such species for HRP because the protein prevents the heme from dimerizing, they cannot be models for HRP compound 0.

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## REFERENCES

1. H. Baek and E. Van Wart, *Biochemistry* **28** (1989) 5714–5719.
2. H. Beak and H. V. Wart, *J. Am. Chem. Soc.* **114** (1992) 718.
3. H. W. Wart and H. E. Zimmer, *J. Anal. Biochem.* **117** (1981) 410.
4. J. E. Falk, *Porphyrins and Metalloporphyrins* (1964) 181.
5. D. Ostovic and T. C. Bruice, *Acc. Chem. Res.* **25** (1992) 314.
6. F. S. Woo, R. E. Rodriguez, A. H. Dale, P. Jones, and H. C. Kelly, *Inorg. Chem.* **31** (1992) 1144.
7. P. Jones, K. Prudhoe, T. Robson, and H. C. Kelly, *Biochemistry* **13** (1974) 4279.
8. H. C. Kelly, D. M. Davies, M. J. King, and P. Jones, *Biochemistry* **16** (1977) 3543.
9. P. Jones, D. Mantle, D. M. Davies, and H. C. Kelly, *Biochemistry* **16** (1977) 3974.
10. P. Jones and D. Mantle, *J. Chem. Soc., Dalton. Trans.* (1977) 1849.
11. S. B. Brown, H. Hatzikonstantinou, and C. G. Herries, *Biochem. J.* **174** (1978) 901.
12. H. C. Kelly, K. J. Parigi, I. Wilson, D. M. Davies, P. Jones, and L. Roettger, *J. Inorg. Chem.* **20** (1981) 1086.
13. P. Jones, D. Mantle, and I. Wilson, *J. Inorg. Biochem.* **17** (1982) 293.
14. H. B. Dunford, T. Araiso, D. Job, J. Ricard, R. Rutter, L. P. Hager, R. Wever, W. M. Kast, R. Boelens, N. Ellfolk, and M. Ronnberg, *The Biological Chemistry of Iron*, D. Reidel, Boston, MA, 1982, p. 337.
15. P. Jones, D. Mantle, and I. Wilson, *J. Chem. Soc., Dalton Trans.* (1983) 161.
16. H. C. Kelly and S. C. Yasui, *Inorg. Chem.* **23** (1984) 3559.
17. R. E. Rodriguez and H. C. Kelly, *Inorg. Chem.* **28** (1989) 589.

## SAŽETAK

### Katalitički međuprodukti modela peroksidaze deuteroferihema u reakciji s *m*-klorperoksibenzojevom kiselinom

*Vera Imper i Harold E. Van Wart*

U reakciji deuteroferihema (DFH) s *m*-kloroperoksibenzojevom (*m*-CPB) kiselinom opaženo je stvaranje međuprodukata čiji optički spektar nalikuje na spoj »0« kod peroksidaze hrena. Da bi se izbjegli mogući problemi nastali uslijed agregacije novonastalih međuprodukata, postotak dimera u reakciji nastojao se održati < 2% tokom svih pokusa. U reakciji između DFH i *m*-CPB u 10 mM fosfatnog pufera sa 1% *v/v* 1,2-dimetoksietana ($I$ = 0.1 M NaCl) pri pH 5.5 i temperaturi od 1 °C nastaje međuprodukt s izrazitom Soret vrpcom kod 350 nm. Iz EPR spektra spomenutog međuprodukta opaženo je da se sastoji jednim dijelom od željeza (IV) s toga je mogućnost stvaranja željezo (III) dimer isključena. Predložena struktura međuprodukta je (Po)Fe(IV)-O-Fe(III)(Po).