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Which Oxidant Is Really Responsible for P450 Model Oxygenation Reactions? A Kinetic Approach**

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The nature of the reactive intermediates participating in the iron(III)-porphyrin-catalyzed oxygenation of organic substrates has been a target of intense experimental and theoretical research in the field of biological and bioinorganic chemistry during the last two decades. While the olefin competitive epoxidation[1] and site-directed mutagenesis[2] investigations resulted in the multiple-oxidants hypothesis, computational studies postulated a high-valent iron(IV)-oxo porphyrin π-cation radical ([(Por$^{+\bullet}$)Fe^{IV}=O], Cpd I) as the sole active species in the catalytic oxygenation reactions.[3] The multiple-oxidants dilemma is especially emphasized in the sulfoxidation mechanism of thioethers: the recent experimental results of Cryle and De Voss support the ferric hydroperoxide species (Cpd 0) as a potent oxidant for sulfoxidation,[4] whereas theoretical calculations of Shaik et al. rule out this possibility demonstrating that Cpd 0 should be a non-reactive species compared to Cpd I.[5] Moreover, experimental work by Nam et al. on the non-heme hydroperoxo complexes showed Cpd 0 to be a very sluggish oxidant.[6]

Very recently, we reported spectroscopic and kinetic information on the formation of [(Por$^{+\bullet}$)Fe^{IV}=O] from a new enzyme mimic of P450 in acetonitrile.[7] In the course of direct kinetic measurements at low temperature we demonstrated the occurrence of a complete P450-like catalytic cycle in which the Cpd I analogue oxidizes *cis*-stilbene leading to the almost complete re-formation of the catalyst.[7] However, the fact that Cpd I is a strong oxidant towards various hydrocarbons can not exclude the involvement of other alternative oxidizing intermediates of which the adduct formed from iron(III) porphyrin and the oxidant appears to be the most discussed candidate[1,2]. Therefore, there is a growing need to examine the reactivity of Cpd 0 towards organic substrates in the course of direct oxidation reactions. A more recent study by Nam et al.[8] has demonstrated that addition of a four-fold excess of *m*-chloroperbenzoic acid (*m*-CPBA) to [Fe^{III}-(TMP)(Cl)] (TMP = *meso*-tetramesitylporphyrin) in mixed CH_3CN/CH_2Cl_2 at −40 °C affords the formation of [(TMP$^{+\bullet}$)Fe^{IV}=O], which displays a high oxidizing activity towards selected organic substrates. Although the [Fe^{III}-(TMP)(*m*-CPBA)] species was proposed as the first intermediate in the catalytic cycle of [Fe^{III}(TMP)(Cl)], spectroscopic evidence for its formation and reactivity was not reported. We now report the first successful description of the reactivity of the intermediate [Fe^{III}(TMP)(*m*-CPBA)] (**1**) in the direct oxidation reactions of organic substrates, as well as under catalytic conditions. Through selection of appropriate reaction conditions, we were able to monitor, spectroscopically, the formation of **1**, whose stability at lower temperatures appeared to be sufficient to examine its reactivity in direct epoxidation and sulfoxidation reactions of selected organic substrates.

Addition of a small excess of *m*-CPBA (1.8:1) to an acetonitrile solution of [Fe^{III}(TMP)(OH)] (the chloride-substituted derivative, [Fe^{III}(TMP)(Cl)], is not suitable as a starting material[9]) at −15 °C resulted in spectral changes that indicated the occurrence of a two step reaction. The first reaction step is characterized by a small but significant absorbance increase in the Soret band with a concomitant shift of about 1–2 nm to longer wavelength, and a substantial absorbance decrease in the range between 440 and 540 nm (Figure 1). This step is followed by a slower reaction associated with a large absorbance decrease in the Soret band and an absorbance increase between 550 and 700 nm. The product of the second reaction displays the characteristic

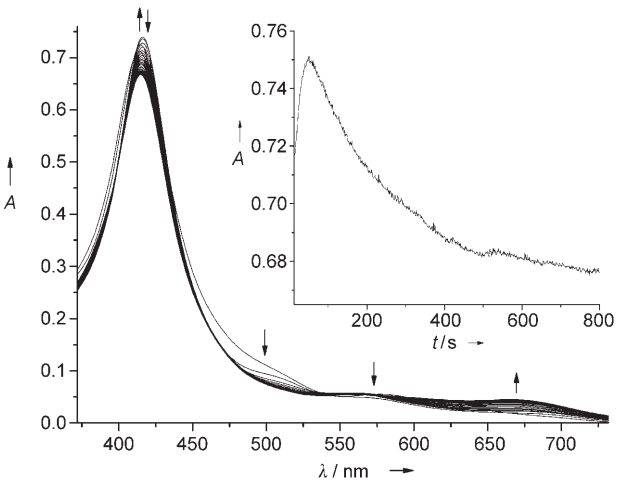

Figure 1. Spectral changes recorded after addition of 1.35×10^{-5} M *m*-CPBA to 7.4×10^{-6} M [Fe(TMP)(OH)] in acetonitrile at −15 °C. Inset: Kinetic trace recorded for this reaction at the Soret band, demonstrating fast formation of **1** and subsequent slow heterolytic cleavage of the O–O bond to form **2**.

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UV/Vis spectrum of a high-valent iron(IV)-oxo porphyrin π-cation radical [($TMP^{+\bullet}$)Fe^{IV}=O] (**2**)[10] (Figure 1).

The visible spectrum of the first intermediate,[10] and this intermediates easy conversion into [($TMP^{+\bullet}$)Fe^{IV}=O] under the chosen reaction conditions, support the assignment of this species as [Fe^{III}(TMP)(*m*-CPBA)]. Addition of sub-equivalent amounts of oxidant to the iron(III) porphyrin solution (0.6:1 based on [Fe(TMP)(OH)]) results in the very slow formation of **1** which remained stable in acetonitrile at −15 °C for at least 500 s (Figure 2).

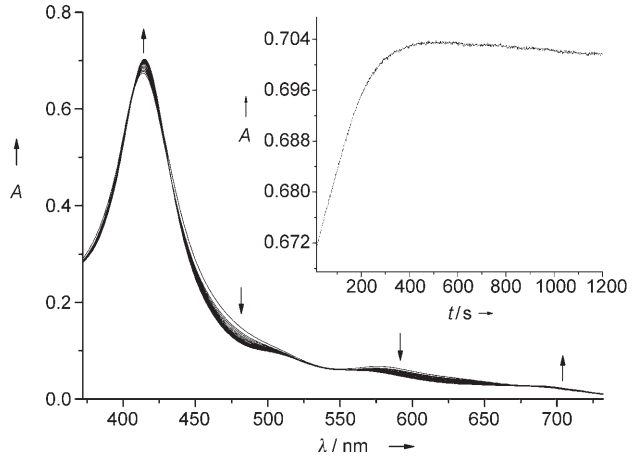


Figure 2. Spectral changes that accompany the formation of **1** in acetonitrile at −15 °C. Experimental conditions: concentration of [Fe(TMP)(OH)] = 6.5×10^{-6} M, concentration of *m*-CPBA = 3.8×10^{-6} M. Inset: Kinetic trace recorded for this reaction at the Soret band.

This observation is consistent with the findings in earlier studies by Groves and Watanabe[10] which demonstrated that the decomposition of the acylperoxoiron(III) porphyrin complex to form the iron(IV)-oxo porphyrin π-cation radical is significantly accelerated by the presence of acid in the reaction medium (proton catalysis favoring and accelerating the heterolytic O–O bond cleavage). In our case the presence of even a very small excess of *m*-CPBA (1:1.2) leads to the irreversible conversion of **1** into **2**. Thus, under conditions of a sub-equivalent amount of *m*-CPBA, heterolytic O–O bond cleavage (and thus the formation of [($TMP^{+\bullet}$)Fe^{IV}=O]) is not acid catalyzed, and leads to significant stabilization of **1**. By analogy, site-directed mutagenesis studies have revealed that the mutant enzymes in which the proton relay was disrupted by changes in the amino acid residues at the active site, were not capable of converting Cpd 0 into Cpd I.[2b,c] Selection of appropriate reaction conditions in our model studies (that is, aprotic solvent, temperature, [Fe(TMP)(OH)]/[*m*-CPBA] ratio) allows us to stabilize both intermediates in the catalytic cycle of [Fe^{III}(TMP)(OH)], that is, **1** and **2**, and enables us to examine their reactivity towards the selected organic substrates *cis*-stilbene and dimethyl sulfide (DMS).

In the course of direct oxygenation reactions, the substrates were injected into the acetonitrile solution of **1** or **2** and the time-resolved spectral changes were recorded with the use of a quartz-glass dip-in detector coupled to a J&M TIDAS diode-array spectrophotometer. The solution of **1** was prepared with a sub-equivalent of *m*-CPBA and its stability was continually monitored by UV/Vis spectroscopy before injection of the substrate. The addition of an excess of *cis*-stilbene to the acetonitrile solution of **1** at −15 °C (Figure S1(a) in Supporting Information) results in a very slow reaction which is not complete within 1200 s at −15 °C. The spectral changes accompanying this reaction are characterized by a slow absorbance decrease of the Soret band with a concomitant shift to shorter wavelengths and appearance of a new band at 510 nm. The visible spectrum of the reaction product is different from that of [Fe^{III}(TMP)(OH)] and points to the formation of [Fe^{III}(TMP)(CH_3CN)$_2$] (**3**) as the final product.[11] The observed pseudo-first order constant for this reaction is a very small, $k_{obs}^{\text{Cpd 0/stilbene}} = 9.4\times10^{-4}\ s^{-1}$ at −15 °C and 1.68×10^{-3} M *cis*-stilbene. Note, the rate of spontaneous decomposition of **1** to **3** without addition of substrate is of the same order of magnitude, $6.2\times10^{-4}\ s^{-1}$. In contrast, addition of the same amount of *cis*-stilbene to a solution of **2** under similar reaction conditions (Figure S1(b) in Supporting Information), results in a much faster reaction with $k_{obs}^{\text{Cpd I/stilbene}} = 1.1\times10^{-1}\ s^{-1}$ at −15 °C. The spectral changes associated with this reaction indicate reduction of **2** to a [Fe^{III}(TMP)] derivative (absorbance increase of the Soret band and absorbance decrease between 550 and 750 nm), whose visible spectrum is consistent with the formation of **3**.

When dimethyl sulfide[12] was added to the acetonitrile solution of **1** or **2**, sulfoxidation of DMS, monitored by the formation of **3**, was much faster for both oxygenating species than the corresponding oxygenation of *cis*-stilbene and resulted in $k_{obs}^{\text{Cpd 0/DMS}} = 3.5\times10^{-3}\ s^{-1}$ at −15 °C and [DMS] = 1.7×10^{-4} M and $k_{obs}^{\text{Cpd I/DMS}} = 0.27\ s^{-1}$ at −35 °C and [DMS] = 1.7×10^{-5} M for **1** and **2**, respectively (see Figure S2(a) and S2(b), respectively, in Supporting Information).

The oxygenation reactions of *cis*-stilbene or DMS by **1** were studied under pseudo-first order conditions for various excesses of substrates. Upon addition of larger concentrations of *cis*-stilbene or DMS to the acetonitrile solution of **1**, the decomposition reaction of the first intermediate appeared to be significantly accelerated (see Figure 3 as an example for the oxygenation of *cis*-stilbene).

The dependence of $k_{obs}^{\text{Cpd 0/stilbene}}$ on [*cis*-stilbene] gave a straight line with a significant intercept (see Figure S3(a) in the Supporting Information) and gave the second-order rate constant, $k^{\text{Cpd 0/stilbene}} = 0.142\pm0.006\ M^{-1}\ s^{-1}$ at −15 °C. The presence of an intercept of $(5.4\pm0.8)\times10^{-4}\ s^{-1}$ points to the occurrence of the parallel spontaneous decomposition of **1** under the reaction conditions (the rate constant in the absence of substrate $6.2\times10^{-4}\ s^{-1}$). The value of the second-order rate constant for the oxygenation of DMS by **1** determined in the same way (Figure S3(b) in the Supporting Information) was found to be $k^{\text{Cpd 0/DMS}} = 9.7\pm0.1\ M^{-1}s^{-1}$ at −15 °C, approximately 70-fold higher than that of *cis*-stilbene.

To compare the reactivity of **1** and **2**, the reaction of **2** was examined for various *cis*-stilbene and DMS concentrations. The values of $k_{obs}^{\text{Cpd I/stilbene}}$ plotted against [*cis*-stilbene] resulted in a straight line without a significant intercept (Figure S4, in the Supporting Information). The second-order rate constant is $k^{\text{Cpd I/stilbene}} = (66\pm2)\ M^{-1}\ s^{-1}$ at −15 °C, which is approximately 470-fold higher than the corresponding

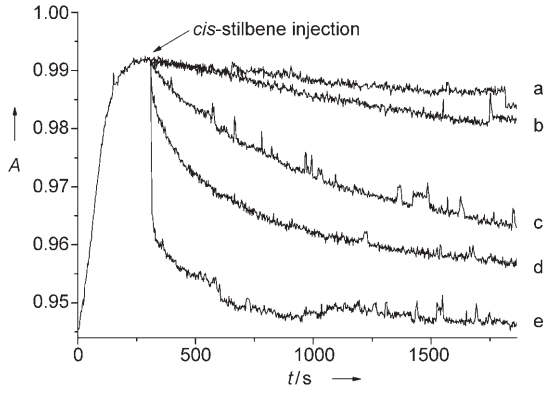
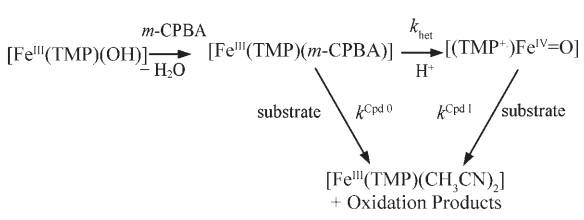


Figure 3. Decomposition of **1** after addition of various *cis*-stilbene concentrations: a) spontaneous decomposition; b) 1.35×10^{-3}; c) 6.76×10^{-3}; d) 1.35×10^{-2}; e) 2.71×10^{-2} M *cis*-stilbene. Experimental conditions: concentration of $[Fe^{III}(TMP)(OH)]=9.3\times10^{-6}$ M, concentration of *m*-CPBA $=5.4\times10^{-6}$ M in acetonitrile at −15 °C. Kinetic traces recorded at the Soret band.

value for **1** ($k^{\text{Cpd 0/stilbene}}$). The value of the second-order rate constant determined for the sulfoxidation reaction of DMS with **2** as oxidative species is extremely high, $k^{\text{Cpd I/DMS}}=(1.7\pm0.1)\times10^{4}\,\text{M}^{-1}\text{s}^{-1}$ at −35 °C (Figure S5 in the Supporting Information). Note, the reactivity of **1** or **2** towards selected organic substrates and the rates of the oxygenation reactions strongly depend on the chemical nature of the substrate used. The direct measurements of the second-order rate constants for both intermediates occurring in the catalytic cycle of $[Fe^{III}(TMP)(OH)]$ reveal that **1** can not compete with **2** in the direct epoxidation reaction of *cis*-stilbene or sulfoxidation of DMS. However, it should be kept in mind that on going from the direct measurements to studies under catalytic turnover conditions, where the substrate is present in the reaction medium right from the start of the catalytic cycle, the scenario is slightly different, that is, not only the relative ratio $k^{\text{Cpd I}}/k^{\text{Cpd 0}}$ plays a decisive role in the substrate oxidation in the competition between Cpd 0 and Cpd I. As illustrated in Scheme 1, **1** formed from $[Fe^{III}(TMP)(OH)]$ and *m*-CPBA in the presence of substrate can react in two different ways, that is, **1** can transfer oxygen to *cis*-stilbene ($k^{\text{Cpd 0}}$) or it can undergo heterolytic O–O bond cleavage to produce the significantly more active intermediate, **2** (k_{het}), which also reacts with the substrate ($k^{\text{Cpd I}}$).

Direct measurements have shown that $k^{\text{Cpd I}}\gg k^{\text{Cpd 0}}$, but the important question to answer is how fast is the heterolytic O–O bond cleavage in comparison to oxygen transfer from the first intermediate to substrate, that is, the ratio $k^{\text{Cpd 0}}/k_{\text{het}}$



Scheme 1. Two competing reaction pathways that the acylperoxoiron(III) porphyrin complex, formed from $[Fe^{III}(TMP)(OH)]$ and *m*-CPBA, can undergo in the presence of substrate.

must be taken into account. The heterolytic O–O bond cleavage in **1** is an acid-catalyzed reaction.[10] Under conditions of a small excess of *m*-CPBA (1:1.8) and in the absence of added substrate, **1** undergoes conversion into **2** within 1000 s with $k_{\text{obs}}{}^{\text{het}}=5.0\times10^{-3}\,\text{s}^{-1}$ in acetonitrile at −15 °C (Figure 1, inset). What effect can be expected in the presence of a substrate? A relative high excess of *cis*-stilbene (1.7×10^{-3} M, 1:161 based on $[Fe^{III}(TMP)(OH)]$, see Figure S6(a) in the Supporting Information) does not disturb formation of **1**, which accumulates in the reaction solution almost to the same amount as during the reaction in the absence of substrate. The formation of **1** is followed by a much slower reaction for which the spectral changes do not indicate formation of **2**, but formation of **3**. At first sight it could be concluded that under these conditions, **1** is formed and then it reacts with *cis*-stilbene to give **3** as the final product (in other words, **1** reacts faster with the substrate than it undergoes heterolytic O–O bond cleavage). However, detailed inspection of the $k_{\text{obs}}{}'$ value for the second slower reaction, reveals that it can not be the reaction between **1** and *cis*-stilbene, which based on the direct measurements should be significantly slower. At [*cis*-stilbene] $=1.7\times10^{-3}$ M the value of $k_{\text{obs}}{}^{\text{Cpd 0}}$ should be about $7.8\times10^{-4}\,\text{s}^{-1}$, which is approximately five-times lower than the value measured for the second reaction in the presence of *cis*-stilbene. To confirm this assumption, the reaction between $[Fe^{III}(TMP)(OH)]$ and *m*-CPBA was carried out under conditions of a sub-equivalent amount of oxidant (to inhibit formation of **2**) and the same excess of *cis*-stilbene. As expected, under such conditions the second reaction after the formation of **1**, is substantial slower (Figure S6(b) in the Supporting Information) with a rate constant corresponding to that measured for the reaction between **1** and *cis*-stilbene. On the basis of these observations, it can be concluded that under conditions of even a very small excess of oxidant (in the studied system the oxidant is also the source of protons), **1** formed in the reaction between $[Fe^{III}(TMP)(OH)]$ and *m*-CPBA is not responsible for the oxidation of *cis*-stilbene, because the heterolytic O–O bond cleavage is significantly faster than oxygen transfer from **1** to the substrate.

That formation of **2** can not be seen in the presence of a large excess of *cis*-stilbene can be accounted for in terms of the very fast reaction between **2** and the substrate. This means that with a small excess of oxidant and a large excess of *cis*-stilbene, the rate-determining step is heterolytic O–O bond cleavage in **1** (giving **2**) and not the oxygenation of substrate by **2** (see Scheme 1). Indeed, the observed rate constant measured for the reaction after the formation of **1** with a small excess of *m*-CPBA and a large excess of *cis*-stilbene ($k_{\text{obs}}{}'=4.3\times10^{-3}\,\text{s}^{-1}$), is in good agreement with that determined for O–O bond cleavage under otherwise identical reaction conditions but in the absence of substrate ($k_{\text{obs}}{}^{\text{het}}=5.0\times10^{-3}\,\text{s}^{-1}$). Note that although heterolytic O–O bond cleavage is the rate-determining step under such conditions, it is still faster than oxygen transfer from **1** to *cis*-stilbene. Thus, under the studied reaction conditions, the active species responsible for oxygenation of *cis*-stilbene is **2** and not **1**.

Convincing evidence that supports these assumptions was provided by an experiment in which the substrate and *m*-CPBA concentrations were kept at a low excess. It was

expected that under such conditions the reaction between **2** and *cis*-stilbene should not be so fast owing to the low [*cis*-stilbene], that is, heterolytic O–O bond cleavage in **1** will not be the rate-determining step in the reaction sequence shown in Scheme 1. Indeed, addition of a small excess of oxidant to the [Fe^{III}(TMP)(OH)] solution containing a small amount of the substrate reveals three subsequent reactions, that is, first **1** is formed, then this complex undergoes heterolytic O–O bond cleavage to form **2**, which then reacts with *cis*-stilbene to form **3** as the final product (Figure S6(c) in the Supporting Information). This experiment clearly shows that even at a very low excess of oxidant, heterolytic O–O bond cleavage of **1** is substantially faster than oxygen transfer from **1** to the substrate.

Based on the values of the observed rate constants determined for each reaction step, it can be calculated that only at [*cis*-stilbene] = 3.1×10^{-2} M (ca. 5000-fold excess based on the porphyrin complex), can *cis*-stilbene oxygenation by **1** compete with heterolytic O–O bond cleavage. This situation means that for the selected conditions where the oxidant concentration is kept in a small excess ([*m*-CPBA] = 1.6×10^{-5} M) and the substrate is present in concentrations higher than 3.1×10^{-2} M, **2** will not be responsible for the oxygenation of *cis*-stilbene, because its formation will be much slower than oxygen transfer from **1** to the substrate. Experimental evidence for this assumption is demonstrated in Figure S7 of the Supporting Information. Injection of 5×10^{-2} M *cis*-stilbene into the solution of **1** prepared from [Fe^{III}(TMP)(OH)] and a small excess of *m*-CPBA (the substrate was injected before **1** was converted into **2**), resulted in a very fast reaction leading to the formation of **3** as the final product. This reaction appears to be much faster than expected for the scenario with **2** as oxygenating agent. Note, that although oxygen transfer from **2** to *cis*-stilbene is much faster than the oxygenation reaction by **1**, it first requires formation of **2**, which is the rate-determining step under such conditions. In other words, the observed reaction can not be faster than heterolytic O–O bond cleavage at the selected *m*-CPBA concentration. Thus, all these findings support that under extreme conditions, such as a several thousand-fold excess of *cis*-stilbene and a very small excess of *m*-CPBA, **1** has a chance to act as an oxygenating agent towards *cis*-stilbene.

The competition scenario seems to be slightly different when a more reactive substrate, such as DMS is present. Since sulfoxidation of DMS by **1** is much faster than the corresponding epoxidation of *cis*-stilbene, it can compete with heterolytic O–O bond cleavage at significantly lower substrate concentrations. In fact, a DMS concentration of approximately 4.5×10^{-4} M is enough to reach conditions at which oxygen transfer from **1** to DMS efficiently competes with the heterolytic cleavage of the O–O bond when the oxidant concentration is kept at a very low excess, that is, [*m*-CPBA] = 1.6×10^{-5} M.

In conclusion, direct kinetic studies on epoxidation and sulfoxidation reactions by **1** and **2** revealed that the oxygenating capability of the iron(IV) oxo porphyrin π-cation radical towards selected organic substrates is orders of magnitude higher than that of the acylperoxoiron(III) porphyrin complex. However, it should be emphasized that although oxygen transfer from **2** to the selected substrates proceeds extremely quickly, under selected reaction conditions where formation of the high-valent iron species is the rate-determining step and the substrate is present in the reaction medium right from the start of the catalytic cycle, care should be taken concerning the relative ratio between the rate of O–O bond heterolytic cleavage leading to the formation of **2** and the rate of oxygen transfer from **1** to the substrate. Note that the formation of **2** in the present study was tuned through the selected reaction conditions to be extremely slow, whereas in biological systems conversion of Cpd 0 into Cpd I is known to be very fast and controlled by the proton release from the amino acid residues at the active site. The presented in vitro results suggest that the high-valent oxo–iron species Cpd I must be the sole active species responsible for in vivo P450 oxygenation reactions under enzymatic turnover conditions.

Experimental Section

Materials: All solutions were prepared in acetonitrile (99.9% AMD CHROMASOLV from Sigma-Aldrich). Fe(III)meso-tetra(2,4,6-trimethylphenyl)porphyrin hydroxide ([Fe^{III}(TMP)(OH)]) was obtained from Frontier Scientific Porphyrin Product. *cis*-Stilbene (96%) and dimethyl sulfide were purchased from Aldrich. *m*-Chloroperoxybenzoic acid was purchased from Acros Organics and purified before use by re-crystallization from hexane.

Low Temperature Stopped-Flow Instrument and Software: Time-resolved UV/Vis spectra were recorded with a Hi-Tech SF-3L low-temperature stopped-flow unit (Hi-Tech Scientific, Salisbury, UK) equipped with a J&M TIDAS 16/300-1100 diode array spectrophotometer (J&M, Aalen, Germany). The optical cell had a light path of 1.0 cm and was connected to the spectrophotometer unit with flexible light guides. 5 mL driving syringes were used. The mixing chamber was immersed in an ethanol bath which was placed in a Dewar flask containing liquid nitrogen. The ethanol bath was cooled by liquid nitrogen evaporation and its temperature was measured by use of a Pt resistance thermometer and maintained at ±0.1 °C by use of a PID temperature-controlled thyristor heating unit (both Hi-Tech). Complete spectra were collected between 372 and 732 nm with the integrated J&M software Kinspec 2.30.

Low Temperature Spectral Measurements: Time-resolved UV/Vis spectra were recorded with a quartz-glass dip-in detector (Spectralytics, Aalen, Germany) coupled to a J&M TIDAS 16/300-1100 diode array spectrophotometer (J&M, Aalen, Germany). The optical dip-in detector had a light path of 1.0 cm and was connected to the spectrophotometer unit with flexible light guides. A 20 mL double-wall reaction vessel was used and thermostatted (±0.1 °C) by a combination of cold methanol circulation (Colora WK 14-1 DS, Lorch, Germany) and an 800 W heating unit. Complete spectra were recorded between 372 and 732 nm with the integrated J&M software Kinspec 2.30.

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[9] The hydroxide-substituted derivative of [Fe^{III}(TMP)] was chosen because of the significantly larger difference between the UV/Vis spectrum of [Fe^{III}(TMP)(OH)] and that of **1** in acetonitrile. For example, the addition of a very small amount of *m*-CPBA to the [Fe^{III}(TMP)Cl] derivative also results in the formation of **1** (data not shown), but its UV/Vis characteristics are very similar to those of [Fe^{III}(TMP)Cl], although not the same. Thus, very small absorbance changes accompanying substitution of chloride by *m*-CPBA in [Fe^{III}(TMP)Cl] make the UV/Vis detection of **1** difficult.

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[11] For comparison, **3** can be obtained after acidification of an acetonitrile solution of [Fe^{III}(TMP)(OH)] by a non-coordinating acid, for example *p*-toluenesulfonic acid (data not shown)

[12] The potential coordinating ability of DMS was checked using UV/Vis spectrophotometry. It was found that DMS does not bind to the iron(III) center of [Fe^{III}(TMP)(OH)] in the DMS concentration range studied.

