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Formation of a Tyrosyl Radical in Xanthine Oxidase[†]

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ABSTRACT: Treatment of xanthine oxidase with ferricenium at high pH gives rise to an EPR signal not previously seen with this enzyme. The signal is apparently isotropic at 9 GHz with a g_{avg} of ≈2 and once generated is stable to pH 6.0, so long as the sample is kept in the dark. Treatment of the signal-giving species with hydroxyurea results in complete loss of the signal, indicating that the signal is radical-based. Pretreatment of the enzyme with iodoacetate has no effect on signal formation with ferricenium. The ferricenium-generated EPR signal shows proton hyperfine coupling that is not lost upon exchange into D_2O and bears considerable resemblance to the tyrosyl radical of the photosynthetic reaction center and other systems. These observations lead us to interpret the new ferricenium-generated EPR signal of xanthine oxidase as arising from a tyrosyl radical, the result of one-electron oxidation of a protein tyrosinate residue. Kinetic parameters for the reductive half-reaction of ferricenium-treated xanthine oxidase with xanthine were determined by stopped-flow spectrophotometry; k_{red} and $K_D^{xanthine}$ (15 s^{-1} and 12 μM, respectively) were essentially unchanged. Addition of 2-hydroxy-6-methylpurine (in the presence of 2 mg/mL catalase and superoxide dismutase) generated the "very rapid" Mo^V EPR signal while preserving the ferricenium-derived EPR signal, providing a further indication that the modified enzyme remains fully functional and the presence of the tyrosyl radical does not impact turnover by the enzyme. Coupling of the two signals was not evident, nor was coupling to the two 2Fe−2S centers or the flavin semiquinone evident. The implications of covalent modifications of proteins mediated by ferricenium are discussed.

Xanthine oxidase, a homodimeric metalloprotein ($M_r \approx$ 300 kDa) containing one molybdenum atom, two 2Fe−2S ferredoxin-type centers, and one FAD[1] per subunit (*1*, *2*), catalyzes the oxidative hydroxylation of xanthine and related heterocycles. Hydroxylation of the substrate and reduction of the enzyme occur at the molybdenum site in the course of the reductive half-reaction of the catalytic sequence (*3*, *4*). Molecular oxygen serves as the acceptor of reducing equivalents generated during the course of catalysis, reacting at the FAD of the enzyme in the oxidative half-reaction (*5*). Because the reductive and oxidative half-reactions occur at separate sites, intramolecular electron transfer between them (mediated by the two iron−sulfur centers) is integral to catalysis (*6*).

A pyranopterin cofactor, consisting of a bicyclic pterin fused to a monocyclic pyran ring, is known to directly coordinate the molybdenum of xanthine oxidase through a dithiolene side chain (*7*, *8*). The pyranopterin cofactor in all likelihood does not directly participate in the catalytic sequence of the molybdenum hydroxylases but has been implicated in mediating electron transfer to other redox-active cofactors (*8*) and/or modulating the reduction potential of the molybdenum (*9*, *10*). In the related enzyme sulfite oxidase, oxidation of the pyranopterin cofactor by ferricyanide has been reported to attenuate the oxidative half-reaction with cytochrome *c*, and this observed rate decrease has been interpreted as arising from compromised electron transfer from the molybdenum site to the heme moiety upon oxidation of the pyranopterin cofactor (*11*). In light of this observation, we have attempted to oxidize the pyranopterin cofactor of xanthine oxidase by treating the enzyme with ferricenium hexafluorophosphate, a strong oxidant. Ferricenium salts have been widely used in studies of biological reactions owing to their ease of preparation and the convenient absorbance change associated with reduction that is useful in enzyme assays (*12*, *13*). However, little is known about the effects of ferricenium−protein interactions or the possible reactivity of ferricenium with proteins. In this work, we have investigated the effect of ferricenium on xanthine oxidase and find no evidence to suggest that the pyranopterin cofactor of xanthine oxidase can be oxidized by ferricenium. Instead, we observe a new EPR signal generated with this reagent under certain reaction conditions. The characteristics of this novel EPR signal lead us to interpret the signal-giving species as a phenoxy radical generated by oxidation of a tyrosinate residue of the protein by the ferricenium ion.

MATERIALS AND METHODS

Xanthine oxidase was purified from unpasteurized cow's milk obtained from the dairy herd of The Ohio State University using the method of Massey et al. (*14*). CM-52 and S-200 column chromatography steps were included at the end of purification to remove contaminating lactoper-

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[1] Abbreviations: AFR, activity-to-flavin ratio; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; FAD, flavin adenine dinucleotide; HMP, 2-hydroxy-6-methylpurine.

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oxidase. Typical AFR values (the ratio of catalytic activity to the absorbance at 450 nm) were ≈140, indicating that the enzyme was approximately 65% active (fully functional enzyme has an AFR of 210 under the standard assay conditions of 1 atm of oxygen, 111 μM xanthine, 0.1 M sodium pyrophosphate, pH 8.5, and 25 °C). Xanthine dehydrogenase was purified from chicken livers by the method reported by Ratnam et al. (*15*).

Ferricenium hexafluorophosphate was prepared by the method of Lehman et al. (*13*), and fresh stock solutions were prepared immediately prior to use in these experiments. Xanthine was purchased from the Sigma Chemical Co. 2-Hydroxy-6-methylpurine (HMP) was purchased from the Alfred Bader Division of the Aldrich Chemical Co. and was recrystallized by dissolving in dilute base, decolorized over activated charcoal, acid precipitated, filtered, and dried. All other reagents and buffers were of the highest quality commercially available and were used without further purification.

The reaction of xanthine oxidase with ferricenium was carried out by incubating the enzyme (approximately 100 μM) with 5 mM ferricenium hexafluorophosphate; the time course of formation of the signal-giving species was determined, and we determined that maximal signal accumulation occurs after 15 min on ice in the dark. The EPR signal of ferricenium-treated xanthine oxidase was found to exhibit modest photosensitivity such that after 10 min in ambient room light much of the signal had been lost. Owing to this light sensitivity, all samples were prepared in the dark. After reaction with ferricenium ion, the mixture was passed through a G-25 column [equilibrated with 0.1 M CAPS buffer and 0.3 mM EDTA (pH 10)] in the dark to rid the protein of ferricenium and the ferricenium-free enzyme was then placed in quartz EPR tubes and frozen in liquid nitrogen. Cysteine residues were blocked by incubating xanthine oxidase in the presence of a 100-fold molar excess of iodoacetate for 1 h at room temperature [in 0.05 M sodium pyrophosphate and 0.3 mM EDTA (pH 8.5)]. The "very rapid" xanthine oxidase EPR signal was generated by reacting xanthine oxidase [100 μM in 0.1 M CAPS buffer and 0.3 mM EDTA (pH 10)] with 5 equiv of HMP for 40 s on ice, whereupon solutions were immediately frozen in quartz EPR tubes in a dry ice/acetone bath and then transferred to liquid nitrogen.

Ultraviolet−visible spectra and the kinetics of ferricenium oxidation of xanthine oxidase were recorded using a Hewlett-Packard 8452 single-beam diode-array spectrophotometer interfaced with a Hewlett-Packard Chemstation computer. Electron paramagnetic resonance spectra were collected on a Brüker Instruments, Inc., ER 300 spectrometer equipped with an ER 035M NMR gaussmeter and a Hewlett-Packard 5352B microwave frequency counter. EPR spectra were simulated using a simulation package developed by G. George at the Stanford Linear Accelerator Center.

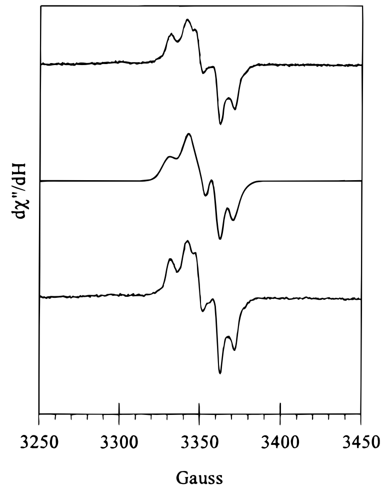

FIGURE 1: EPR spectra of ferricenium-treated xanthine oxidase in H_2O (top spectrum) and in D_2O (bottom spectrum). Spectra were recorded at 150 K, with a 9.45 GHz microwave frequency, a 2 mW power, and a 2 G modulation amplitude. The simulation of the EPR signal of ferricenium-oxidized xanthine oxidase is shown in the middle. Parameters used for the simulation are as follows: g, 2.015; g_{avg}, 2.012; first average A value, 14 G; second average A value, 8.8 G; and half-line width, 5.2 G.

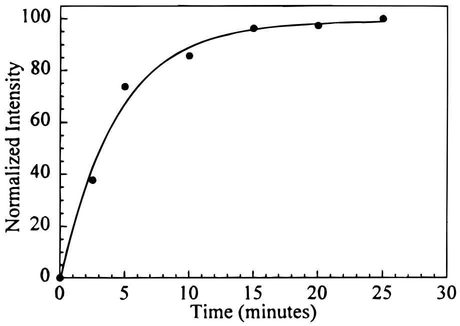

FIGURE 2: Time course of formation of the signal-giving species formed from ferricenium-mediated oxidation of xanthine oxidase (●) and the single-exponential fit (—). The rate of appearance of the EPR signal is 0.23 min^{-1}. The reaction and EPR spectral collection conditions were the same as in Figure 1. Spectral intensities were determined by double integration of the EPR spectra and normalized to the enzyme concentrations for each sample.

RESULTS

Figure 1 (upper spectrum) shows the EPR signal observed upon reaction of xanthine oxidase with ferricenium at pH 10 (the sample was prepared as described in Materials and Methods). The split isotropic signal is centered at $g \approx 2$ and is generally reminiscent of the tyrosyl radical signals observed in photosystem II (*16*) and bovine liver catalase (*17*). The middle spectrum of Figure 1 shows the simulated spectrum. The good agreement between the observed and simulated spectra strongly indicates that only a single paramagnetic center is generated by reaction with ferricenium. Double integration of the signal using the "very rapid" Mo^{V} EPR signal observed upon reaction of xanthine oxidase with HMP as an internal standard (see below) indicates approximately one spin center per enzyme molybdenum. Also shown in Figure 1 (lower spectrum) is the spectrum of the ferricenium-derived signal in D_2O buffer. The sample was exchanged into D_2O by passage through a G-25 column hydrated in D_2O buffer [0.1 M CHES and 0.1 M KCl (pD 9.6)]. The EPR spectrum of the sample prepared in D_2O is virtually identical to the spectrum of the sample prepared in H_2O, exhibiting a split isotropic resonance centered at $g \approx 2$, indicating that the paramagnetic center lacks exchangeable protons. Shown in Figure 2 is the time course of formation of the signal-giving species (solid circles) determined by incubating xanthine oxidase with 5 mM ferricenium hexafluorophosphate (on ice and in the dark) for increasing times. Intensities were determined by double integration and

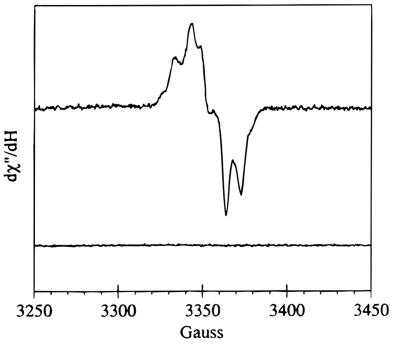

FIGURE 3: EPR spectrum of the signal formed after incubation of xanthine oxidase with ferricenium (top spectrum). Adding hydroxyurea to the sample completely quenches the signal resulting from ferricenium treatment (bottom spectrum), indicating that the signal is radical-based. Spectra were recorded under conditions identical to those in Figure 1.

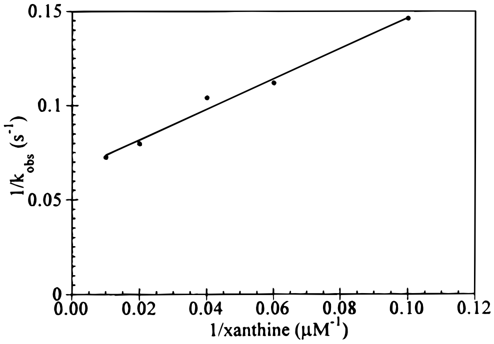

FIGURE 4: Rate of tyrosine oxidation of xanthine oxidase determined from a plot of k_{obs} vs ferricenium concentration. The value for the rate of tyrosine oxidation is 0.25 M^{-1} s^{-1} at room temperature. Additionally, the rate constant for tyrosyl radical reduction is 7.24 × 10^{-6} M^{-1} s^{-1}. The reaction was monitored at the peak absorbance of reduced ferricenium (618 nm).

normalized to the concentration of xanthine oxidase. Also shown is the single-exponential fit (solid line) giving a rate of appearance of the signal of 0.23 min^{-1} with maximal signal accumulation after an incubation for 15 min under these reaction conditions and decays significantly (by approximately 80%) over a period of 10 min in ambient light (data not shown). Addition of hydroxyurea to 1 mM to ferricenium-treated xanthine oxidase (upper spectrum, Figure 3) results in complete loss of the EPR signal (lower spectrum, Figure 3), indicating that the signal-giving species is radical-based. To rule out the possibility that the signal may arise from a sulfinyl radical formed upon cysteine oxidation by the ferricenium ion, cysteine residues were alkylated with iodoacetate prior to ferricenium treatment. Alkylation of cysteine residues had no effect on the EPR signal formed following ferricenium treatment (data not shown). The characteristics of the ferricenium-generated EPR signal lead us to assign it as a tyrosyl radical generated upon ferricenium-mediated oxidation. As seen with other tyrosyl radicals, splitting of the isotropic radical resonance arises from coupling to both the phenolic and methylene protons.

The pH dependence of signal formation was investigated by incubating xanthine oxidase at pH values ranging from 6 to 10 with 5 mM ferricenium hexafluorophosphate for 15 min. Each reaction was stopped by passage through a G-25 column equilibrated in identical buffer such that the original pH was maintained. Maximal signal generation occurred at pH 10, although once generated, the signal was stable indefinitely down to pH 6 (data not shown). These results are consistent with facile tyrosine oxidation by ferricenium from a parent tyrosinate anion. The rate constant for oxidation of the tyrosine residue at pH 10 was determined by conducting a family of experiments wherein the rate of reduction of ferricenium at varying absolute concentrations of oxidant was monitored at the peak absorbance of ferricenium at 618 nm. Figure 4 shows the plot of k_{obs} versus ferricenium concentration, and from the slope of this plot, a forward rate constant of 0.25 M^{-1} s^{-1} is obtained. The small finite *y*-axis intercept indicates that the oxidation is reversible, with a reverse rate constant of 7.24 × 10^{-6} M^{-1} s^{-1}.

To investigate the influence of tyrosine oxidation on the activity of the molybdenum center, ferricenium-oxidized enzyme was allowed to react with HMP at pH 10, which leads to the accumulation of substantial amounts of the "very rapid" EPR signal (Figure 5, upper spectrum). In these experiments, which were performed under anaerobic conditions, including catalase and superoxide dismutase (2 mg/mL) in the reaction mix proved to be necessary for preserving the tyrosyl radical EPR signal. Presumably, superoxide and/or peroxide generated in the course of the aerobic turnover of HMP by the enzyme leads to quenching of the ferricenium-derived EPR signal. EPR spectra were collected at both liquid nitrogen and helium temperatures, and deconvolution of the observed spectra indicates that the very rapid and tyrosyl radical signals are essentially unchanged compared with standard samples consisting of only one or the other signal, implying that there was no detectable splitting of one signal by the other. However, line broadening of <1 or 2 G would be difficult to determine with confidence. The fact that the tyrosyl radical and the "very rapid" species can coexist in the enzyme indicates that the former cannot be quenched by electron transfer from the canonical redox-active centers of the enzyme. It should also be pointed out

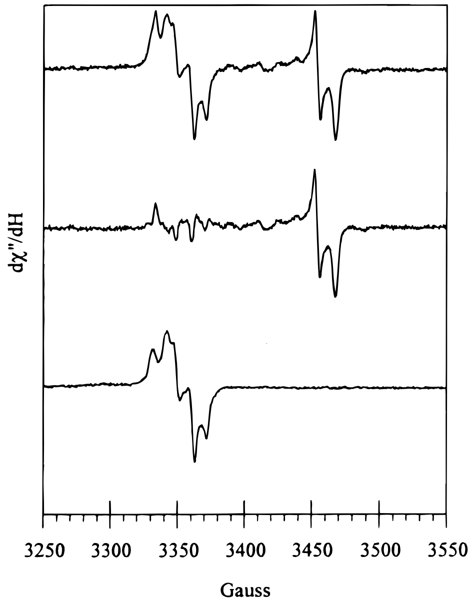

FIGURE 5: EPR spectra of ferricenium-treated xanthine oxidase in the presence of the "very rapid" EPR (Yrad − Mo^{V}) signal (top spectra) and the subtraction results of the (Yrad − Mo^{V}) minus the tyrosyl radical signal (middle spectra) and the (Yrad − Mo^{V}) minus the "very rapid" signal (bottom spectra). Spectra were recorded under conditions identical to those in Figure 1.

that a comparison of the signal intensity resulting from the tyrosyl radical to that of the HMP-generated "very rapid" Mo^V signal (known to amount in ∼80% of the functional molybdenum centers of the sample in the Mo^V state) suggests that the integrated intensity of the tyrosyl signal corresponds to approximately one spin per subunit, indicating that a single tyrosine is particularly sensitive to oxidation by the ferricenium ion.

In an effort to establish whether the tyrosyl radical interacts with either the Fe−S or flavin centers of xanthine oxidase, two additional experiments were performed. In the first, ferricenium-treated xanthine oxidase at pH 6.0 was mixed with 1 equiv of xanthine in an EPR tube and promptly (less than 2 s) frozen in a dry ice/acetone bath. Under these conditions, the equilibrium distribution of reducing equivalents within the enzyme will be such that Fe−S II and FAD are reduced and EPR-active. In the second experiment, ferricenium-treated enzyme was reacted with 5 equiv of xanthine at pH 8.5. Under these conditions, the flavin is reduced to the hydroquinone and the reduced iron−sulfur centers are the primary EPR-active centers. Neither sample gave evidence of strong magnetic interaction between the tyrosyl radical and either of the Fe−S centers or the flavin semiquinone.

Because a tyrosyl residue has been implicated in the NAD^+ binding site of xanthine dehydrogenase (*18*) (an enzyme closely related to xanthine oxidase that utilizes NAD^+ rather than dioxygen as its oxidizing substrate), we used the redox-inert NADH analogue 3-aminopyridine adenine dinucleotide (AAD) (*19*) in an experiment aimed at determining whether the ferricenium-oxidized tyrosyl radical of xanthine oxidase was near the NAD^+ binding site. Incubation of ferricenium-oxidized xanthine oxidase (90 μM) with a 10-fold stoichiometric excess of AAD did not perturb the tyrosyl radical EPR signal, suggesting that the ferricenium-oxidized site giving rise to the EPR signal is not near the NAD^+ binding site (data not shown). Treating xanthine dehydrogenase with ferricenium results in an EPR signal identical to that seen with xanthine oxidase post-ferricenium treatment; subsequent AAD addition to xanthine dehydrogenase has no perturbatory effect on the EPR signal, illustrating both that xanthine dehydrogenase is sensitive to ferricenium treatment in a fashion analagous to that of xanthine oxidase and that the tyrosyl radical thus formed is not the tyrosyl implicated in NAD^+ binding.

The observation that the enzyme retains activity at the molybdenum site, as shown by the formation of the catalytic intermediate giving rise to the very rapid EPR signal upon reaction with HMP, indicates that ferricenium oxidation of xanthine oxidase has no substantial effect on the catalytic capability of the molybdenum site. To confirm this, the reductive half-reaction of ferricenium-treated xanthine oxidase with xanthine was examined by stopped-flow spectrophotometry. The observed kinetic parameters obtained from the dependence of ferricenium-oxidized enzyme reduction on the concentration of xanthine ($k_{red} = 15\ s^{-1}$ and $K_d^{xanthine} = 12\ \mu M$) were not significantly different from literature values for native enzyme, leading us to conclude that the presence of the tyrosyl radical generated by reaction with ferricenium does not affect catalytic turnover of xanthine oxidase.

DISCUSSION

These results demonstrate that at high pH ferricenium hexafluorophosphate readily oxidizes a tyrosyl residue of xanthine oxidase to a phenoxy radical. The resulting ferricenium-oxidized protein exhibits an isotropic EPR signal with a *g* value typical of an organic radical ($g_{avg} \approx 2$) that is dependent on high pH for its formation, is moderately light sensitive, and is lost upon treatment with hydroxyurea. Further, formation of the EPR signal upon ferricenium oxidation is insensitive to alkylation with iodoacetate prior to ferricenium treatment. In conjunction with the observation that no loss of signal splitting is seen upon exchange with D_2O, the simulated EPR spectrum of the tyrosyl radical in xanthine oxidase closely resembles those of other tyrosyl radicals such as those in photosystem II (*16*) and bovine liver catalase (*17*). The signal intensity of the tyrosyl radical compared to that of the "very rapid" Mo^V signal generated with HMP (known to give rise to ∼80% of the functional molybdenum centers of the sample in the Mo^V state) and the good agreement between the observed and simulated EPR signals (Figure 1) suggest that a single tyrosyl is sensitive to oxidation and is quantitatively converted to the tyrosyl radical. The new radical species does not appear to interact strongly with any of the canonical redox-active centers of xanthine oxidase when these are made paramagnetic. This observation, along with full retention of enzyme activity upon tyrosine oxidation, rules out a sulfite oxidase form of the pyranopterin cofactor at the molybdenum center as the signal-giving species. We conclude that the pyranopterin moiety of xanthine oxidase is not susceptible to oxidation by reagents such as ferricenium hexafluorophosphate, in all likelihood due to physical inaccessibility [in the crystal structure of the related enzyme aldehyde oxidoreductase from *Desulfovibrio gigas* (*8*), the pyranopterin cofactor is found to be largely inaccessible to solvent].

Generally, ferricenium is used not as an oxidizing agent per se but rather as an oxidizing substrate in assays where the concentrations of both protein and ferricenium are quite low and the assay time course is short. It is apparent from these results that, while ferricenium does oxidize the susceptible tyrosine residue of xanthine oxidase under certain conditions, it does so at a rate (0.25 $M^{-1}\ s^{-1}$) that is not likely to significantly affect an enzyme assay utilizing ferricenium as an oxidizing substrate. However, when ferricenium is used as a direct oxidizing agent, where concentrations of both ferricenium and protein are high as in these experiments, it is clear that oxidation of amino acid side chains can occur. This work indicates that, while ferricenium remains an extremely useful reagent for use with redox-active proteins, caution must be exercised in its application so that undesirable oxidation of amino acid side chains can be avoided.

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