KINETIC STUDIES OF ELECTRON TRANSPORT REACTIONS AT LOW TEMPERATURES IN XANTHINE OXIDASE

Alan Van Heuvelen

Physics Department, Box 3D

New Mexico State University, Las Cruces, New Mexico 88003

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SUMMARY. Samples of rapidly frozen xanthine oxidase reduced with xanthine have been warmed between -78°C and -50°C. EPR measurements of oxidation - reduction processes at these temperatures have revealed a new EPR signal which appears to be a disulfide radical involved in xanthine hydrolysis. Other EPR signal changes indicate that at pH 6.5 enzyme reduction by xanthine is rate limiting and at pH 8.5 or higher that some step following enzyme reduction is rate limiting. Evidence is presented for the lack of anaerobicity in most rapid freeze apparatus, the oxygen entering the samples during rapid freeze quenching in isopentane.

INTRODUCTION. Xanthine oxidase has a molecular weight of 380,000 and appears to have two independent active centers each containing one molybdenum ion, one FAD and two distinct iron-sulfer centers (1). A substrate such as xanthine deposits two electrons (and perhaps a proton) with the Mo(VI) ion on the enzyme and is then hydrolyzed to form uric acid. The two electrons given the enzyme equilibrate rapidly between the various enzyme prosthetic groups before being picked up at the flavin by oxygen to form H_2O_2 (2-4). The rate of appearance and disappearance of different paramagnetic species ($\mathrm{Mo}^{5+}_{\mathrm{VR(very\ rapid)}}$, $\mathrm{Mo}^{5+}_{\mathrm{R(rapid)}}$, $\mathrm{Fe/S}^{\circ}_{\mathrm{I}}$, $\mathrm{Fe/S}^{\circ}_{\mathrm{II}}$ and FADH°) are representative of these enzyme reduction and oxydation processes.

Reported herein are EPR measurements and interpretations of the changing electron distributions due to oxidation-reduction processes in rapid freeze samples as these samples are warmed for various times at temperatures of -78°C to -50°C. The specific changes depend on pH, temperature and on rapid freeze delay time before quenching.

METHODS AND MATERIALS. Xanthine oxidase was prepared according to the procedure of Massey et al. Xanthine was purchased from Sigma Chemical Corporation. Buffers used included 0.1 M phosphate (pH 6.3), 0.1 M pyrophosphate (pH 8.5) and 0.1 M glycine (pH 10.1). A Varian V-4502 EPR spectrometer with a modified Varian temperature control device was used to measure EPR spectra of samples rapidly frozen in an apparatus similar in design to that developed by Ballou (5). Rapid freeze samples were warmed in slush baths (6) after which EPR spectra were recorded under conditions described by Edmondson et al (2). We were unable to use liquid helium for observing the Fe/S^{o}_{II} ($g_{av} = 2.01$) EPR signal. Some samples were run continuously in the EPR spectrometer at temperatures from -78°C to -33°C. Both methods of warming gave consistent results. EPR signal integrations were made using Cu(II)-EDTA as a standard (the errors in these integrations - especially for Fe/S^{o}_{I} - can be large).

SULFUR RADICAL EPR SIGNAL. Figure 1 shows the development of a new EPR signal observed in rapid freeze, slow warm samples of xanthine oxidase reduced by xanthine at pH 6.3. The signal grew in amplitude as 10,29 and 57 msec rapid freeze shots were warmed at -50°C for periods of ten or more hours but did not appear in pH 8.5 or 10.3 samples. This new signal overlaps with flavin and molybdenum EPR signals, making it difficult to determine its shape. The signal does have a derivative maximum at $g = 2.025$ and the midpoint between this maximum and what appears to be a derivative minimum is at about $g = 2.015$. The shape and unusual g-value suggest that the signal is a sulfur radical (7-10).

The new EPR signal is observed only in slow warm experiments during which a substrate can reduce the enzyme and perhaps be hydrolyzed, but after either or both events the substrate cannot leave the active site due to the frozen environment. Thus the new signal could be due to a disulfide radical bonded from the enzyme to the oxidized or hydrolyzed substrate. These observations are consistent with the reduction mechanism proposed by Massey et al (11). The appearance of the new EPR signal only at low pH's must be due to the pH dependence of oxidation-reduction potentials of other electron reduction centers on the enzyme.

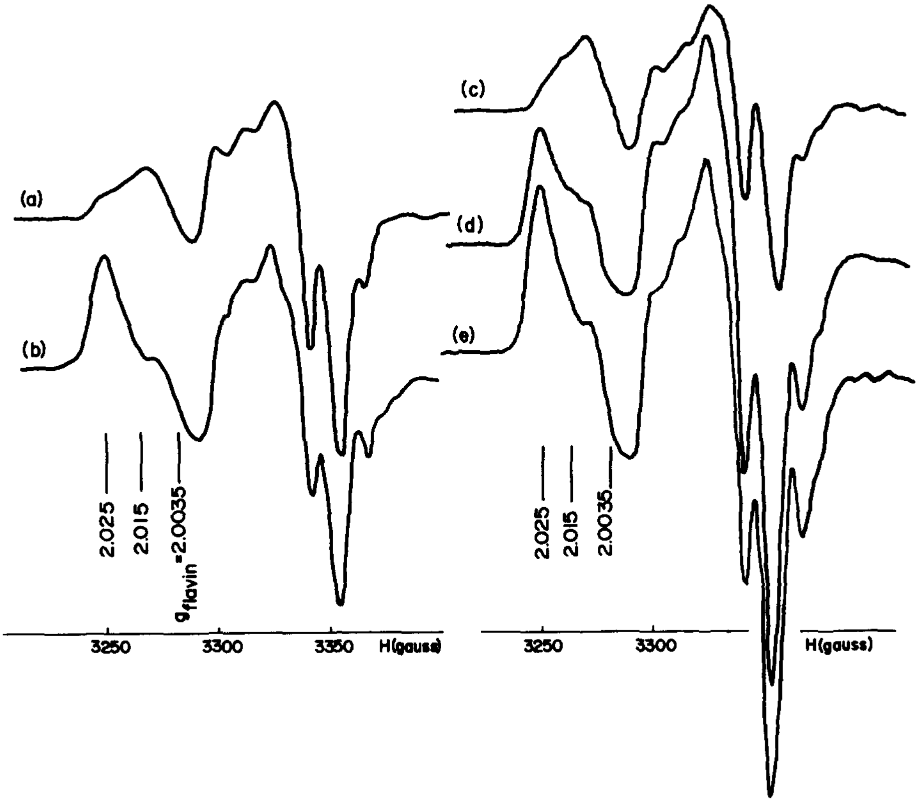

Figure 1. EPR spectra of pH 6.3 rapid freeze samples at various stages of warming at -50°C. The growth of an EPR signal at $g = 2.15$ can be seen. (a) 29 msec rapid freeze shot with no warming (modulation setting = 1600, gain setting = 125,27 mwatts power, 9.21 GHz, time constant 1.0 sec, sample temperature 123°K). (b) Same as (a) only warmed for 1500 minutes at -50°C. (c) 10 msec rapid freeze shot with no warming (EPR settings same as (a) only gain = 250). (d) Same as (c) only warmed at -50°C for 150 minutes. (e) Same as (c) only warmed at -50°C for 1500 minutes.

OXIDATION IN "ANAEROBIC" RAPID FREEZE SAMPLES. Most EPR signal amplitude changes shown in Figures 2 and 3 (and seen in other samples not reported) reflect oxidation processes rather than reduction processes even though samples were prepared anaerobically. E.g. the Fe/S_I EPR signal decreased significantly in both samples in Figure 2, a behavior which can only occur if the enzyme is losing electrons (4). Similar effects were seen in all but the early rapid freeze shots.

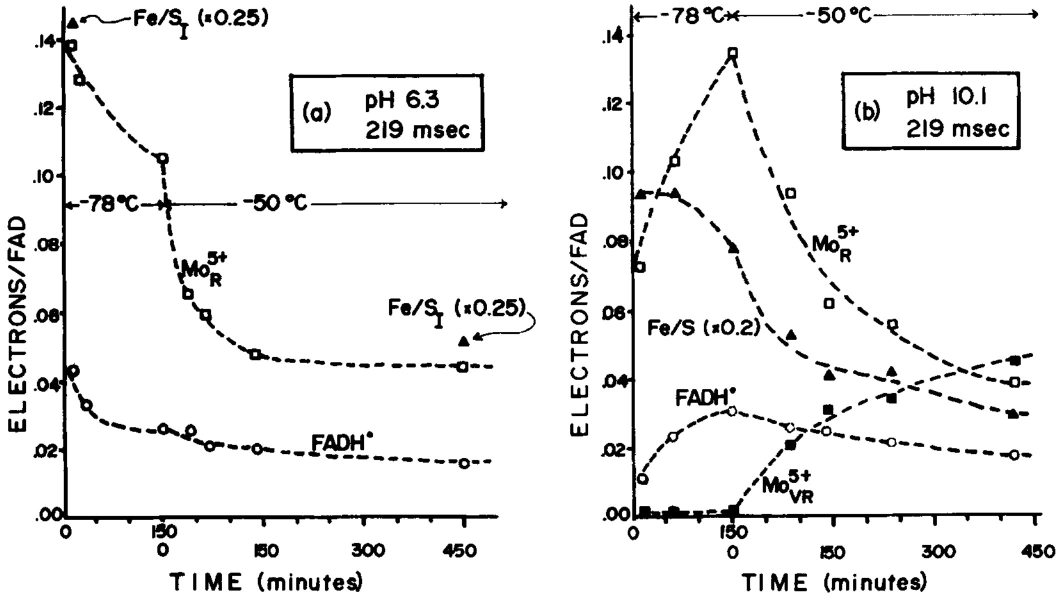

Figure 2. (a) EPR signal amplitude changes as a 219 msec pH 6.3 rapid freeze shot is warmed for various times at -78°C and -50°C. (b) Same as (a) only for a pH 10.1 rapid freeze sample.

The preparation and mixing parts of the rapid freeze apparatus keep samples at less than 10^{-6} M in oxygen. A layer of oxygen free water containing dithionite placed above samples to isolate them from atmospheric oxygen did not alter this reoxidation. Rapid freeze quenching occurs in isopentane held at -140°C. Oxygen is very soluble in isopentane [more than ten times that in water (12)] and we believe this is the source of oxygen in our samples.

Most rapid freeze experiments reported in the literature used a quenching technique similar to ours. In these experiments there can be considerable reoxidation of rapid freeze samples in the 5-7 msec quenching time in isopentane. It should be noted that the 5-7 msec is an effective time for the reactants if kept at their mixing temperature (usually 23°C). Thus for an enzyme like xanthine oxidase whose fast phase of reoxidation by oxygen to form hydrogen peroxide is 6 msec (3), we expect roughly 50% of the enzymes to lose two electrons during rapid freeze quenching. This reoxidation during quenching

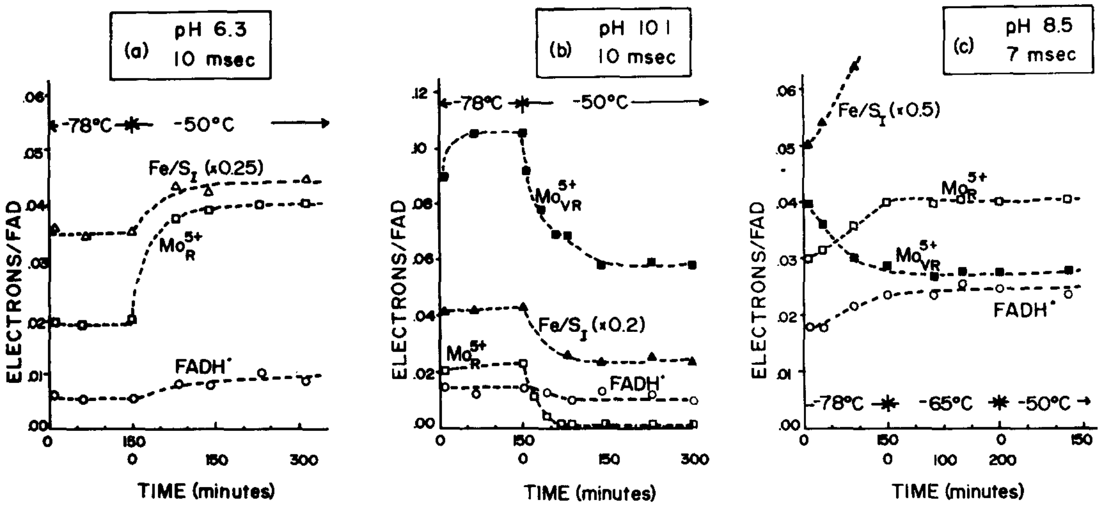

Figure 3. (a) EPR signal amplitude changes as a 10 msec, pH 6.3 rapid freeze shot is warmed for various times at different temperatures. (b) Same as (a) except sample was rapidly frozen at 10 msec in pH 10.1 buffer. (c) Same as (a) except sample was rapidly frozen at 7 msec in pH 8.5 buffer.

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can explain the fact that significant Mo^{5+} and FADH° EPR signals are observed in long delay, rapid freeze shots of xanthine oxidase reduced by excess substrate (2), whereas we should see only fully reduced enzymes with diamagnetic Mo^{4+} and $FADH_2$ centers for these long delay shots.

Olson et al (3,4) have described the reoxidation of fully reduced xanthine oxidase (six electrons) in terms of a fast phase in which five electrons are removed and a slow phase in which the last electron is removed with much greater difficulty. The EPR signal amplitudes in Figures 2 and 3 after warming at -50°C for 300 minutes approach a steady state which is nearly the same for all rapid freeze shots at a particular pH (this was true for other samples not shown here). Evidently this steady state electron distribution is representative of one electron reduced enzymes left after the fast phase of reoxidation. EPR integrations, although not decisive, are consistent with this interpretation.

ENZYME REDUCTION. Figure 3(a) shows all EPR signals increasing as a 10 msec, pH 6.3 sample is warmed at -50°C. This is clearly an enzyme reduction process whereby the enzyme receives two electrons per active site from a substrate and probably loses one electron to oxygen in the fast reoxidation process discussed earlier. The temperature dependence for the rate of increase of the EPR signals in Figure 3(a) when extrapolated back to room temperature is the same as the substrate turnover rate. This implies that at pH 6.3 enzyme reduction is probably a rate limiting step in the whole turnover process.

Figures 3(b) and (c) show slow warm EPR signal changes of 10 msec, pH 10.1 and 7 msec, pH 8.5 samples. There is a slight increase in the $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ EPR signal in Figure 3(b) at -78°C followed by a decrease in all signals at -50°C. The $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ signal of the 7 msec shot [Figure 3(c)] decreases at -78°C and at -65°C whereas there are small increases in the $\mathrm{Mo}_{\mathrm{R}}^{5+}$ and FADH° EPR signals at these temperatures. Reduction of the enzyme by the first substrate can only increase the amplitude of an EPR signal. By 7 msec only about 15 percent of the active sites could have been reduced by more than one substrate (assuming a reduction rate of 885 min^{-1}). This implies that there are two consecutive events occurring in higher pH reduction by the first substrate. The temperature dependence of the rate of disappearance of the $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ signal at low temperatures extrapolates back to a value at room temperature which is very close to the turnover rate for the enzyme reaction (13). The activation energy for this $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ disappearance process is 10.7 ± 1 kcal/mole (13). It would seem that the process leading to the $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ signal decrease must represent a rate limiting step in high pH substrate turnover.

As the 219 msec, pH 10.3 shot is warmed at -50°C [Figure 2(b)] a $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ signal developes. Evidently this signal appears as the enzyme is reoxidized from six to one electron. Thus it seems that the $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ signal does not have to be produced by reduction but is just one of several equilibrium sites on the enzyme for one or two electron reduced enzyme. $\mathrm{Mo}_{\mathrm{VR}}^{5+}$ and $\mathrm{Mo}_{\mathrm{R}}^{5+}$ signals have been shown to differ by the absence or presence, respectively, of a proton (14,2).

The above experiments are consistent with a reduction process as follows:

(1) At higher pH the deprotonated substrate can rapidly donate an electron pair (no proton) to the enzyme forming Mo^{4+} or a small amount of the Mo^{5+}_{VR}, $\overset{o}{\mathrm{Fe}}/S_{II}$ pair. (2) After the substrate is hydrolyzed or removed slightly from the molybdenum, protons can equilibrate with the deprotonated Mo^{4+} or Mo^{5+}_{VR} centers leading to the development of the Mo^{5+}_{R} and other EPR signals. Evidently the protonated Mo^{4+} is energetically compatible for transferring electrons to other centers (FAD and Fe/S_I) whereas the deprotonated Mo^{4+} interacts better with the Fe/S_{II} center. Turnover at higher pH's seems to be limited by the step following enzyme reduction, a step which removes a hindrance for protons equilibrating on the molybdenum. This mechanism is consistent with that of Olson et al (4). At lower pH's the proton coordinated at the active site on the xanthine competes with the Mo^{6+} for the electron pair and slows reduction.

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