Magnetic Interactions in Milk Xanthine Oxidase†

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ABSTRACT: The relaxation behavior of the EPR signals of Mo^V, FAD semiquinone, and the reduced Fe/S I center was measured in the presence and absence of other paramagnetic centers in milk xanthine oxidase. Specific pairs of prosthetic groups were rendered paramagnetic by poising the native enzyme or its desulfo glycol inhibited derivative at appropriate potentials and pH values. Magnetic interactions were found between the following species: Mo–Fe/S I (100-fold increase in microwave power required to saturate the Mo^V EPR signal at 103 K when Fe/S I is reduced as opposed to oxidized), FAD–Fe/S I and FAD–Fe/S II (70-fold increase in power required to saturate the FADH· EPR signal at 173 K when either Fe/S center is reduced), and Fe/S I–Fe/S II (2.5-fold increase in power to saturate the reduced Fe/S I EPR signal at 20 K when Fe/S II is reduced). The Mo–Fe/S I interaction was also detected as a reduced Fe/S I induced splitting of the Mo^V EPR spectrum at 30 K. No splittings of the FADH· or Fe/S center spectra were detected. No magnetic interactions were found between FAD and Mo or between Mo and Fe/S II. These results, together with those of Coffman & Buettner [Coffman, R. E., & Buettner, G. R. (1979) *J. Phys. Chem. 83*, 2392–2400], were used to estimate the following approximate distances between the electron carrying prosthetic groups of milk xanthine oxidase: Mo–Fe/S I, 11 ± 3 Å; Fe/S I–Fe/S II, 15 ± 4 Å; FAD–Fe/S I, 16 ± 4 Å; FAD–Fe/S II, 16 ± 4 Å. A model for the arrangement of these groups within the xanthine oxidase molecule is suggested.

It is common for enzymes which catalyze oxidation–reduction reactions to contain multiple electron carrying groups. Problems of current interest include the mechanisms of electron transfer and the steric relationships between such groups [see Palmer & Olson (1980) for a short review].

Milk xanthine oxidase is one of the more thoroughly studied soluble enzymes containing multiple electron carriers [see Bray (1975, 1980)]. It is a dimer of M_r 140 000 identical subunits, each of which contains one Mo center (with its associated pterin cofactor; Johnson et al., 1980), one FAD, and two spectroscopically distinct Fe_2S_2 centers, termed Fe/S I and Fe/S II. During xanthine oxidation by O_2, electrons enter the enzyme via the Mo center (which is reduced by substrate from the Mo^{VI} to the Mo^{IV} oxidation state), pass one at a time within the enzyme centers (with the Mo being sequentially oxidized to the Mo^V and Mo^{VI} states), and leave the enzyme via the FAD moiety (with $FADH_2$ being oxidized to FADH· and FAD) (Olson et al., 1974a,b). Each of the enzyme electron carriers can exist in a paramagnetic state (Mo^V, FADH·, reduced Fe/S I, and Fe/S II) during the catalytic cycle; each of these paramagnetic species yields a characteristic EPR spectrum (Bray, 1975).

Olson et al. (1974a,b) showed that, under a variety of experimental conditions, internal electron transfer processes in xanthine oxidase are rapid when compared to maximum rates of electron entry and egress from the molecule during the catalytic cycle. Thus, it has not been possible to strictly define a pathway for internal electron transfer within xanthine oxidase, with the kinetic order of appearance and disappearance of electrons in the various carriers, following reaction with reducing and oxidizing substrates, being determined solely by their relative reduction potentials. Figure 1 summarizes the current knowledge of the possible electron transfer pathways in xanthine oxidase, as well as the possible spatial relationships between the four electron-carrying prosthetic groups of the enzyme.

It has now become possible, by making use of the differential pH dependence of the various enzyme centers (Barber & Siegel, 1982) and the kinetic stability of the paramagnetic Mo^V state in certain ligated forms of the enzyme (Bray, 1980), to design experiments in which all of the possible pairwise magnetic interactions between enzyme centers (see Figure 1) can be studied by using EPR spectroscopy. Measurement of the strength of the magnetic interaction between two centers can, in favorable circumstances, yield information as to their spatial relationship (e.g., how far apart they are from one another).

Several reports indicating the presence of magnetic interaction between prosthetic groups in xanthine oxidase and related molybdo–iron flavoproteins have already appeared. Thus, Beinert & Hemmerich (1965), in one of the first applications of EPR relaxation techniques to oxidation–reduction enzymes, showed that the relaxation behavior of the FAD semiquinone in xanthine oxidase strongly suggested that the flavin was interacting with one or more metal centers in the enzyme. Rajagopalan et al. (1968) showed that the relaxation rate of Mo^V in aldehyde oxidase varied with time and method of enzyme reduction. They concluded that the Mo^V was interacting with the Fe/S center(s).

Lowe et al. (1972) have reported the only evidence which describes a magnetic interaction between two specific centers in xanthine oxidase: Mo and Fe/S I. The Mo^V EPR spectra of several differently ligated forms of the Mo center, recorded at temperatures of 50 K or less, were found to be split in a nearly isotropic fashion when enzyme molecules contained both Mo^V and reduced Fe/S. The temperature dependence of the phenomenon, its association in ELDOR spectroscopy with a species exhibiting the relaxation properties characteristic of Fe/S I (Lowe & Hyde, 1975), and the finding of line broadening in the g_1 feature of Fe/S I (spectra recorded at 4.2 K) in enzyme molecules containing Mo^V (Lowe & Bray, 1978) all pointed to the presence of a specific Mo–Fe/S I interaction. These studies did not exclude the possibility of there being additional Mo interactions with other enzyme groups (including Fe/S II). Lowe & Bray (1978) estimated Mo–Fe/S I distance to be 20–25 Å. Coffman & Buettner

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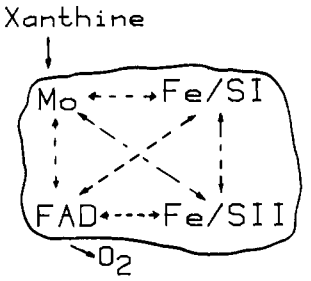

FIGURE 1: Possible electron-transfer pathways and pairwise magnetic interactions in a subunit of xanthine oxidase.

(1979) reevaluated the Lowe and Bray data and concluded that the Mo–Fe/S I distance was in fact 8–14 Å.

In the present study, we looked by EPR spectroscopy for each of the six possible pairwise interactions between electron-carrying centers in the xanthine oxidase molecule. In addition to the Mo–Fe/S I magnetic interaction, positive evidence was obtained for interactions between Fe/S I and Fe/S II, FAD and Fe/S II, and FAD and Fe/S I. No interaction was detected between Mo and Fe/SII or between FAD and Mo. These findings have enabled us to propose a model for the possible spatial arrangement of prosthetic groups in xanthine oxidase.

Experimental Procedures

Milk xanthine oxidase was isolated from fresh cream as described by Barber & Siegel (1982). The activity/A_{450} ratio for the native enzyme used in this work was 131 at 23.5 °C. Enzyme concentrations used in various experiments ranged from 50 to 100 μM (total enzyme), estimated from the A_{450}, assuming E_{mM} = 72 L mmol^{-1} cm^{-1} (Avis et al., 1956). Total active-center concentrations (functional and nonfunctional) were taken as twice the enzyme concentration. Concentrations of FAD semiquinone and reduced Fe/S centers, determined by quantitation of EPR spectra by the procedures described by Barber & Siegel (1982), were divided by the concentration of total active centers to yield the fraction of reduced flavin and Fe/S centers. Concentration of Mo^V species in desulfo enzyme were also expressed relative to the concentration of total enzyme active centers (although in this case none were functional). The proportion of active sites which were functional in native enzyme preparations was estimated from the activity/A_{450} ratio, with the limiting value of this ratio for fully functional enzyme taken as 197 (Massey & Edmondson, 1970). Mo^V (rapid) species concentrations [see Bray (1975)] were expressed relative to the concentration of functional active centers in native enzyme samples.

Nonfunctional desulfo xanthine oxidase was prepared as described by Massey & Edmondson (1970). The desulfo glycol inhibited form of xanthine oxidase, exhibiting the stable Mo^V signal termed "resting II" (Bray, 1980), was prepared by treatment of reduced desulfo enzyme with ethylene glycol according to the procedure of Lowe et al. (1976).

Buffers used in this work were of the zwitterionic type described by Good (1966) in order to minimize changes in solution pH on freezing of samples prior to EPR analysis (Williams-Smith et al., 1977).

Samples were poised at specific reduction potentials by addition of sodium dithionite to solutions of enzyme and mediator dyes under an Ar atmosphere in an apparatus of the type described by Dutton (1971). Samples were removed with a gas-tight Hamilton syringe, transferred to EPR tubes flushed with Ar, and quickly frozen in liquid N_2 for EPR analysis. [See Cammack et al. (1976) and Barber & Siegel (1982) for details.]

EPR spectra were recorded on a Varian E9 spectrometer equipped with a variable temperature accessory and by using 100-kHz modulation. Measurements below 50 K were made by using a liquid He cryostat (Air Products). For details of signal measurement and quantitation for the various centers of xanthine oxidase, see Barber & Siegel (1982).

For the microwave power saturation measurements, EPR spectra were recorded over a 0.01–200-mW range of microwave power. The saturation curves for all of the paramagnetic species in xanthine oxidase could be satisfactorily obtained in this range by variation of the temperature. The microwave power incident on the sample was taken as the value of the instrument power setting P_0. The absolute value of the microwave magnetic field H_1 at the sample is difficult to estimate as it depends upon P_0Q, where Q depends upon the geometry of the cavity, the sample, and the quartz sample Dewar. Values of $P_{1/2}$ used in the saturation studies are therefore dependent upon the particular instrument used. We estimate the microwave field component to be approximately 0.04 G when the microwave power was 1 mW. Since all studies were performed with the same EPR spectrometer, this does not influence the results, except for absolute estimates of T_1, but it may affect a comparison of $P_{1/2}$ values in this study with values obtained in other laboratories.

Data of EPR signal amplitude (S) as a function of incident microwave power (P) were plotted, and curves were calculated to fit the data (using a Hewlett-Packard 9825A computer and 7225A graphics plotter) from the semiempirical equation given by Beinert & Orme-Johnson (1967):[1]

$$S = k\sqrt{P}/[1 + P/P_{1/2}]^{0.5b} \quad (1)$$

where b, the "inhomogeneity parameter", varies from 1.0 for inhomogeneously broadened lines to 4.0 for the homogeneous case. In the plots of log ($S/P^{1/2}$) vs. log P presented in this work, the curves tend to a slope of $0.5b$ under conditions of saturation. Saturation curves for species containing more than one relaxing component were constructed by adding together individual saturation curves obtained for each of the components in various proportions until the best fit was obtained.

Saturation behavior of Mo^V was determined by measurement of the overall g_2 signal amplitude (above and below the base line) as a function of microwave power. FAD semiquinone saturation behavior was determined by measuring the peak-to-peak signal amplitude for samples containing little or no Mo^V signal or the signal height above the base line for samples with intense Mo^V features. Both methods were found to yield similar results. Fe/S II was monitored by using the height of the g_1 feature above the base line. The height of the g_2 feature above the base line was used to monitor Fe/S I in saturation studies.

Computer simulation of EPR spectra was performed with the use of numerical integration procedures described by Salerno & Ohnishi (1980).

[1] The power saturation of inhomogeneously broaded lines has been treated by Portis (1953) and Castner (1957). Recently Blum & Ohnishi (1980) considered the effects of a rhombic **g** tensor and extended sample size on the saturation of inhomogeneously broadened species. An expression describing the saturation of rhombic species was not obtained in closed form even for point samples, but they were able to successfully attack the problem by using computer simulations. Their results show that the expression used by Beinert & Orme-Johnson (1967) to fit saturation data is strictly valid only in the limiting cases of completely homogeneous or completely inhomogeneous lines. However, since our data can be reasonably well fit by eq 1 in the limited range of power available to us, we have used this expression in a descriptive way, since the parameters involved do describe changes in the shapes of the curves observed.

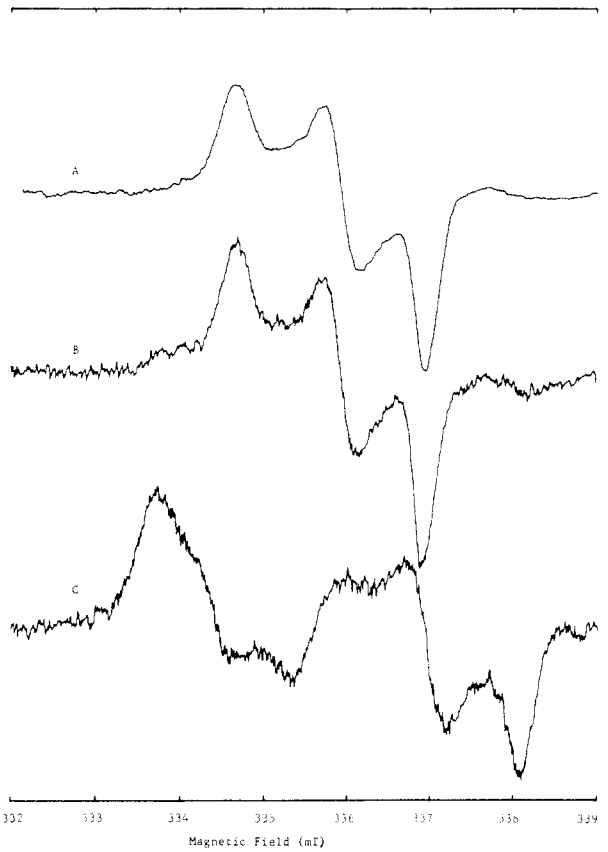

FIGURE 2: EPR spectra of Mo^V in the presence and absence of reduced Fe/S centers in desulfo glycol inhibited xanthine oxidase. Enzyme, in 50 mM Ches, pH 9.8, was poised at defined reduction potentials, and samples were removed anaerobically. EPR spectra were recorded at 30 K, 0.1 mW of power, and a modulation amplitude 2.5 G. (A) All centers other than Mo^V oxidized. (B) Enzyme poised at −300 mV (Fe/S II 70% reduced and Fe/S I 10% reduced). (C) Enzyme poised at −480 mV (Fe/S I and II both reduced).

Results

Molybdenum. The reduction potentials of the Mo center in xanthine oxidase are generally more negative than those of the FAD and Fe/S centers (Barber & Siegel, 1982). A complexed form of the enzyme has been described by Lowe et al. (1976), in which a large proportion of the Mo is in the Mo^V state, and this state is kinetically stable, i.e., resistant to oxidation or reduction of the Mo. This is the desulfo glycol inhibited enzyme, in which the terminal sulfur atom ligated to the Mo in the native enzyme (Bordas et al., 1980) has been replaced by an oxygen and a $HOCH_2C(O)-$ moiety (derived from ethylene glycol) has become attached to the Mo (Bray, 1980). The EPR spectrum of this Mo^V species is particularly useful for detecting magnetic interactions with other paramagnetic centers, since it is both narrow in line width and uncomplicated by proton hyperfine structure.

Figure 2 shows a series of EPR spectra recorded at 30 K on samples of desulfo glycol inhibited xanthine oxidase (in 50 mM Ches,[2] pH 9.8) which had been poised at defined solution potentials, in order to achieve differing degrees of reduction of the Fe/S I and Fe/S II centers. Spectrum A was obtained from a sample of enzyme in the absence of reducing agents; the characteristic narrow rhombic Mo^V EPR spectrum is observed. Spectrum B was obtained from an enzyme sample poised at a potential of −300 mV. Since the E_m values for Fe/S I and Fe/S II at pH 9.8 are −365 and −282 mV, respectively, (Barber & Siegel, 1982), this sample should have Fe/S II largely in the reduced state and Fe/S I largely oxidized. Quantitation of the iron–sulfur EPR spectra for this enzyme sample showed that indeed 70% of the Fe/S II was reduced while 90% of the Fe/S I was oxidized. The Mo^V spectrum of this sample is little changed from that in which both Fe/S centers are fully oxidized. Spectrum C was obtained from a sample of enzyme poised at a potential of −480 mV. Under these conditions, both Fe/S I and II were found to be greater than 95% reduced. The Mo^V spectrum is clearly altered upon Fe/S I reduction, with a large splitting observed at each g value. These results confirm the finding of Lowe et al. (1972) that the Mo center of xanthine oxidase interacts with the Fe/S I center. They also show that there is no magnetic interaction between the Mo and Fe/S II centers which can be detected by this method.

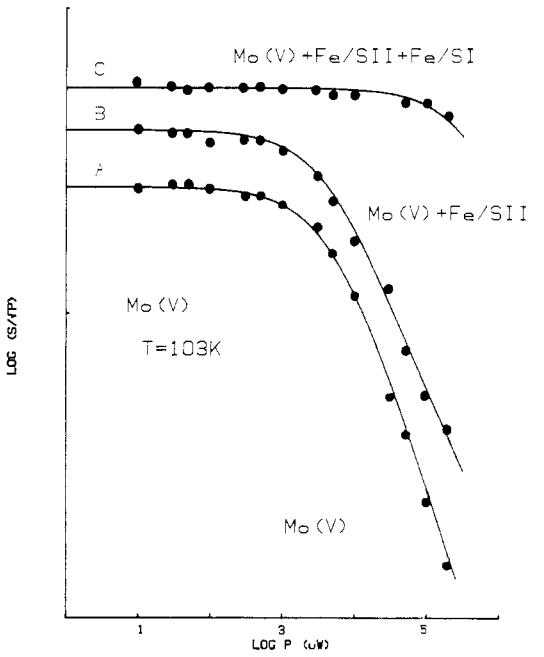

FIGURE 3: Microwave power saturation behavior of the Mo^V EPR signal in desulfo glycol inhibited xanthine oxidase in the presence and absence of reduced Fe/S centers. The curves refer (bottom to top) to samples A, B, and C of Figure 2. EPR spectra were recorded at 103 K and 2.5-G modulation amplitude. Experimental points were fit by eq 1, using one or two relaxing components, with the parameters described in the text.

Interactions between paramagnetic centers can also be detected when the juxtaposition of a relatively rapidly relaxing center nearby to a slowly relaxing center increases the observed relaxation rate of the latter center. This phenomenon can be readily studied experimentally with the EPR spectrometer by comparing the relative ease with which the signal intensity due to the slowly relaxing center can be saturated with incident microwave power in the absence vs. in the presence of the rapidly relaxing paramagnetic center. Figure 3 shows the microwave power saturation behavior at 103 K of the Mo^V species for the enzyme samples of Figure 2. All of the curves are fit by curves with $b = 1.5$ and varying $P_{1/2}$. The Mo^V in the enzyme in which all other centers are oxidized exhibited a $P_{1/2}$ of 5 mW. The Mo^V in the enzyme with both Fe/S centers reduced exhibited a $P_{1/2}$ of 500 mW; i.e., there was an increase of 2 orders of magnitude in the microwave power required to saturate the Mo^V at the temperature used. The saturation behavior of the Mo^V center in the presence of 70% reduced Fe/S II and 10% reduced Fe/S I was fit well by a

[2] Abbreviations: bicine, *N,N*-bis(2-hydroxyethyl)glycine; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

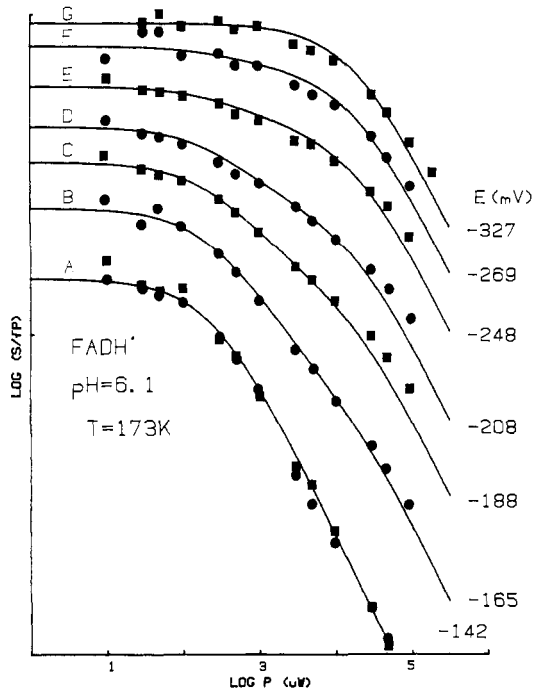

FIGURE 4: Microwave power saturation behavior of the FAD semiquinone EPR signal in xanthine oxidase poised at several reduction potentials. Native enyzme, in 50 mM Mes, pH 6.1, was poised at the indicated potentials, and samples were removed for EPR analysis. EPR spectra were recorded at 173 K and 2.5-G modulation amplitude. Experimental points were fit by Eq 1, using one or two relaxing components, with the parameters described in the text.

composite curve consisting of two independently saturating Mo^V components: 90% of the Mo^V exhibiting a $P_{1/2}$ of 5 mW and 10% exhibiting a $P_{1/2}$ of 500 mW.

FAD. At pH 6.1, substantial amounts of xanthine oxidase FAD can be converted to the semiquinone form. The E_m for the $FAD/FADH_2$ couple is −215 mV at this pH, while the E_m values for Fe/S I and II, respectively, are −280 and −250 mV (Barber & Siegel, 1982). Thus, it is possible to prepare enzyme samples containing both amounts of FAD semiquinone EPR signal adequate for microwave power saturation studies and differing proportions of reduced Fe/S I and Fe/S II by poising the enzyme at a series of solution potentials at pH 6.1. Figure 4 shows the microwave power saturation behavior of the FAD semiquinone EPR signal in such a series of enzyme samples. Curve A was obtained for enzyme at a potential of −142 mV. EPR spectra of this sample taken at 21 K showed no detectable reduction of Fe/S I and no more than 5% reduction of Fe/S II. No Mo^V could be detected. The saturation behavior of this sample could be fit by eq 1 with $b = 1.0$ and $P_{1/2} = 0.25$ mW. Curve G was obtained from a sample reduced at a potential of −327 mV. Examination of the EPR spectra of this sample at 173 and 21 K showed it to contain 98% of the Fe/S II and 85% of the Fe/S I in the reduced form, as well as 24% of the functional Mo as Mo^V. The saturation behavior of this sample could be fit by eq 1 with $b = 1.0$ and $P_{1/2} = 18$ mW. Curves B–F, obtained at intermediate potentials, showed the FAD semiquinone saturating as a mixture of two components, one ("not relaxed") with $P_{1/2} = 0.25$ mW and the second ("relaxed") with $P_{1/2} = 18$ mW.

Figure 5A shows the fraction of the FAD semiquinone which exhibited the relaxed saturation behavior at each of the potentials examined between −142 and −269 mV, together with the fractions of Fe/S I and Fe/S II measured to be in the reduced state at each of these potentials. It can be seen that there is some correlation between the amount of Fe/S II reduced and the amount of FAD semiquinone in the relaxed

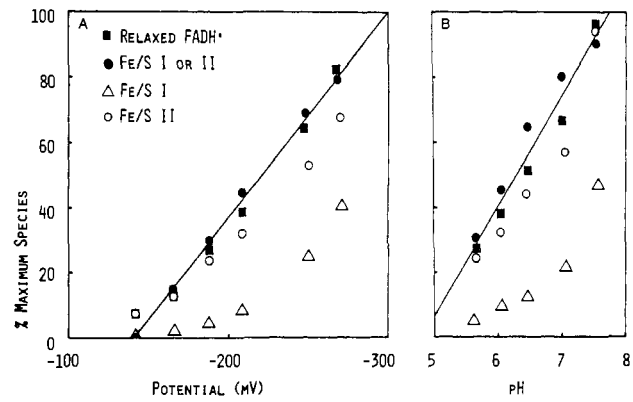

FIGURE 5: Correlation of the fraction of FAD semiquinone in the "relaxed" state with the fraction of Fe/S centers in the reduced state. See text for details. (■) Fraction of FAD semiquinone EPR signal saturating with $P_{1/2} = 18$ mW. (△) Fraction Fe/S I reduced. (○) Fraction Fe/S II reduced. (●) Fraction of enzyme subunits which contain either reduced Fe/S I, reduced Fe/S II, or both Fe/S centers reduced.

state. However, at a number of potentials where there is only partial reduction of either Fe/S center, the proportion of FAD semiquinone which is relaxed is significantly greater than the fraction of any one Fe/S center which is reduced. If it is assumed, as suggested by the results of Barber & Siegel (1982), that Fe/S I and Fe/S II are reduced independently in xanthine oxidase, then one can calculate the fraction of enzyme subunits in which any Fe/S center reduction (whether it be Fe/S I, Fe/S II, or both) has occurred from the equation $F_{either} = F_I + F_{II} - F_I F_{II}$, where F_I and F_{II} represent the fractions of Fe/S I and Fe/S II, respectively, which are in the reduced state at a given potential. Figure 5A shows that there is good agreement between the fraction of FAD semiquinone in the relaxed state at any potential and the fraction of enzyme subunits in which any Fe/S center has been reduced.

Studies of the microwave power saturation behavior of the FAD semiquinone EPR signal were also performed on enzyme samples reduced at a number of pH values in the pH range 5.7–10.9. Each sample was poised at a potential at which maximum conversion of FAD to the semiquinone occurred at that pH (see Barber & Siegel (1982) for details; the pH values and buffers used were those indicated for native xanthine oxidase in Table I of their paper). As shown in Figure 6, the saturation characteristics of the FAD semiquinone signal changed markedly as the pH was raised from 5.7 to 7.7. At higher pH values (8.9, 9.3, 9.8, and 10.9 were examined), the FAD semiquinone saturation behavior was not significantly different from that shown for pH 7.7. These curves could be fit readily by assuming that they are due to mixtures of the two FAD semiquinone species (with $P_{1/2} = 0.25$ and 18 mW, respectively) defined by curves A and G of Figure 4. The fraction of FAD semiquinone in the relaxed state as a function of pH is shown in Figure 5B, together with the fractions of Fe/S I and II and the fraction of enzyme subunits which contain either or both Fe/S centers reduced at any pH. Again, the best correlation is obtained when the amount of relaxed FAD semiquinone is compared with the amount of enzyme subunits in which any Fe/S center reduction has occurred. These results indicate the presence of magnetic interactions between FAD and both Fe/S centers of xanthine oxidase.[3]

[3] The possibility must be considered that FADH· could be relaxing through a mechanism involving participation of the Mo^{IV} ($S = 1$) oxidation state of xanthine oxidase. However, this is clearly not the case in the experiments of Figures 4–6, since the potentiometric data of Barber & Siegel (1982) clearly show that negligible amounts of Mo^{IV} are formed at the pH values and potentials used in these experiments.

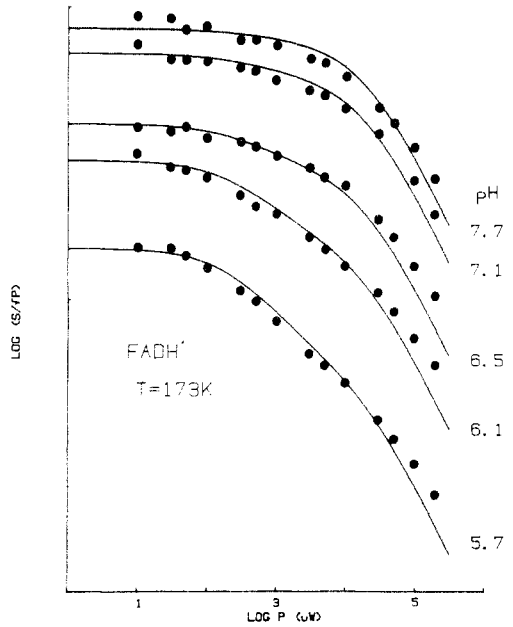

FIGURE 6: Microwave power saturation behavior of the FAD semiquinone EPR signal in xanthine oxidase as a function of pH. Native enzyme samples, buffered at the indicated pH values, were poised at potentials resulting in maximum conversion of FAD to semiquinone [see Barber & Siegel (1982) for details] and samples removed for EPR analysis. EPR spectra were recorded at 173 K and 2.5-G modulation amplitude. Experimental points were fit by eq 1, using two relaxing components, with the parameters described in the text. Buffers (all at 50 mM) used were the following: Mes, pH 5.7, 6.1; Pipes, pH 6.5, 7.1; bicine, pH 7.7.

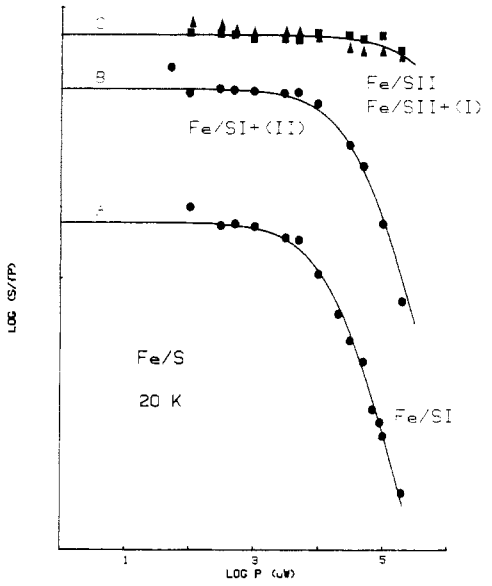

FIGURE 7: Microwave power saturation behavior of each Fe/S center in xanthine oxidase with the other Fe/S center oxidized or reduced. Native enzyme, in 50 mM Mes, pH 5.7, was poised at defined reduction potentials and samples removed anaerobically. EPR spectra were recorded at 20 K and 10-G modulation amplitude. (A) Enzyme poised at −170 mV (Fe/S I 5% reduced and Fe/S II 8% reduced). The microwave power saturation behavior of the Fe/S I EPR signal is shown. (B) Enzyme poised at −370 mV (both Fe/S centers over 95% reduced). The microwave power saturation behavior of the Fe/S I EPR signal is shown. (C) The microwave power saturation behavior of the Fe/S II EPR signal in the samples of curves A and B is shown. Experimental points were fit by eq 1, with the parameters described in the text.

For examination of the possibility of interaction between FADH· and Mo^V, a sample of desulfo glycol inhibited xanthine oxidase (with 48% of its Mo in the Mo^V state), in 50 mM Mes, pH 6.1, was poised at a potential of −142 mV. Under these conditions, FAD semiquinone is formed, but there is no reduction of Fe/S I and only slight reduction of Fe/S II. The microwave power saturation behavior of the resulting FAD semiquinone was identical with that shown in curve A of Figure 4 for native enzyme poised at the same potential (no Mo^V present). Since the Mo^V relaxes more than an order of magnitude faster than the FAD semiquinone (in the absence of reduced Fe/S) under these conditions, the absence of relaxation enhancement of the FAD semiquinone species by the Mo^V can be considered significant. There is thus no evidence for Mo^V–FADH· interaction in xanthine oxidase.

No splitting of the FAD semiquinone EPR signal was detected under any of the experimental conditions used in this work.

Iron–Sulfur Centers. In order to detect interaction between Fe/S I and Fe/S II by the microwave power saturation technique, one must examine the saturation behavior of reduced Fe/S I, the more slowly relaxing species, in the presence of various amounts of reduced Fe/S II present in the same enzyme subunit. Under most experimental conditions, the reduction potential of Fe/S I is considerably more negative than that of Fe/S II (Cammack et al., 1976). However, Barber & Siegel (1982) have recently found that the potentials of both centers are pH dependent and that at pH 5.7, the E_m values for Fe/S I and II differ by only 10 mV (−260 and −250 mV, respectively).

Curve A of Figure 7 shows the microwave power saturation behavior of the reduced Fe/S I EPR signal in an enzyme sample, in 50 mM Mes, pH 5.7, poised at −170 mV. This sample contained 5% of the Fe/S I and 8% of the Fe/S II in the reduced state. The data for this sample were fit by eq 1 with $b = 2.0$ and $P_{1/2} = 20$ mW. Curve B shows the saturation behavior of the Fe/S I signal in a sample poised at −370 mV, at which potential both Fe/S centers were over 95% reduced. This data was fit was $b = 2.0$ and $P_{1/2} = 50$ mW. Thus, there is significant relaxation of the Fe/S I EPR signal when the Fe/S II center is also reduced.[4]

As expected, when the same samples were examined for saturation behavior of the reduced Fe/S II EPR signal, the resulting curves (Figure 7, curve) were indistinguishable despite the greatly differing levels of Fe/S I reduction, with both samples being fit by eq 1 with $b = 2.0$ and $P_{1/2} = 500$ mW. No splitting of the EPR spectrum of either Fe/S center was detected in any of the experiments of this work.

[4] In the experiment of Figure 7, the samples in curves A and B differed not only in the level of reduction of Fe/S II but also in the amounts of reduced Mo species present. Thus, in curve A the Mo was almost totally in the oxidized Mo^{VI} state ($S = 0$), while in curve B the Mo was predominately in the reduced Mo^{IV} state ($S = 1$) (Barber & Siegel, 1982). Since Mo^{IV} could in principle be an effective relaxer of Fe/S centers, we investigated the power saturation behavior of reduced Fe/S I in samples of xanthine oxidase in which the level of Fe/S II reduction was kept constant while the Mo center was kept either nearly fully oxidized or fully reduced. In an enzyme sample in 50 mM Ches–1 mM EDTA buffer, pH 9.8, poised at −357 mV, Fe/S I and Fe/S II were 48% and 94% reduced, while the Mo center was over 99% in the Mo^{VI} state based on the reported E_{m1} and E_{m2} values of −505 and −439 mV, respectively, for xanthine oxidase Mo in the buffer used (Barber & Siegel, 1982). In a second enzyme sample in the same buffer, but poised at −596 mV, the Fe/S I and Fe/S II centers were both fully reduced, while the Mo center was calculated to be over 99% in the Mo^{IV} state. (No Mo^V was detected by EPR in either sample.) The saturation behavior of Fe/S I at 20 K was identical in both enzyme samples, yielding curves indistinguishable from that in Figure 7 (curve B), with $b = 2.0$ and $P_{1/2} = 50$ mW. We can conclude that the difference in saturation behavior of Fe/S I in curves A and B of Figure 7 is due to the difference in reduction state of Fe/S II and not the presence or absence of Mo^{IV}.

Discussion

The results presented in this paper clearly demonstrate magnetic interactions between the following centers of xanthine oxidase: Mo–Fe/S I, FAD–Fe/S II, FAD–Fe/S I, and Fe/S I–Fe/SII. No such interactions could be detected between the pairs Mo–Fe/S II and FAD–Mo.

Lowe et al. (1972) previously demonstrated spin coupling between Mo and Fe/S I. Their analysis of the nearly isotropic splittings observed with the Mo^V EPR spectrum in the presence of reduced Fe/S I led Lowe & Bray (1978) to conclude that the centers are probably 20–25 Å apart. That analysis is strictly appropriate, however, only for systems in which the interacting species exhibit greatly dissimilar g values ($\Delta g\beta H \gg J$). Since in fact the g tensors of Mo^V and reduced Fe/S I show considerable overlap (as do the g tensors for all of the paramagnetic species considered in this work), mixing of states by terms in $S_1^+S_2^-$ and $S_1^-S_2^+$ arising from the isotropic exchange term $-2J\cdot\vec{S}_1\cdot\vec{S}_2$ becomes important in at least some orientations. Only for those orientations in which the g values are equal is no splitting from exchange terms to be expected. Thus, dipolar contributions do not have to be simply related to the anisotropy of the observed splitting as claimed. From the overall magnitude of the splittings we expect, as suggested by Coffman & Buettner (1979), that the Mo and Fe/S centers are probably 8–14 Å apart, rather than the larger distance previously suggested by Lowe & Bray (1978), although computer simulations would be needed to distinguish the relative dipole and exchange components even with the assumption of predominately isotropic exchange.

Since no splitting was observed in the FAD–Fe/S and Fe/S–Fe/S interactions, the coupling in these cases is expected to be weaker and the distance greater than the estimate given for Mo–Fe/S I. We shall now present some considerations which will enable us to make estimates of these distances based on the relaxation enhancement data reported in this work.

Several processes can contribute to the enhanced relaxation of an intrinsically slowly relaxing species through coupling to a rapidly relaxing species (Abragam, 1961). Dipolar coupling between spins has been used to account for the enhanced relaxation of nuclear spin by paramagnetic metal ions. Similar descriptions of the relaxation produced by interactions coupling two electron spins can be used to estimate dipolar coupling strengths and thus distances between spins.

If two $S = {}^1/_2$ paramagnets with spins $\vec{S}_1$ and $\vec{S}_2$ are in a magnetic field $\vec{H}_0$, in the absence of spin coupling, the spin Hamiltonian is just

$$H_{\text{Zeeman}} = \beta(\vec{S}_1\cdot\hat{g}_1\cdot\vec{H}_0 + \vec{S}_2\cdot\hat{g}_2\cdot\vec{H}_0)$$

where g_1 and g_2 are the **g** tensors of the two species and β is is the Bohr magneton. The spins are quantized with respect to H_0 and the states of the system are $[++\rangle$, $[+-\rangle$, $[-+\rangle$, and $[--\rangle$, where the plus and minus describe the orientation of S_1 and S_2 with respect to H_0.

The exchange interaction may be written $-2\vec{S}_1\cdot\hat{J}\cdot\vec{S}_2$ where J contains both isotropic and anisotropic contributions. For species with modest g anisotropy (such as the centers observed in this work), the isotropic part of J will be dominant.

The dipolar interaction may be written as $g_1g_2\beta^2[(\vec{S}_1\cdot\vec{S}_2)/r^3 - 3(\vec{S}_1\cdot\vec{r})(\vec{S}_2\cdot\vec{r})/r^5]$, where $\vec{r}$ is the vector between S_1 and S_2. This expression is often decomposed as

$$H_{\text{dipolar}} = A + B + C + D + E + F$$

where $A = g_1g_2\beta^2(1 - 3\cos^2\theta)S_{1z}S_{2z}/r^3$, $B = g_1g_2\beta^2(1 - 3\cos^2\theta)(S_1^+S_2^- + S_1^-S_2^+)/(4r^3)$, and C–F are terms containing combinations of the operators S_z, S^+, and S^-, which connect states differing in energy by at least one Zeeman splitting ($g_1\beta H_0$ or $g_2\beta H_0$).

The overall spin coupling Hamiltonian is then

$$H_{ss} = \vec{S}_1\cdot\hat{J}\cdot\vec{S}_2 + A + B + C + D + E + F$$

with $\vec{S}_1\cdot\hat{J}\cdot\vec{S}_2 = S_{1z}\cdot\hat{J}\cdot S_{2z} + S_{1x}\cdot\hat{J}\cdot S_{2x} + S_{1y}\cdot\hat{J}\cdot S_{2y}$ The dipolar terms C–F connect states differing by at least one Zeeman splitting term. In the time-independent case, these terms will thus have little effect if the Zeeman terms are much larger than the dipolar terms. This is true for distances greater than approximately 4 Å. The B terms connect states which differ only by the difference in Zeeman energies of the two spins. These terms will contribute to splittings if the separation between the $[+-\rangle$ and $[-+\rangle$ states is not much greater than the magnitude of B. The A term contributes a first-order dipole splitting of $2A$ but does not mix the Zeeman eigenstates.

Relaxation effects are contributed by terms B–F (Bloembergen, 1949; Abragam, 1955). Hyde & Rao (1978) have estimated the relative strength of the three processes depending on the B, CD, and EF terms for relaxation of free radicals by a series of paramagnetic metal ions. When the metal ions were fast relaxing and when the difference between the Larmor frequencies of the ion and the radical were large, the CD term often dominated. When the ion relaxed more slowly and when the Larmor frequencies were comparable, the B term was much more important.

The ratio of the relaxation rates is always $T_1/[2[1 + (w_2 - w_1)^2T_1^2]]:3T_1/[2[1 + w_2^2T_1^2]]:3T_1/[1 + (w_1 + w_2)^2T_1^2]$ where w_1 and w_2 are the Larmor frequencies of the "fast" and "slow" species, respectively; T_1 refers to the "fast" species, and the processes depend on the B, CD, and EF terms, respectively.

The EPR detectable species in xanthine oxidase have only modest anisotropies and have g nearly equal to 2.0. Interactions between such species are characterized by $|w_1 - w_2|$ of 3×10^8 s^{-1} or less. The fastest induced relaxation possible for a given dipolar coupling strength would occur when T_1 of the faster relaxing species is approximately 3×10^{-9} s. This is almost entirely contributed by the B term, which remains dominant until T_1 is $\leq 2 \times 10^{-10}$ s. The contributions from the CD and EF terms are negligible for $T_1 \geq 10^{-9}$ s, and the "CD" and "EF" processes are nearly 2 orders of magnitude weaker at their respective maxima ($T_1 = 10^{10}$ s and 5×10^{-11} s) than the "B" process is at its maximum ($T_1 = 10^{-8}$ s). As $w_1 - w_2$ decreases, the "B" process becomes faster for short T_1. While not changing significantly for T_1 much smaller than 3×10^{-9} s, "B" processes still dominate until $T_1 \leq 2 \times 10^{-10}$ s.

The origin of the B term dependent relaxation can be understood in terms of the S^+S^- and S^-S^+ operators; the exchange terms in S^+S^- and S^-S^+ will also contribute. If, for a particular orientation of H_0 with respect to S_1 and S_2 (we consider the **g** tensors to be fixed with regard to each other), $g_1\beta H_0 = g_2\beta H_0$, these flip-flop terms will completely mix the degenerate $[+-\rangle$ and $[-+\rangle$ states. Even if the $[+-\rangle$ and $[-+\rangle$ states are not degenerate, considerable mixing will result as long as the g values do not differ by too much. In the absence of coupling, the $[++\rangle \leftrightarrow [-+\rangle$ and $[+-\rangle \leftrightarrow [--\rangle$ transitions were characteristic of S_1; the $[++\rangle \leftrightarrow [+-\rangle$ and $[-+\rangle \leftrightarrow [--\rangle$ transitions were characteristic of S_2. After the two spins are coupled all the transitions have both S_1 and S_2 character since the two intermediate states have been mixed. If $[+-\rangle$ and $[-+\rangle$ differ in energy by at least a few times the coupling strength, we can estimate the strength of the relaxation pathway this effect provides for the more slowly relaxing of the two species by using the perturbation theory. Letting $\vec{S}_2$ be the slower of the two, the fractional amount of S_1 character

in the predominantly S_2 transitions is $[K/(2\Delta Z)]^2$, where K is the strength of the flip-flop term and ΔZ is the difference in Zeeman energy of the $[+-\rangle$ and $[-+\rangle$ states. This arises from the mixed character of the new eigenstates, which are $[\alpha\beta\rangle = C_1[+-\rangle + C_2[-+\rangle$ and $[\beta\alpha\rangle = C_1[-+\rangle + C_2[+-\rangle$. If $\Delta Z \gg K$, $C_1 \sim 1$ and $C_2 \sim K/(2\Delta Z)$. Since the transition probability depends on the square of the matrix elements connecting, for example, $[++\rangle$ and $[\alpha\beta\rangle$, the relaxation rate of the pathway for $|w_1 - w_2| \gg 1/T_1$ is $1/T_x \sim (2/T_1)[k/(2\Delta Z)]^2$. Here, T_1 refers to the spin–lattice relaxation time of S_1. When $1/T_1$ is greater than or equal to $|w_1 - w_2|$, the lifetime broadening becomes an important consideration and $1/T_x \sim T_1K^2/[2[1 + (\Delta Z T_1)^2]]$. When $\Delta Z = 1/T_1$, the rate of the process is maximal for a given ΔZ, and $1/T_x = T_1/K^2/4 = (1/T_1)[K/(2\Delta Z)]^2$.

Clearly, the efficiency of such a process depends strongly on the relative orientation of the **g** tensors of two interacting anisotropic species. The orientation problem, together with the problem of separating dipolar and exchange terms, leaves the parameters describing the interaction hopelessly underdetermined in terms of a unique solution or an accurate distance estimate. We can, however, readily see what approximate magnitudes of coupling strengths are needed to account for the observed effects.

These considerations suggest a strategy for setting limits on the distances between paramagnetic centers in xanthine oxidase. First, where observable splittings are detected in EPR spectra, estimates of distance can be made from spectral simulations, as was done by Coffman & Buettner (1979) for Mo and Fe/S I in xanthine oxidase. In the absence of line-shape changes, a lower limit for distance can be set by using computer simulation routines to determine how much interaction would be necessary to provide readily observable changes in line shape. We note that neither "magic angle" hypotheses nor exchange terms can cancel dipolar terms in *all* orientations. Rapid averaging processes (e.g., rotational) could do so but seem unlikely to be significant at the cryogenic temperatures used in the experiments reported in the present work.

Second, we use our best estimates for T_1 of the more rapidly and more slowly relaxing species to estimate the distance, with the assumption that dipolar terms dominate at distances greater than 12 Å. When both species are significantly anisotropic, this estimate depends on the orientation of the **g** tensors and is consequently less reliable than in the cases in which anisotropy resides primarily with one species.

Third, we assign to T_1 of the rapidly relaxing species the value $(w_1 - w_2)^{-1}$, a situation which would produce the fastest cross-relaxation. We then assume that the **g** tensor orientation (where this is important) is also optimal, and we use our best estimate of the lower bound of T_1 for the more slowly relaxing species to set an upper bound on the distance. This estimate depends only on our ability to set a lower bound for T_1. Since the relationship between the cross-relaxation time and the distance between centers is proportional to r^{-6}, an error in T_{1x} of a factor of 5 (which corresponds roughly to a T_1 error of the same factor in these cases) would lead to only a 30% error in the upper limit for r, if the optimization assumptions are correct. In most cases, we can make good arguments against this; usually, our T_1 lower bound would have to be too small by 30-fold to produce a distance 30% greater than our upper bound.

Fourth, if no magnetic interactions at all can be detected, we assume unfavorable orientations, take the least favorable estimate for T_1 which is still reasonable, and then estimate how far apart the centers must be to show no interaction. In such cases we obtain only a conservative lower limit for distance, typically in the region of 20 Å. The upper bound is, of course, undefined.

The saturation behavior of the paramagnetic centers in xanthine oxidase reported in the present work is consistent with a two-center model for each interacting pair. It is not consistent with the results expected for multiple spin flip "spin diffusion" processes. In each case, the enhanced relaxation of a slowly relaxing species depends on the redox state of *one* other center at a time. For example, the relaxation of FADH· by reduced Fe/S II does not depend on the reduction states of Mo or Fe/S I (although there is evidence that Fe/S I, when reduced, can *independently* promote relaxation of FADH·). The enhanced relaxation of Mo^V or FADH· is not fast enough to mediate the interaction between Fe/S I and Fe/S II even if these species were present in sufficient quantities to do so (there was in fact a negligible amount of either Mo^V or FADH· present in experiments involving both fully reduced Fe/S I and II).

In the case of FADH·, the evidence indicates interaction with Fe/S II and strongly suggests interaction with Fe/S I, but is inconsistent with spin diffusion, since Fe/S II relaxation is not enhanced by Fe/S I reduction. (In any case, this would only lead to even faster relaxation of FADH· in enzyme molecules in which Fe/S II was already reduced, and not increase the proportion of molecules in which relaxation is enhanced by Fe/S I reduction.)

We have seen that the Mo–Fe/S I interaction results in a very short Mo^V relaxation time at 103 K; the $P_{1/2}$ using the power saturation equation of Beinert & Orme-Johnson (1967) is 500 mW. The inhomogeneity parameter was difficult to evaluate because high enough microwave power to completely saturate the sample was not available. T_1T_2 must be 10^{-14} s^2 or less to account for the observed saturation. If T_1 were much less than 10^{-7} s, lifetime broadening would be observed; in the limit $T_1 = T_2$. Thus we can estimate $T_1 \sim 10^{-7}$ s or a few times less. The spectra of Mo^V and Fe/S I overlap. The anisotropy of the spectra will cause the $[+-\rangle$ and $[-+\rangle$ states to be separated by 0.003–0.005 cm^{-1} on the average, depending on the relative orientations of the two **g** tensors. There must be some orientations where these states are degenerate; here a very small interaction will mix these states completely. The onset of saturation will, however, be determined by the orientations in which the states are well separated. We know from studies on lifetime broadening of the Fe/S I EPR signal that T_1 for this iron–sulfur center is on the order of 10^{-8} cm^{-1} at 103 K. We can therefore estimate the magnitude of the flip-flop term to be 0.001–0.002 cm^{-1} by diagonalizing the 2 × 2 Hamiltonian matrix of the $[+-\rangle$ and $[-+\rangle$ states. This is close to the coupling strength implied by the previously observed splitting, although not only the magnitudes but also the relative signs of the dipolar and exchange terms remain unknown. The calculation therefore correctly predicts the magnitude of the coupling from the saturation characteristics, although it should be recognized that less information is obtained in saturation experiments than is contained in spectra showing line-shape modification.

The FAD semiquinone saturation characteristics depend on the reduction state of Fe/S I and Fe/S II, with the flavin becoming more difficult to saturate by a factor of approximately 70 when either Fe/S center becomes paramagnetic. Comparing the experimental results to the theoretical curves described by Blum & Ohnishi (1980), we see that the saturation curve is characteristic of a nearly completely inhomo-

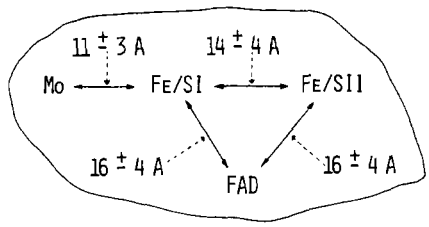

FIGURE 8: Model showing distances between prosthetic groups in a xanthine oxidase subunit estimated from magnetic interaction measurements.

geneously broadened line. It is thus difficult to estimate T_2. In the limiting case, $T_1 = T_2$, and so that the observed saturation can be accounted for, the Fe/S centers must provide a cross-relaxation mechanism with a characteristic time of 10^{-6} s at 173 K. T_2 cannot be more than a few times shorter than this, or more homogeneous character would have been observed. Consequently, T_1 cannot be more than a few times *longer* than the limiting case. At 173 K, Fe/S II is tremendously lifetime broadened. Extrapolating line-width data taken at a lower temperature (100 K and less), we could estimate the T_1 of Fe/S II as approximately 10^{-9} s. The spectra of the FAD semiquinone and reduced Fe/S II overlap. Therefore, at some orientations the $[+-\rangle$ and $[-+\rangle$ states must be degenerate. The average separation of the two states at X band over all orientations is only about 0.01 cm^{-1}. The optimal T_1 for cross-relaxation is therefore about 3×10^{-9} s; we calculate $2K \sim 0.00023$ cm^{-1}. For $T_1 \sim 10^{-9}$ s, a more reasonable figure, we find $2K = 0.0003$ cm^{-1}. Since we were unable to observe any splitting or broadening in the EPR spectra, we can place an upper limit on $2K$ of 0.0008 cm^{-1} from spectral simulations. For such weak coupling, we except dipolar terms to predominate, since exchange terms fall off exponentially with distance at about an order of magnitude per bond length (Coffman & Buettner, 1979). The rough value of K would then be consistent with a distance FAD and Fe/S II of 15 Å by using a point dipole approximation. Since the electron is not localized, the r^{-3} weighting gives somewhere between a center-to-center and edge-to-edge distance. The errors in estimation of T_1 from saturation data and those introduced by using an average value for the separation of the $[+-\rangle$ and $[-+\rangle$ states instead of numerically integrating over all orientations of magnetic field are considerable; but these are compensated for to some extent by the overall r^{-6} dependence of the calculation. While we obviously cannot accurately measure distances by this method, we can conclude with reasonable confidence that the data are consistent with a FAD–Fe/S II distance of 12–20 Å.

Similar results follow from consideration of the interaction between FAD and Fe/S I. The strength of the relaxation pathway is apparently similar, and while the **g** tensor anisotropy is slightly less than that for FAD–Fe/S II, the relaxation time of Fe/S I is somwhat longer. The FAD–Fe/S I distance therefore probably falls into the same 12–20-Å range.

At 20 K, Fe/S I relaxes faster after reduction of Fe/S II. The T_1 for Fe/S I at this low temperature is of the same order as that of the FAD at 173 K in the presence of reduced Fe/S II. It is more difficult to saturate Fe/S I than flavin, but the line has more homogeneous character, and therefore T_2 is shorter. The T_1 of Fe/S II at 20 K is of course much longer than at 173 K. The Fe/S I–Fe/S II interaction is much more difficult to treat than the other interactions discussed. Both **g** tensors are significantly anisotropic, so that, unlike the FAD–Fe/S case, the $[+-\rangle$ and $[-+\rangle$ separation depends critically on the relative orientations of the Fe/S centers in the molecule. Fortunately, the absence of observable splitting or broadening lets us set a lower bound of approximately 10 Å. In the most favorable case, the **g** tensors would be roughly parallel. The sepration of the $[+-\rangle$ and $[-+\rangle$ states would then be typically about 0.003–0.005 cm^{-1}. The relaxation pathway provided for Fe/S I by the interaction is about a factor of 20 slower than the relaxation of Fe/S II; thus $2K$ would be about 0.008 cm^{-1}. This would correspond to a distance of about 13 Å if the interaction were dipolar in nature and we assume $1 - 3\cos^2\theta \sim 1$. At this distance we expect some exchange contribution, which may either add or subtract from the dipolar term. Since we can observe enhanced relaxation at all three Fe/S I turning points (although spectral overlap does not permit detailed power saturation plots of all three), we can discount the possibility of the magnitude of the angular factor $(1 - 3\cos^2\theta)$ not being of the order of unity. The Fe/S I–Fe/S II distance probably lies in the range 11–18 Å, with the coupling being somewhat stronger than the FAD–Fe/S couplings but weaker than the Mo–Fe/S I interaction.

We were unable to observe any effects of interaction between FAD semiquinone and Mo^V. Because of the relatively long Mo^V relaxation time, the relaxation effects would be rather weak in any case, although they should have been readily observable given Mo–FAD distances similar to those estimated for FAD–Fe/S. Similarly, we would have expected to see relaxation enhancement of Mo^V by the fast relaxing reduced Fe/S II species if these centers were as close as 15–20 Å.

The distance estimates obtained in this investigation suggest a spatial arrangement of the prosthetic groups in xanthine oxidase subunits shown in Figure 8. The results suggest that the Mo, Fe/S I, and Fe/S II centers should be placed in a linear array, while the FAD is placed in a position to interact with both Fe/S centers but not with the Mo center. The relationship between the FAD and Fe/S I must clearly be considered tentative, since the relaxation enhancement of the flavin due to Fe/S I was always detected experimentally under conditions where there was already a large amount of relaxation enhancement due to reduced Fe/S II.

Coughlan et al. (1979) showed that the Mo and both Fe/S centers of chicken liver xanthine dehydrogenase were retained on a polypeptide fragment of M_r 65 000 following partial proteolytic cleavage of the enzyme. (A polypeptide of this size can be expected to possess a minimum diameter, if spherical, of 50–55 Å.) The FAD was not found in this fragment. Nagler & Vartanyan (1976), on the basis of similar but less extensive experiments with milk xanthine oxidase, also concluded that the Mo and Fe/S centers of that enzyme were located on a separate enzyme domain from the FAD. These findings are consistent with the prosthetic group arrangement of Figure 8 based solely on magnetic interactions within xanthine oxidase.

References

Abragam, A. (1955) *Phys. Rev. 98*, 1729.

Abragam, A. (1961) *Principles of Nuclear Magnetism*, Clarendon, Oxford.

Avis, P. G., Bergel, F., & Bray, R. C. (1956) *J. Chem. Soc.*, 1219.

Barber, M. J., & Siegel, L. M. (1982) *Biochemistry* (preceding paper in this issue).

Beinert, H., & Hemmerich, P. (1965) *Biochem. Biophys. Res. Commun. 18*, 212–220.

Beinert, H., & Orme-Johnson, W. H. (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A., Malm-

strom, B. G., & Vangaard, T., Eds.) pp 221-247, Pergamon Press, Oxford.
Bloembergen, N. (1949) *Physica (Amsterdam) 15*, 386.
Blum, H., & Ohnishi, T. (1980) *Biochim. Biophys. Acta 621*, 9-18.
Bordas, J., Bray, R. C., Garner, C. D., Gutteridge, S., & Hasnain, S. S. (1980) *Biochem. J. 191*, 499-508.
Bray, R. C. (1975) *Enzymes, 3rd Ed. 12*, 299-419.
Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol. 51*, 107-165.
Cammack, R., Barber, M. J., & Bray, R. C. (1976) *Biochem. J. 157*, 469-478.
Castner, T. G. (1957) *Phys. Rev. 115*, 1506-1515.
Coffman, R. E., & Buetter, G. R. (1979) *J. Phys. Chem. 83*, 2392-2400.
Coughlan, M. P., Betcher-Lange, S. L., & Rajagopalan, K. V. (1979) *J. Biol. Chem. 254*, 10694-10699.
Dutton, P. L. (1971) *Biochim. Biophys. Acta 226*, 63-80.
Good, N. E. (1966) *Biochemistry 5*, 467-477.
Hyde, J. S., & Rao, K. V. S. (1978) *J. Magn. Reson. 29*, 509-516.
Johnson, J. L., Hainline, B. E., & Rajagopalan, K. V. (1980) *J. Biol. Chem. 255*, 1783-1786.
Lowe, D. J., & Hyde, J. S. (1975) *Biochim. Biophys. Acta 377*, 205-210.
Lowe, D. J., & Bray, R. C. (1978) *Biochem. J. 169*, 471-479.
Lowe, D. J., Lynden-Bell, R. M., & Bray, R. C. (1972) *Biochem. J. 130*, 239-249.
Lowe, D. J., Barber, M. J., Pawlick, R. T., & Bray, R. C. (1976) *Biochem. J. 155*, 81-85.
Massey, V., & Edmondson, D. (1970) *J. Biol. Chem. 245*, 6595-6598.
Nagler, L. G., & Vartanyan, L. S. (1976) *Biochim. Biophys. Acta 427*, 78-90.
Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974a) *J. Biol. Chem. 249*, 4350-4362.
Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974b) *J. Biol. Chem. 249*, 4363-4382.
Palmer, G., & Olson, J. S. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., Ed.) pp 189-220, Pergamon Press, Oxford.
Portis, A. M. (1953) *Phys. Rev. 91*, 1071-1078.
Rajagopalan, K. V., Handler, P., Palmer, G., & Beinert, H. (1968) *J. Biol. Chem. 243*, 3797-3806.
Salerno, J. C., & Ohnishi, T. (1980) *Biochem. J. 192*, 769-781.
Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. S., & Vincent, S. P. (1977) *Biochem. J. 167*, 593-600.

Determination of the Stoichiometry of Electron Uptake and the Midpoint Reduction Potentials of Milk Xanthine Oxidase at 25 °C by Microcoulometry†

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ABSTRACT: Microcoulometric titrations of several forms of milk xanthine oxidase at 25 °C (bicine buffer, pH 7.7) showed that full reduction of the native enzyme (with a functional Mo center, FAD, and two Fe_2S_2 centers) required six electrons, while reduction of the desulfo enzyme (with FAD, two Fe_2S_2 centers, and a nonfunctional Mo center) required four electrons, and reduction of the desulfo enzyme with its flavin alkylated (with two Fe_2S_2 centers and both nonfunctional FAD and Mo) required two electrons. From these data, the following reduction potentials were determined: Mo^{VI}/Mo^{V}, -375 mV; Mo^{V}/Mo^{IV}, -405 mV; FAD/$FADH_2$, -280 mV; Fe/S $I_{ox/red}$, -320 mV; Fe/S $II_{ox/red}$, -230 mV. Each of these potentials was shifted significantly in a negative direction (25-75 mV) when native enzyme was titrated coulometrically at pH 8.9 (Ches buffer), in agreement with the pH dependence predicted for these potentials by the results of Barber & Siegel [Barber, M. J., & Siegel, L. M. (1982) *Biochemistry* (first paper of three in this issue)]. Pyrophosphate caused positive shifts in the Mo^{V}/Mo^{IV} and Fe/S $II_{ox/red}$ potentials from values found in zwitterionic buffers. The potentials for Mo^{V}/Mo^{IV} and Fe/S $II_{ox/red}$ determined by coulometric titration at 25 °C were 25-30 mV more negative and 25-45 mV more positive, respectively, than the values determined by EPR analysis of enzyme frozen after being poised at defined potentials at 25 °C, whereas the Mo^{VI}/Mo^{V}, FAD/$FADH_2$, and Fe/S $I_{ox/red}$ potentials obtained by the two methods were in good agreement. It can be concluded that the prosthetic groups of xanthine oxidase possess differing entropies of reduction [$dE_m/dT = \Delta S/(nF)$], and it is therefore not strictly valid to combine results obtained from EPR analysis of some groups in frozen samples with optical spectroscopic measurements of other groups at 25 °C in order to determine the reduction state of the various enzyme centers during titrations and during turnover.

Milk xanthine oxidase is a dimer of apparently identical M_r 140 000 subunits, each containing one FAD, one Mo center (with an associated pterin cofactor; Johnson et al., 1980), and two spectroscopically distinct Fe_2S_2 centers, termed Fe/S I and Fe/S II (Bray, 1975). There has been controversy in the literature as to the exact number of electrons which can be taken up by xanthine oxidase. If each Fe/S center takes up one electron and the FAD and Mo centers two each, then xanthine oxidase should take up six electrons per functional half-molecule. Edmondson et al. (1972) and Olson et al. (1974a) observed close to this stoichiometry in reductive ti-

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