Studies of Aldehyde Oxidase by Electron Paramagnetic Resonance Spectroscopy

I. SPECTRA AT EQUILIBRIUM STATES*

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SUMMARY

Aldehyde oxidase from rabbit liver was studied by electron paramagnetic resonance (EPR) spectroscopy. Reduction by substrate elicited three types of signals characteristic of free radicals, molybdenum(V), and a non-heme iron complex, respectively. The properties of these signals are described, and the representation of each species in the corresponding signal is quantitatively assessed. Under the specific conditions which yielded a maximum for each of the signal types, 24% of the flavin was in the semiquinone form (g = 2.00), 25% of the iron was in the reduced form responsible for the signal of g = 1.94, and 15 to 20% of the molybdenum was in the Mo(V) state with a signal at g = 1.97. Anaerobic titration of the enzyme with dithionite indicated that reduction of only 1 iron out of each 4 (per flavin) results in the appearance of the g = 1.94 signal, in agreement with the quantitative recovery in the EPR signal.

When titration of the enzyme with N-methylnicotinamide was followed by EPR spectroscopy, partial formation of flavin semiquinone and accumulation of reducing equivalents in the non-heme iron complex was observed during consumption of the first 5 to 6 electrons per flavin. Extensive reduction of molybdenum ensued after an additional 1 to 2 eq. Spectrophotometry suggests that the enzymic components which accept electrons from substrate and are not detectable by EPR spectroscopy are that portion of the flavin which is fully reduced and the remainder of the non-heme iron. Rapid titrations, insufficient to permit equilibration between enzyme and substrate, showed migration of reducing equivalents from molybdenum toward flavin and non-heme iron with increasing time. These experiments show that intramolecular electron transfer can be effectively stopped at a low temperature. Measurements of electron spin relaxation of reduced enzyme suggest intramolecular magnetic interactions among the various paramagnetic species generated during the titrations.

Aldehyde oxidase purified from rabbit liver contains 2 moles of FAD, 2 g atoms of molybdenum, and 8 g atoms of non-heme iron per mole of enzyme of molecular weight of about 280,000 (1). In addition, these preparations contain variable amounts of a material tentatively identified as coenzyme Q10 (1). Studies on the inhibition of this enzyme by numerous inhibitors, including Amytal and antimycin A, suggested a linear sequence of at least four electron carriers (2, 3). Spectrophotometric studies showed that a non-heme iron complex is a major chromophoric component of aldehyde oxidase as well as of milk xanthine oxidase and dihydroorotate dehydrogenase of Zymobacterium oroticum (4). When reducing substrate is added, anaerobically, to aldehyde oxidase, there occurs profound bleaching in that portion of the absorption spectrum attributed to the non-heme iron complex. Studies of the substrate-binding site of aldehyde and xanthine oxidases yielded strong presumptive evidence for the presence of molybdenum at the substrate reactive site of these enzymes (5). If it is assumed that FAD and Mo(VI) in the enzyme are reducible to FADH2 and Mo(V), respectively, and that 1 electron can be accepted per iron atom present, up to 7 electrons per active center may be accommodated by the identified electron carriers of the enzyme. It was hoped that EPR spectroscopy would permit assessment of the extent of enzyme reduction by substrate, as well as identification, within the enzymic transport system, of the location of the electrons so acquired.

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The abbreviation used is: EPR, electron paramagnetic resonance.
The properties of the EPR signals of milk xanthine oxidase and the kinetics of their appearance and disappearance during oxidation or reduction have been described in some detail (6, 8). As expected from the similarity of xanthine and aldehyde oxidases, EPR studies on aldehyde oxidase yielded results generally analogous to those obtained with xanthine oxidase. However, many differences in the detailed behavior of the two enzymes were noted. The properties of the signals observed with aldehyde oxidase at equilibrium states and attempts at their quantitative evaluation will be the subject of this paper. Kinetic studies and a general discussion are presented in the accompanying paper (9).

METHODS

The preparation of aldehyde oxidase from rabbit liver has been described previously (1). Unless specifically mentioned otherwise, the enzyme and all additions were dissolved in 0.05 M phosphate buffer at pH 7.5, containing 0.1 mM EDTA. The basic techniques of rapid mixing, freezing, and producing anaerobic conditions have been described and discussed in a series of papers (7, 8, 10-13). Several improvements in instrumentation and technique were introduced in the present work. The rapid mixing apparatus was driven by an electric servomotor (14). The polyethylene hoses of the rapid mixing system were replaced by hoses of 1.2-mm wall thickness, and an improved mixing chamber and a double exit spray nozzle were used (12). Longer reaction times, of the order of seconds, were produced by the technique described elsewhere (13).

Throughout this series of papers, moles or electron equivalents of substrate or dithionite are given per mole of enzyme-bound flavin, i.e. per active center, not per mole of enzyme, and enzyme concentrations are also expressed in active center molarities.

The anaerobic titration of aldehyde oxidase was conducted according to a procedure developed in collaboration with Dr. B. F. van Gelder. For each “point” on the titration curve, a separate tube containing enzyme and the appropriate amount of substrate was used. Dithionite was used as a solid diluted to weighable quantities by potassium chloride. The tubes had side arms, the contents of which could be mixed without entering the quartz tube attached beneath, in which the EPR measurements were to be made. The top portion of the tube ended in a standard taper joint into which a high vacuum stopcock could be fitted. Joints and stopcocks were greased with Apiezon N. Enzyme, 0.30 ml, and 0.020 ml of a substrate solution were placed in the side arms of the tubes. The tubes were successively evacuated with an aspirator pump and filled with nitrogen purified by a pyrogallol train, and were finally evacuated twice with a forepump to a pressure of 1 to 2 mm of mercury. The enzyme and substrate solutions (or dithionite-KCl), at room temperature, were then well mixed in the upper part of the tube, and part of the mixture (0.2 ml) was permitted to run into the extremum of the sealed quartz tube so that a column of liquid at least 2 cm high was obtained. The solution was frozen in the quartz part approximately 2 min after mixing. Changes in fluid volume due to repeated evacuation were determined by direct measurement of the height of the liquid columns after thawing. Since the samples in the EPR tubes were kept under vacuum and the lower quartz portion of the tubes was immersed in liquid nitrogen, all water in the tubes was condensed in that lower portion, so that a measurement of the liquid column in matched tubes afforded a valid comparison of total liquid volumes at the time of mixing. In the titration experiments, when insufficient time was allowed for equilibrium (Figs. 12 and 13), enzyme and substrate solutions were mixed with the rapid mixing freeze apparatus (7, 11, 12, 14), injected into nitrogen-filled anaerobic tubes, and frozen by immersion in a bath of liquid isopentane at -140° as described previously (13). When these samples were thawed and refrozen, they were immersed in a warm water bath and subsequently in the isopentane bath.

The techniques utilized for EPR spectroscopy have been described in a series of publications (7, 10, 15). All spectra were recorded at low temperature (130° to 180°). Throughout this work the conventional “magic T” circuit was replaced by a circuit incorporating a microwave circulator and a bypass arm for crystal bias (16). This made it possible to operate reproducibly at low microwave powers as well as permitting high power operation. Since at low temperature the free radical and molybdenum signals of aldehyde oxidase are relatively easily saturated with power, the use in this work of the modified microwave circuit was of considerable advantage. Saturation curves of signals were determined by varying the microwave power (P) over a 104 to 105-fold range of power and observing signal height (S). Log S/√P is plotted on the ordinate against log P on the abscissa. Since log √P = 1/2 log P, the abscissa scale, if divided by 2, also gives log √P. Since signal height varies with √P outside the range of saturation, a straight line parallel to the abscissa should be found at nonsaturating powers. With increasing saturation the curves will curve toward the abscissa. The characteristic power at half saturation (P/2) is found in this type of plot by the intersection of the extrapolated linear portions of the curves (cf. Reference 17).

For the sake of optimal signal intensity or resolution, different instrumental conditions were often chosen in the observation of the individual component signals of the oxidase spectrum, and these are specifically mentioned in each instance. In order to conform to more general usage, the derivative spectra presented herewith have been inverted by 180°, as compared to previous publications (7, 10). For quantitative evaluation, the method of double integration was used. In most instances the integrations were performed by an automatic electronic integrator, which will be described elsewhere.

The following standards were used for comparison: for iron, the copper-EDTA standard; and for molybdenum, the copper and phantom standard. The values for molybdenum concentration found by comparison with the copper standard were approximately 1.5-fold higher than those arrived at by comparison with the pitch sample. The values reported here refer to an average derived from comparison with both standards. Corrections were applied for differences in g values (19). Since this method of correcting does not apply when g2≠g3= g1, an approximation was made by assuming an average value for the two closest lying g values. The accuracy and precision of the integration procedure have been discussed previously (7, 15, 20). Measurement of g values was performed as previously de-
Fig. 1. EPR spectra (right) and corresponding saturation curves (left) for the molybdenum component of aldehyde oxidase in different states. The bottom curves were obtained from 0.42 mM untreated enzyme. The enzyme, 0.42 mM, was then mixed in the rapid mixing apparatus with an equal volume of 20 mM N-methyl-nicotinamide and frozen 110 msec after mixing (center curves). An analogous mixture was injected into a tube without isopentane and left for 30 min at room temperature before freezing (top curves). The EPR spectra were obtained at a power of 1 milliwatt, 3-gauss modulation amplitude, scanning rate of 63 gauss per min, and -178°C. The time constant throughout this work was kept at 0.3 sec. The broad signal to the right (approximately 100 gauss from the main Mo(V) signal) in the two upper curves is due to the iron component of the enzyme. The saturation curves were obtained at the same modulation amplitude and scanning rate, and at -172°C. Saturation curves are plotted as described in the text. Note that the values shown on the abscissa are the logarithm of the actual powers (P) used, i.e. before extraction of the root (\sqrt{P}). The peaks designated by the numbers are found at the following field values (in gauss) at a microwave frequency of 9044.5 MHz: 1, 3265; 2, 3279.7; 3, 3296.5; 4, 3311.1; 5, 3215.4; 6, 3229.3; 7, 3245.3; 8, 3254.4; 9, 3271.8; 10, 3291.0; 11, 3316.0; 12, 3279.1; 13, 3286.9.

Results and Discussion

The EPR signals obtained with oxidized and reduced enzyme are shown in Fig. 1. Qualitatively, these resemble the signals obtained with xanthine oxidase with g values of 2.005, 1.97, and 1.93. These are attributed to a free radical, Mo(V), and a non-heme iron complex (22, 23), respectively.

Free Radical Signal—This signal has a peak to peak width of 16 gauss at a modulation amplitude of 3 gauss and is relatively easily saturated with microwave power (24). This signal has been observed only in states of partial reduction of enzyme; during neither reduction by substrate nor reoxidation thereafter has the intensity of this signal exceeded 5% of that expected were all of the flavin in the semiquinone state. During titration with dithionite, signals accounting for as much as 24% of the total flavin were recorded. Since ubiquinone has also been found in liver aldehyde oxidase, there is the possibility that radicals from this species might arise at certain oxidation states. Because the g values of free radical signals are similar and the line width of protein-bound species resembles that of over-modulated model systems, there are available no satisfactory criteria by which to distinguish between flavin and ubiquinone radicals under these conditions. The kinetics of appearance and disappearance of the free radical signals, observed at different times in the reaction with substrate, gave no indication of different free radical species. Measurements of saturation with increasing microwave power did, however, indicate the presence of different radical species. Since this observation has also been made with flavoproteins not containing ubiquinone (23), it is not evidence for ubisemiquinone formation. Moreover, the substrate-induced radical signal intensity is very weak, and overlap with the much stronger molybdenum and iron signals renders meaningful studies of the radical difficult.

Molybdenum Signals of Resting Enzyme—Unexpectedly, “resting” aldehyde oxidase exhibited a small signal at g = 1.97, indicative of Mo(V), as shown in the bottom curve of Fig. 1. At high protein concentration, the hyperfine structure in this signal could be resolved as shown in Fig. 2. As indicated by markers, several periodically occurring structures, separated by approximately 61 gauss, are apparent, and five of the six expected hyperfine lines of one set (g1) are clearly evident. The resolution in this case, as in Fig. 3, is not as good as that obtained with the “γ, δ” signal of reduced xanthine oxidase (Fig. 5 of Reference 7), where g1 was sufficiently separated from g2 and g3, so that overlap of hyperfine structure from the different lines and from the radical signal did not interfere. The two periods of 61 gauss observed at low field and one at high field are, however, sufficiently clear to indicate that the observed
signal is due to molybdenum, as suggested by the g value. Hyperfine splittings of approximately 75 gauss have been observed with Mo(ClO) at low temperature (25). In view of the presence of molybdenum in the enzyme, we attribute this and other signals at g = 1.97 to Mo(V). Reasons for this assignment in preference to Mo(III) were given previously (7).

Fig. 1 also shows the response of the Mo(V) signal in the resting enzyme to increasing microwave power. The signal is readily saturated at -175° at powers greater than 1 milliwatt. The magnitude of the Mo(V) signal of the resting enzyme corresponds to that expected if approximately 3 to 4% of the total molybdenum of the enzyme were in the pentavalent state. As will be shown below, even in the presence of excess reducing substrate, only approximately 15 to 20% of enzymic molybdenum can be accounted for as Mo(V) from the observed signal.

Molybdenum Signals of Reduced Enzyme—When a substrate is added to aldehyde oxidase, a rapid increase of the signal at g = 1.97 is observed. The shape of the signal changes, as is evident from a comparison of the middle curve of Fig. 1 (110 msec after mixing with substrate) and the bottom curve (resting enzyme). The Mo(V) spectrum obtained initially on reduction is very similar to the spectrum obtained with xanthine oxidase approximately 1 sec after reduction with xanthine at pH 8.3 (cf. Fig. 4 of Reference 7). At g = 2.005, a free radical signal is superimposed, and the broad signal at high field (g = 1.93) is due to a non-heme iron complex. As shown in Fig. 3, at higher protein concentrations, hyperfine structure could be resolved for an enzyme sample in a state similar to that of Fig. 1, middle curve.

**Fig. 2.** EPR spectra of "resting" aldehyde oxidase (1 mM), showing hyperfine structure of Mo(V) signal. The spectra were recorded at a 1-milliwatt power, 3-gauss modulation amplitude, scanning rate of 100 gauss per min, and -160°. The lower curve was obtained with the aid of a Varian C-1024 time-averaging computer and is shown at approximately 30-fold higher amplification than the upper curve. The amplitude of the main signal therefore exceeds the chart. At 9044.5 MHz, the following field values (in gauss) correspond to the numbers: 1, 3264.0; 2, 3286.1; 3, 3077.0; 4, 3143.2; 5, 3206.0; 6, 3275.5; 7, 3267.0; 8, 3415.5.

**Fig. 3.** EPR spectra analogous to those of Fig. 2, recorded after addition of 1 M N-methylnicotinamide to the same preparation and freezing after 30 sec; recorded at a 14-milliwatt power, 7.5-gauss modulation amplitude, a scanning rate of 100 gauss per min, and -163° for the upper two curves, and -134° for the bottom curve. The two lower curves were obtained at an amplification 27-fold higher than that used for the top curve. The two lower curves illustrate the overlap of the iron with the molybdenum signal, which is minimized by raising the temperature. The peaks designated by the numbers are found at the following field values (in gauss) at a microwave frequency of 9028.5 MHz: 1, 3263.6; 2, 3286.1; 3, 3077.0; 4, 3143.2; 5, 3206.0; 6, 3275.5; 7, 3267.0; 8, 3415.5.

The top curve of Fig. 3 shows the spectrum of the enzyme at low instrumental amplification and -163°. The two lower curves were obtained under analogous conditions, except for instrument sensitivity 27 times higher. In addition, the bottom curve was recorded at -134°. At the high gain, the main signal at g = 1.97 exceeds the chart of the recorder and only the wings are visible; the relative vertical displacement of the wings is correct, however. It will be seen that at the lower temperature the intensity of the iron signal at high field increases and the signal consequently overlaps and distorts the molybdenum signal. Since the amplitude of the iron signal rises sharply with decreasing temperature, the mutual interference of the molybdenum and iron signals is very pronounced at liquid nitrogen temperature (-196°). At high instrument sensitivity (Fig. 3, lower curves), and particularly at the elevated temperature (bottom curve), hyperfine structure of...
the molybdenum signal is clearly seen at both low and high field. Again periods indicating a 61-gauss splitting, as seen in Fig. 2, are apparent. Overlap of the hyperfine structure lines from $g_x$ and $g_z$ interferes with a complete resolution. As pointed out above, the wings of the signal are not located on a horizontal line, even at $-134^\circ$, where the interference by the iron signal is small. It appears, therefore, that a very broad signal centered at $g = 2$ is overlapping with the molybdenum, iron, and flavin signals. Since this broad signal has not been regularly observed with all samples, it is likely that it originates from a contaminant. A detailed interpretation of these asymmetrical and complicated spectra is not possible at this time. Unless hyperfine structure from nuclei other than $^{95}$Mo and $^{97}$Mo are superimposed, the number of lines observed in the molybdenum spectrum of the reduced enzyme cannot be explained in terms of a single molybdenum species, even if rhombic symmetry is assumed. Recently, however, Bray, Knowles and Meriwether (26) showed convincingly in experiments with $^{95}$Mo-labeled xanthine oxidase that proton hyperfine structure is indeed superimposed on hyperfine structure from molybdenum nuclei. This possibility should therefore also be considered with aldehyde oxidase in attempts to explain the multiplicity of lines.

The top spectrum of Fig. 1 was obtained when the enzyme was exposed to N-methylnicotinamide for more than 1 min. At this state, the free radical signal is very small, and additional structure appears on the main peak of the molybdenum signal.

On the left side of Fig. 1, the saturation curves for the corresponding EPR signals are shown. The bottom, center, and top lines of the left and right parts of the figure belong to the same species. It is apparent that the molybdenum species appearing on rapid reduction is less readily saturated than that in the resting enzyme, and the species observed on prolonged reduction (top curve) even less so. The molybdenum complexes responsible for the three types of signals are different, therefore, or are composed of species in different proportions, as is also apparent from the signal shapes. As will be shown below, the species corresponding to the top and bottom lines are not readily oxidized and may therefore be catalytically inactive. It may also be noteworthy that, from the kinetic experiments reported in the following paper (6), different substrates produce molybdenum signals of somewhat different shape and saturation behavior.

Iron Signal—As reduction of aldehyde oxidase proceeds, a broad signal appears at high field (27), as has also been observed with xanthine oxidase (7). The signal appears at a $g$ value of 1.93, has a component at low field ($g = 2.02$), and is very temperature-dependent. Fig. 4 shows a spectrum of dithionite-reduced aldehyde oxidase taken at $-248^\circ$. The low field component of the prominent iron signal is easily seen, since both the molybdenum and radical signals are weak after addition of dithionite and at the saturation with microwave power which was employed. The properties of the type of signal shown in Fig. 4 have been described and discussed previously (22, 23, 25, 28-32). Because of its properties and the presence of non-heme iron in all preparations which exhibited this signal under reducing conditions, this signal was tentatively attributed to iron (28, 29, 32); this assignment has received additional support (30, 31, 33-37). However, the original assumption (28, 29) that the iron is initially present in the ferric and reduced to the ferrous form has to be qualified or abandoned. The four different models of the responsible iron complex which have been considered (31, 33, 36, 37) involve molecular orbital considerations and do not permit an adequate description of the oxidation-reduction process in terms of classical valence theory. For the present discussion it appears sufficient to state that the enzymic iron complex can accept electrons while disregarding the exact localization of these electrons. Only one of the models (37) requires that at least 2 iron atoms be present in the reduced form of the enzyme, of which 1 atom is ferric, whereas the other models in their simplest form require (31, 33), or are flexible (36) so as to allow, uptake in the complex of 1 electron per iron atom. This has obvious implications for the quantitative assessment of the iron signal.

The iron signal is seen in the top and center curves of Fig. 1 and is particularly evident in the center curves of Figs. 3 and 4. The experiment depicted in the center curve of Fig. 3 differs from that of the bottom curve only in the sample temperatures, $-105^\circ$ for the center curve and $-134^\circ$ for the bottom curve. The intensity of the iron signal increases 4- to 5-fold within this temperature range, whereas the molybdenum signal changes very little. Comparison of the two curves reveals that at the lower temperature the molybdenum signal is distorted by the iron signal and hyperfine lines of the molybdenum signal are superimposed on the iron signal. This was previously observed with xanthine oxidase (7), but the two lower curves of Fig. 3 offer a better demonstration of this phenomenon. This situation will be seen also in the spectra obtained in the titration experiment (Fig. 8) to be described below; at low substrate concentrations (which elicit no change in the molybdenum signal, whereas the radical and iron signals appear), the two peaks on the iron signal are not very pronounced. The iron signal does, however, show a separation of $g_x$ and $g_z$, under appropriate conditions, and this separation is close to the 61-gauss spacing of the molybdenum hyperfine.

Since this type of signal is generally referred to as the "$g = 1.94$ signal," according to the $g$ value most frequently found with this type of compound, this same general designation will be used here, or it will be called simply the iron signal.

Between $-197^\circ$ and $-248^\circ$, the iron signal increases 30-fold in height.
structure. A similar separation of \( g_z \) and \( g_y \) has been seen with other iron flavoproteins which show the \( g = 1.94 \) signal but do not contain molybdenum, such as dihydroorotic and succinic dehydrogenases. This overlap of molybdenum hyperfinestructure with lines of the iron makes quantitative evaluation of the iron signal difficult, particularly when the molybdenum signal is relatively intense as compared to the iron signal. For evaluation of the iron signal, therefore, spectra were recorded at the lowest convenient temperatures and high modulation and power, conditions that lead to power saturation and modulation broadening of the molybdenum signal while increasing the iron signal.

The difficulties in quantitatively estimating iron concentration from the signal have been discussed previously (7). On the assumption that the non-heme iron complex producing the \( g = 1.94 \) signal contains 1 unpaired electron per iron atom, the signal elicited in aldehyde oxidase by an excess of N-methyl- nicotinamide represents 10 to 15% of the total iron concentration. On addition of dithionite, the iron signal increases further, so that approximately 20 to 25% of the total iron is accounted for. Low recoveries of iron in the \( g = 1.94 \) signal have been the general rule (7, 25, 30). Signals of this type are very temperature-dependent and are practically unmeasurable at sample temperatures higher than \(-100^\circ\) (23, 32). One of us has recently observed that these signals are greatly intensified in the range of \(-200^\circ \mbox{ to } -250^\circ\) (cf. Fig. 4), and similar signals can be seen in materials previously thought not to yield a \( g = 1.94 \) signal (34). Studies of the temperature dependence of the \( g = 1.94 \) signals of succinic dehydrogenase and the non-heme iron protein from Azotobacter (30) eliminate the possibility that, for these proteins, the low recovery can be explained by the assumption that under the conditions of our measurements a large percentage of the intensity is located in the wings of the signal and therefore escapes detection. There remains the possibility that material which is not in the state that produces the \( g = 1.94 \) signal in the temperature range between liquid nitrogen and room temperature assumes the proper configuration to do so at very low temperature.

However, as will be shown below in the experiment illustrated by Fig. 7, approximately 1 electron equivalent of dithionite per enzyme-bound flavin of aldehyde oxidase produces the maximal signal at \( g = 1.94 \). This would mean that only 1 iron atom out of 4, in the models of References 31 and 33, or perhaps 2 according to the model of References 36 and 37, are involved in the structure producing this signal. This brings the "recoveries" into a reasonable range, and the remaining discrepancies may well lie within the limits of error of our integration of this signal. In confirmation, integration of the signal shown in Fig. 4, which had been obtained at \(-245^\circ\), resulted in recoveries of approximately 25%. Thus, the repeatedly observed low "recoveries" of reduced iron calculated from the \( g = 1.94 \) signal are apparently not due to experimental shortcomings but have a distinct meaning in terms of the structure of these non-heme iron complexes.

**EPR Spectroscopy of Flavin-free Aldehyde Oxidase—Rajagopalan and Handler showed (4) that the flavin components of aldehyde and xanthine oxidases can be removed by precipitation from 50% methanol. The optical absorption spectra of the molybdenum-iron proteins so obtained were identical with those derived by differential spectrophotometry. These spectra were attributed to the enzymic iron complexes and proved to be strikingly similar to the absorption spectrum of spinach ferredoxin, a non-heme iron-protein (4, 38). It was of interest, therefore, to observe the EPR spectra of these flavin-free metalloproteins in both their oxidized and reduced states. Since the proteins so prepared lack their original enzymic activities, reduction was accomplished by treatment with dithionite. Fig. 5 depicts the EPR spectra of the molybdenum-iron-protein obtained from aldehyde oxidase. Fig. 5A shows the signal with instrument settings suitable for observation of molybdenum; Fig. 5B illustrates the effect of dithionite reduction with settings optimized for observation of iron. Particularly noteworthy is the fact that, by analogy with observations with the "resting," native enzyme, a molybdenum(V) signal was also exhibited by the flavin-free protein; this signal represented approximately 30% of the maximum \( g = 1.97 \) signal which has been observed after anaerobic reduction of the native enzyme by excess substrate. Upon addition of dithionite, the usual iron signal emerged at \( g = 1.99 \), while the molybdenum signal decreased, as is generally observed when aldehyde and xanthine oxidases are reduced by...
enzymic electron transport sequence closest to the substrate. Both aldehyde and xanthine oxidases are inactivated when incubated with their normal reducing substrates in the presence of methanol in concentrations of 0.5 to 2.0 M. Since there was no major change in the EPR spectrum of the inhibited enzymes, it appeared of interest to study the EPR spectra of the reduced states of these enzymes in the presence of proton hyperfine structure which they would have to be ascribed to the presence of different chemical species. However, that proton hyperfine structure may indeed be involved was made very likely by recent experiments with xanthine oxidase.

In Fig. 6 is presented the EPR spectrum of reisolated methanol-inactivated aldehyde oxidase in the absence of any additional reductant (Fig. 6B). As in the native enzyme (Fig. 6A), the molybdenum signal observed from methanol-inhibited aldehyde oxidase centers about \( g = 1.97 \). The intensities and spacings of the lines are such that the multiplicity cannot be explained in terms of the usual hyperfine structure lines of the molybdenum isotopes. In the absence of proton hyperfine structure they would have to be ascribed to the presence of different chemical species. Therefore, that proton hyperfine structure may indeed be involved was made very likely by recent experiments with xanthine oxidase.

Analogous data were obtained with xanthine oxidase. Fig. 6C shows the EPR spectrum of the reisolated, methanol-inactivated xanthine oxidase. Again, a signal at \( g = 1.97 \), presumably attributable to a form of Mo(V), is observed in the methanol-inactivated preparation. Upon dithionite treatment, an iron signal appears (not shown) which is identical with that elicited by dithionite reduction of the native enzyme. In contrast to aldehyde oxidase, reisolated methanol-inactivated xanthine oxidase also exhibited a signal at \( g = 2.00 \) (between \( 1 \) and \( 8 \) in Fig. 6), indicating the presence of an unidentified free radical. As observed repeatedly with flavoproteins, immediate and complete reduction of radical species cannot always be expected on addition of dithionite. The presence of this signal may therefore not be of any significance. Except for this radical signal, the EPR spectra of methanol-inactivated aldehyde and xanthine oxidases are quite similar (see also Reference 26). It should be noted that resolution of all the splittings in the xanthine oxidase spectrum could not be expected because of the relatively high power and modulation amplitude which had to be employed.

It follows from the spectra of Fig. 5, \( A \) and \( B \), that the environment of the molybdenum moiety of methanol-inactivated aldehyde oxidase is drastically changed from that in the native enzyme (Figs. 1 and 2). Thus, both methanol-inactivated xanthine and aldehyde oxidases must contain a species of Mo(V) which differs significantly from those present in the resting and reduced states of the native forms of these enzymes. These findings are compatible with (although no proof for) the previous suggestion (5) that inactivation by methanol in the presence of reducing substrate may involve substitution of methanol (or a methoxyl ion) for water (or hydroxyl ion) on the molybdenum atom itself. Finally, it should be noted that the molybdenum signal observed in reisolated, methanol-inactivated aldehyde oxidase is of the order of 50% of the signal elicited by substrate reduction of the native enzyme.

### Figure 6

![EPR spectra of aldehyde and xanthine oxidases after inactivation by 2 M methanol](image)

**A.** Spectrum of untreated aldehyde oxidase; **B.** spectrum of the reduced form of aldehyde oxidase; **C.** spectrum of the reduced form of xanthine oxidase. The protein concentration was similar to that used in Fig. 5. The spectra were obtained at a 1-milliwatt power, 3-gauss modulation amplitude, a scanning rate of 60 gauss per min, and a frequency of 9044.5 MHz. For **A**: 1, 3220.1; 2, 3237.4; 3, 3243.9; 4, 3261.6; 5, 3266.4; 6, 3278.1; 7, 3290.2; 8, 3307.8; 9, 3314.5; and for **B**: 1, 3221.9; 2, 3228.6; 3, 3246.0; 4, 3265.0; 5, 3288.9; 6, 3300.5; 7, 3309.2; 8, 3328.4. The peaks, designated by the numbers, are located at the following field values (in gauss), at a frequency of 9044.5 MHz: for **A**: 1, 3220.1; 2, 3237.4; 3, 3243.9; 4, 3261.6; 5, 3266.4; 6, 3278.1; 7, 3290.2; 8, 3307.8; 9, 3314.5; and for **B**: 1, 3221.9; 2, 3228.6; 3, 3246.0; 4, 3265.0; 5, 3288.9; 6, 3300.5; 7, 3309.2; 8, 3328.4.

This finding further strengthens the conclusion that the \( g = 1.94 \) signal reflects the presence of iron and not of flavin in the diverse group of proteins which have shown this signal. Since there was no major change in the EPR spectrum of the reduced iron complex, the state and manner of binding of the metal in the flavin-free preparation must be similar to, if not identical with, that of the native protein.

**EPR Signals of Methanol-inactivated Aldehyde and Xanthine Oxidases**—Both aldehyde and xanthine oxidases are inactivated when incubated with their normal reducing substrates in the presence of methanol in concentrations of 0.5 to 2.0 M (5). Since this inactivation was retarded significantly by increasing the concentration of substrate, it was concluded that inactivation probably represented an alteration of that component of the enzymic electron transport sequence closest to the substrate itself. When these enzymes, thus inhibited, were reisolated by ammonium sulfate precipitation, they exhibited absorption spectra which did not differ from those of the respective native, oxidized enzymes, indicating that the flavin and iron components of the inhibited enzymes were in their oxidized states. Accordingly, it appeared of interest to study the EPR spectra of the inhibited enzymes which had been inhibited by methanol in the presence of their respective substrates and to examine also the effect of dithionite treatment on the EPR spectra of these preparations.
fore, to observe the behavior of these components by EPR spectroscopy in the course of titrations of the enzyme by reducing substrate and by dithionite. The procedure is described under "Methods."

The data obtained in a titration with dithionite are summarized in Fig. 7, which presents the changes in the intensities of the radical, molybdenum, and iron signals in arbitrary units. A tentative evaluation in terms of the quantities of the individual electron acceptors involved is given in Table I. It can be seen in Fig. 7 that flavin and the non-heme iron were titrated simultaneously. The molybdenum signal broadened after addition of 3 eq and finally increased in amplitude, but decreased again after excess of dithionite. It is experiments of this type that have yielded the largest radical signals yet obtained with aldehyde oxidase, amounting to as much as 24% of the total bound flavin of the enzyme. In the experiment of Fig. 7, the point of 2 eq corresponds to 12% of the flavin. The balance sheet of reducing equivalents added compared with equivalents recovered (Table I) shows that not all electrons introduced were recovered and that the unrecovered portion increased from 0.5 to somewhat more than 1 electron per flavin as 1 to 4 equivalents were added. Several reasons for this discrepancy may be offered. First, the curves do not go through the origin, suggesting that something other than the enzyme is serving as electron acceptor early in the titration, such as residual oxygen. This oxidant oxidized 0.35 eq of dithionite, corresponding to 0.6 µl of air under the conditions of this experiment. Second, aldehyde oxidase may contain several potential electron acceptors which are not detectable by EPR. It is possible, therefore, that during titration of EPR-detectable non-heme iron, flavin, and molybdenum, EPR-undetectable non-heme iron and molybdenum also accept electrons to some extent. This possibility is emphasized by the titrations with substrate shown in two subsequent figures (Figs. 9 and 10). Third, of course, the over-all accuracy of these difficult experiments and the quantitative evaluation of EPR signals may be partly responsible for the observed discrepancies.

The titrations show that 3 electron equivalents per bound flavin reduce more than half of the flavin and 80% of the non-heme iron species that produces the 1.94 signal, in addition to a small portion of the molybdenum. In view of the likelihood that some EPR-undetectable species were titrated simultaneously, the data are plausibly interpreted by assuming that only 1 electron per flavin, i.e. per active site, suffices to produce the maximal 1.94 signal, thus indicating that only 1 or, at most, 2 (37) of the 4 iron atoms per active site is responsible for the 1.94 signal of the reduced enzyme. This interpretation is further supported by quantitative evaluation of the observed 1.94 signal by double integration, which accounts for 25% of the total iron, or 100% of 1 iron present in aldehyde oxidase. Similar observations have been made with the succinic dehydrogenase (39).

An anaerobic titration with substrate is shown in Figs. 8 through 10. Fig. 8 presents photographic records of the original traces. The results of a preliminary trial of such a titration have previously been reported (2); the present results, obtained by improved procedures, are in substantial agreement with the earlier report. In Fig. 9 the changes in signal height are plotted as a percentage of the maximal signal observed in Fig. 8. Maximal molybdenum signal was obtained by observation in the presence of excess substrate; maximal iron signal was elicited by treatment with dithionite; and maximal radical signal, as expected, on partial reduction.

The course of such a titration, as it may be followed spectrophotometrically, has been described previously (2). This procedure permits estimation of the extent of reduction of the iron and flavin moieties as substrate was added. After addition of 1, 2, or 3 moles of substrate per enzyme active center (corresponding to 1 flavin, 1 molybdenum, and 4 iron atoms), the

![Fig. 7. Anaerobic titration of aldehyde oxidase with dithionite. The enzyme concentration was 0.217 mM. Signal intensities in arbitrary units, corrected for blank signals, are plotted on the ordinate with respect to electron equivalents added per mole of bound flavin on the abscissa. The curves indicate the following species: O-O, free radical; - - , non-heme iron complex; A-A, molybdenum. The conditions of EPR spectroscopy were: power, 0.1 milliwatt; modulation amplitude, 3 gauss; scanning rate, 100 gauss per min for molybdenum and radical signals; and 23 milliwatts, 14 gauss, and 400 gauss per min for non-heme iron. The temperature was -118°.](http://www.jbc.org/)}
Fig. 8. EPR spectra obtained during anaerobic titration of aldehyde oxidase with N-methylnicotinamide. The enzyme used was 0.177 mm with respect to bound flavin. The titration procedure is described in the text. EPR spectra were recorded at a microwave power of 0.45 milliwatt, a modulation amplitude of 3 gauss, and a scanning rate of 100 gauss per min for free radical and molybdenum (left-hand side of figure), and at a microwave power of 23 milliwatts, a modulation amplitude of 14 gauss, and a scanning rate of 400 gauss per min for non-heme iron (right-hand side of figure). The temperature was -175°. As indicated in the text, the spectra shown are direct copies of the original. Corrections have to be applied for quantitative evaluation. The figures on the left margin indicate the number of electron equivalents added per mole of bound flavin, i.e. per active center.

extent of bleaching indicated that all of the substrate had been oxidized and that 2, 4, or 6 electrons had entered the enzyme system. Oxidation of a fourth mole of substrate, however, appeared to be incomplete, as a small amount of further bleaching at both 450 m& and 550 m& (less than 10% of total) could be achieved by subsequent addition of dithionite. Particularly noteworthy were the facts that the first pair of electrons appeared to reduce a pair of iron atoms, while flavin reduction at this point of the titration was almost negligible, and that at no point in the titration was any absorption at 620 m& apparent, so that this procedure gave no indication of formation of a flavin semiquinone of the neutral or A type (40). Absorption by the non-heme iron makes the detection of the anionic or B type impossible (cf. Reference 41).

In the present study, such a titration was again performed, under maximum precautions to ensure anaerobic conditions. The data were then used to calculate the extent of iron reduction from the change in absorbance at 550 m& and that of the flavin from the change at 450 m&. The latter was possible by first subtracting the decrement due to iron bleaching and on the basis of the following assumptions: (a) all iron atoms have the same absorptivity, (b) no optically detectable semiquinone formation is involved, and (c) the absorbance at 450 m& due to enzyme-bound oxidized or fully reduced flavin is the same as that of free FAD or FADH2. The results of this spectrophotometric titration are presented in Fig. 10; for comparison, the results of the EPR experiment of Figs. 8 and 9 are also incorporated, and will be discussed jointly. Whereas the changes in signal height in Fig. 9 have been plotted without reference to absolute quantities, the plot presented in Fig. 10 rests on the assumption that 1 electron equivalent accepted by an iron or molybdenum atom will produce the maximal g = 1.94 and 1.97 signals. Although this assumption appears reasonable on the basis of the results reported in this paper, it is not necessarily correct. The plot of Fig. 10 shows the following. The first 3 electron equivalents reduce the flavin almost completely. On an absolute scale the radical concentration is almost within

\[ \text{Fig. 9. Anaerobic titration of aldehyde oxidase with N-methylnicotinamide. Signal amplitude, for all curves corrected for blank signals, as a percentage of maximal amplitude observed with each type of signal, is plotted on the left-hand ordinate with respect to electron equivalents added per mole of bound flavin (abscissa). The curves indicate the following species: } - - - - , \text{ free radical; } -- -- --, \text{ non-heme iron complex; } - - - - - - - - , \text{ molybdenum; } - - - - - - - - - - - - , \text{ normalized amplitudes of the molybdenum signal measured at high power (182 milliwatts). These values refer to the right-hand ordinate and are taken from Fig. 11. Details of the titration procedure and measurements are given in the text and the legend to Fig. 8. The signal amplitudes were corrected for the amplitude of the signal found in samples of the resting enzyme. It should be recalled that there is an appreciable molybdenum signal observed in the resting enzyme.} \]
the limits of error of the determination of FADH₂. Hence, if the radical were a flavin semiquinone, the corresponding optical absorbance changes would not be detectable. Accordingly, definitive assignment of the origin of the radical signal was not possible. One of the first 3 electron equivalents per flavin entering the enzyme is taken up by the non-heme iron complex, as indicated by the rise of the signal at \( g = 1.94 \) and the decrease of absorbance at 550 nm. The most significant aspect is that the absorbance at 550 nm continued to decrease as additional reducing equivalents were added, while the \( g = 1.94 \) signal did not increase correspondingly. When the main change at 550 nm was completed, after addition of 5 to 6 electron equivalents per flavin, the signal of Mo(V) appeared. Again it appears that only part of the iron need be reduced to elicit a maximal \( g = 1.94 \) signal, whereas most or all of the non-heme iron complex (or complexes) is represented in the optical absorption spectrum with similar absorptivity per atom.

Maximal reduction of molybdenum was accomplished within 1 or 2 eq after a total of 6 had been consumed per active center. These results indicate that the iron and flavin oxidation-reduction systems are at higher potential than the molybdenum system.

Although the height of the molybdenum signal evident with the oxidized enzyme did not change during the initial phases of the titration, signal broadening occurred, indicating a gradual increase in Mo(V), amounting to at most 20%, concomitant with pronounced changes in saturation behavior. The saturation of the molybdenum signals with increasing microwave power is shown in Fig. 11; the signal heights at high power (182 milliwatts), which can be taken as an indication of saturation, are incorporated in Fig. 9 (right-hand ordinate). Saturation of the molybdenum signal decreased early in the titration, although the signal intensity indicated that electrons are retained by only a very small fraction of the molybdenum atoms. Saturation then assumed a constant value and showed no significant further change as the molybdenum was reduced. It is interesting to note that the maximum in the saturation curve (solid curve in Fig. 9) coincided with the maximal development of the free radical signal and that the saturation curve leveled off at the break in the titration curve of the non-heme iron component. This may mean that the broadening of the initial molybdenum signal and its increased relaxation are due to the appearance of neighboring paramagnetic centers, i.e. flavin.
Fig. 12. EPR spectra obtained on anaerobic “rapid titration” of aldehyde oxidase with N-methylnicotinamide. Enzyme, 0.93 mM, was mixed anaerobically with an equal volume of 0.93 mM N-methylnicotinamide. Samples were frozen at 1.5 sec (B), 10 sec (C), and 40 sec (D); A and E are controls. A was mixed with buffer only in the absence of substrate, and E was mixed with 40 mM N-methylnicotinamide and frozen after 40 sec. The microwave power, modulation amplitude, and temperature for recording radical and iron complex, and that in fact relaxation is at its maximum when flavin radical is maximally developed.

Experiments similar to that described by Figs. 8, 9, and 11 were performed in which insufficient time was allowed for attainment of equilibrium among electron carriers. It was hoped that, by this procedure, electrons might be trapped, so to speak, on their path to the final electron acceptor. In order to achieve significant reduction of electron carriers in the enzyme with titrating quantities of substrate, reaction times of the order of seconds had to be allowed. Enzyme and substrate solutions were therefore mixed with the rapid mix-freeze apparatus as described above. Fig. 12 shows an experiment in which N-methylnicotinamide, corresponding to 2 electron equivalents per flavin, was added to the enzyme. Curve A represents the EPR signal of the untreated enzyme; Curve B, the state reached after approximately 1.5 sec. It is evident that, at this stage, reduction of the iron complex and flavin was relatively small, but the molybdenum signal increased significantly and assumed the shape typical of reduction. In other, similar experiments (cf. Fig. 13), no reduction of flavin and iron has been observed at early times, whereas the molybdenum signal underwent the same changes shown here. Curve C (Fig. 12) shows the state of a similar system at 10 sec. At this stage, when more substrate molecules had had opportunity to react, electrons entered the flavin and the iron complex, rendering the iron and radical signals much more conspicuous, while the molybdenum signal decreased slightly. As yet more time was allowed (Curve D), the solution was depleted of substrate; since there had been sufficient substrate to deposit, on the average, 4 electrons per enzyme molecule (2 per bound flavin), the Mo(V) component lost electrons to the flavin and the iron complex and the Mo(V) signal reverted to its original state. This is in agreement with the results of the equilibrium experiment shown in Fig. 9, in which, at 2 electron equivalents per flavin, the Mo(V) signal of the resting enzyme remained unchanged. Analogous results have been obtained when this experiment was performed with a single sample, which was carried through the various stages by freezing and thawing (Fig. 13). Curve E (Fig. 12) shows, for comparison, the signals obtained 40 sec after addition of an excess of substrate. The iron signal is close to the maximum obtainable by substrate reduction, the radical signal is somewhat decreased, presumably because of extensive full reduction of the flavin, and the molybdenum signal shows the shape of the reduced species, but is relatively small because of the “fading” phenomenon, which will be discussed in the following paper (see Fig. 3 of Reference 9).
Fig. 13. EPR spectra obtained on anaerobic rapid titration of aldehyde oxidase. Enzyme, 0.4 mM, was mixed anaerobically with an equal volume of 1 mM N-methylnicotinamide. The mixture was frozen at approximately 1.5 sec (Curve B) and thereafter was rapidly thawed and refrozen within approximately 10 sec.

Fig. 14. Saturation curves of Mo(V) signals at 160° obtained in the experiment of Fig. 12. The curves were plotted and evaluated as for Fig. 1.

These experiments, in which both the number of electrons and the reaction time were limited, have established the intramolecular passage of electrons from substrate via molybdenum to flavin and the iron complex. The drainage of electrons from the molybdenum toward the other carriers is particularly evident in comparing Curves B and D of Fig. 12 and B and C of Fig. 13. These results agree completely with the pattern of events during operation of the enzyme suggested by the other experiments (Curve C). A and D are controls: A, untreated enzyme; D, enzyme frozen at 2 min after addition of an excess of substrate. Presentation of spectra and conditions of recording are the same as those of Fig. 12, except that the temperatures were different: -176° for molybdenum and -196° for the g = 1.94 signal.

The spin relaxation of the radical obtained in these rapid reaction experiments showed no significant change, while the relaxation behavior of the molybdenum signal fully agreed with that observed in the titration experiment of Figs. 9 and 11. Saturation with increasing microwave power is plotted in Fig. 14. When the signals of Fig. 12 are compared with the saturation curves of Fig. 14, it appears that spin relaxation of the molybdenum signal is a function of the intensity of the g = 1.94 signal, i.e. of the paramagnetism of the iron complex. Additional discussion of the spin relaxation data reported here and in the following paper (9) will be found in the following paper (9) in conjunction with other relevant findings.

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REFERENCES

Studies of Aldehyde Oxidase by Electron Paramagnetic Resonance Spectroscopy: I. SPECTRA AT EQUILIBRIUM STATES
K. V. Rajagopalan, Philip Handler, Graham Palmer and Helmut Beinert


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