Electron Paramagnetic Resonance and Potentiometric Studies of Arsenite Interaction with the Molybdenum Centers of Xanthine Oxidase, Xanthine Dehydrogenase, and Aldehyde Oxidase: A Specific Stabilization of the Molybdenum(V) Oxidation State†

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ABSTRACT: Alterations in the Mo(V) electron paramagnetic resonance spectra of milk xanthine oxidase (XO), chicken liver xanthine dehydrogenase (XDH), and rabbit liver aldehyde oxidase (AO) have been obtained following aerobic incubation with sodium arsenite. The time-dependent inactivation of the milk enzyme was found to result in an intense, rhombic spectrum with g values of $g_1 = 2.0062$, $g_2 = 1.9689$, and $g_3 = 1.9432$, following controlled reduction with dithionite. The spectrum exhibited a complex, four-line nuclear hyperfine pattern due to interaction of the ^{75}As nucleus with ^{96}Mo, with coupling constants of $A_1 = 1.15$, $A_2 = 1.70$, and $A_3 = 2.05$ mT, respectively. Similar g and A values were obtained for the corresponding Mo(V) spectra from AsO_2^--treated XDH and AO. The Mo(V) spectrum of XO–AsO_2^- in D_2O showed no nuclear hyperfine coupling to exchangeable protons. Controlled dithionite reduction of the AsO_2^--inhibited XO in the presence of xanthine showed that the substrate effected significant changes in the Mo(V)–AsO_2^- EPR spectrum. Although AsO_2^- binding to the Mo centers of desulfo and formaldehyde-inhibited xanthine oxidase caused small changes in the Mo(V) EPR spectra of these species, no nuclear hyperfine coupling to the As nucleus was detected. Potentiometric titrations of XO in the presence of arsenite revealed the midpoint potentials of the FAD and Fe/S centers to be unchanged from those of the native enzyme. However, the potentials of the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples were shifted (control values given in parentheses) to values of −130 (−373) mV and −465 (−377) mV, respectively, at pH 7.7. Similar shifts in potential were obtained for the Mo center in aldehyde oxidase. These results indicate a specific stabilization of the Mo(V) oxidation state following AsO_2^- binding to these enzymes. Arsenite inactivation did not eliminate the Mo(V)–Fe/S I magnetic interaction typical of this class of Mo-containing enzymes.

Peters & Sanadi (1961) originally revealed that arsenite inhibits the aerobic conversion of xanthine to uric acid catalyzed by the complex molybdenum–FAD–Fe/S protein milk xanthine oxidase (XO).[1] For a detailed review of this and other enzymes containing the Mo–pterin type of active center, see Bray (1975, 1980). Rajagopalan & Handler (1964) found arsenite inhibition of the related enzymes chicken liver xanthine dehydrogenase (XDH) and rabbit liver aldehyde oxidase (AO). For either XO or XDH this inhibition has been found to be dependent upon the oxidation state of the enzyme and incubation time, to be noncompetitive with respect to the reducing substrate, and to be essentially irreversible. In contrast, for AO, AsO_2^- inactivation has been demonstrated to be competitive, very rapid, and readily reversible (Rajagopalan & Handler, 1964; Coughlan et al., 1969). Absorbance changes, maximal at 380 nm, were associated with AsO_2^- reaction with XDH (Coughlan et al., 1969). The extent of these changes was correlated with the amount of cyanolyzable sulfur present in the enzyme preparation. It was thus proposed that Mo=S rather than Mo=O is required for AsO_2^- reaction with XDH. Binding of AsO_2^- at or near the Mo center has been supported by preliminary EPR studies of XDH (Johnson & Rajagopalan, 1978), which showed the Mo(V) spectrum to be perturbed from its usual "rapid" line shape in native functional enzyme to a broader species exhibiting additional, largely unresolved, hyperfine splittings in the presence of AsO_2^-. Some evidence was provided that AsO_2^- inactivation of XDH does not itself preclude either substrate or product binding to the Mo center of that enzyme.

Recently, Hille et al. (1982) have indicated that the Mo(V) EPR signal from arsenite-treated xanthine oxidase appears during the initial phases of reductive titrations using dithionite and persists even in the presence of excess reducing equivalents. This result suggests that in AsO_2^--inactivated enzyme the Mo potentials may be shifted so as to allow the Mo to be reduced before the flavin and Fe/S prosthetic groups of the enzyme. Such a change in potential could be used to provide a rigorous test of the mechanism of Olson et al. (1974) that the kinetics of electron flow in XO during catalysis are apparently controlled by the equilibrium reduction potentials of the prosthetic groups; i.e., electron flow between the groups is sufficiently rapid to permit their equilibration at all times.

In the present work, we have examined the effects of AsO_2^- on the Mo(V) EPR spectra of XO, XDH, and AO. Both functional enzyme and a variety of derivatives of XO have been investigated. These results indicate that AsO_2^- is closely bound to the Mo center in functional enzyme containing the Mo=S group but that it is also bound, though in a somewhat different fashion, at the Mo center in enzyme containing Mo=O. Studies of the reduction potential of various prosthetic groups in XO and AO show that AsO_2^- binding greatly stabilizes the Mo(V) oxidation state of these enzymes compared to either the Mo(VI) or Mo(IV) states and that the potentials of the FAD and Fe/S groups are not significantly affected. The magnetic interaction between Mo(V) and reduced Fe/S I,

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[1] Abbreviations: EPR, electron paramagnetic resonance; XO, xanthine oxidase; XDH, xanthine dehydrogenase; AO, aldehyde oxidase; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EDTA, ethylenediaminetetraacetic acid; Fe/S or Fe_2S_2, the binuclear iron–sulfur centers termed individually Fe/S I and Fe/S II.

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characteristic of the three Mo–flavin–Fe/S hydroxylases, is still present following AsO_2^- modification of the Mo center.

Materials and Methods

XO was purified from fresh, unpasteurized cream as described by Barber & Siegel (1982a) and had an AFR value of 114 corresponding to 58% functionality (Bray, 1975). Cyanide-treated (desulfo) XO and "glycol-inhibited" desulfo-XO were prepared by the methods of Massey & Edmondson (1970) and Lowe et al. (1976), respectively. Formaldehyde-inhibited XO was prepared as described by Pick et al. (1971). Chicken liver XDH was isolated by using a combination of previously published methods (Rajagopalan & Handler, 1964; Cleere & Coughlan, 1975). The AFR of the preparation was 234, equivalent to 72% functionality. AO was prepared from fresh rabbit livers according to the procedure of Rajagopalan et al. (1962) and contained 30% functional centers. All enzyme samples were maintained in 50 mM Bicine buffer, containing 1 mM EDTA, pH 7.7, unless otherwise noted, except for the samples in D_2O where the pD = 7.7 (Glasoe & Long, 1960). Enzyme D_2O buffer was prepared by repetitive dialysis.

A general proceedure was adopted for the preparation of arsenite-treated enzyme. Samples of protein in the concentration range 10–100 μM functional active sites were incubated, aerobically, with 2 mM sodium arsenite at the desired pH for 15 min, following which reduction was accomplished by either anaerobic addition of excess dithionite or addition of redox mediators and poising the sample at a controlled potential prior to freezing in liquid nitrogen. Inclusion of sodium arsenite did not alter the pH of the buffer solutions when measured either at 298 K or upon freezing to 77 K, as determined by the technique of Williams-Smith et al. (1977).

Potentiometric titrations were performed as described by Barber & Siegel (1982a). Enzyme concentrations (in terms of functional active centers) and mediator concentrations were 34 and 33 and 12 and 11 μM for XO and AO, respectively. As for previous studies, the mediators were added in two groups to assist in monitoring changes in the concentration of the FADH· free radical.

EPR spectra were recorded by using a Varian E9 spectrometer operating at 9.1 GHz and using 100-kHz modulation. For Mo(V) and FADH· signals, spectra were obtained at 173 K by using 5-mW microwave power and 0.25-mT modulation amplitude. Fe/S signals were obtained at 20 K by using 1-mW power and 1-mT modulation. Fe/S II was also monitored by using 100-mW power. EPR spectra were quantitated as described by Barber & Siegel (1982a) using CuEDTA as an integration standard. g and A values were obtained by comparison of experimental and computer-stimulated spectra using a modified version of the program of Lowe (1978) using a Hewlett-Packard HP9825A computer interfaced to an H-P7225A graphics plotter.

Results

Incubation of native XO, aerobically with sodium arsenite (final concentration 2 mM) at pH 7.7, was found to inhibit its ability to catalyze the oxygen-dependent oxidation of xanthine (final concentration 0.1 mM) to uric acid monitored at 295 nm. As the preincubation period with AsO_2^- was increased, the activity of the enzyme was found to drastically decrease, nearly total inhibition being obtained after 30 min. A semilogarithmic plot of the inhibition data yielded a pseudo-first-order reaction half-time of 6.6 min.

The EPR spectrum of arsenite-treated XO in 50 mM Bicine buffer, pH 7.7, poised at a controlled potential of −230 mV

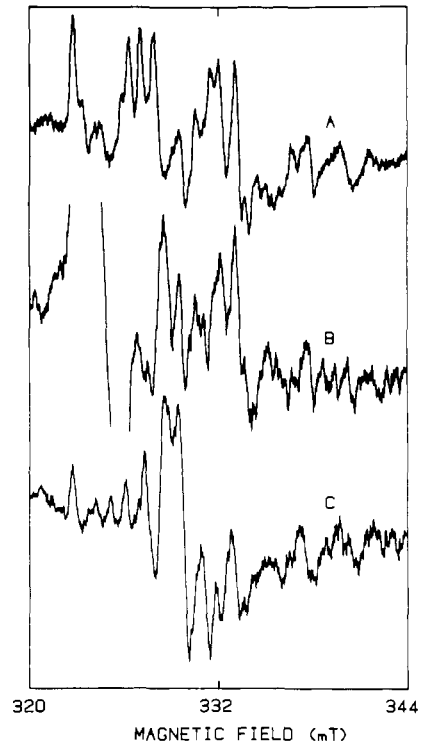

FIGURE 1: Comparison of the Mo(V) EPR spectra from xanthine oxidase, xanthine deydrogenase, and aldehyde oxidase treated with arsenite. (A) Xanthine oxidase (81 μM functional sites) was incubated with $NaAsO_2$ for 15 min. Indigodisulfonate (40 μM) and safranine T (40 μM) were added and the enzyme was poised at −178 mV by using dithionite. (B) Xanthine dehydrogenase (30 μM functional sites) was treated as described in (A) and poised at −220 mV. (C) Aldehyde oxidase (12 μM functional sites) was treated as described in (A) and poised at −260 mV.

is shown in Figure 1. A complex, rhombic spectrum was obtained that could be ascribed to the formation of a Mo(V)–AsO_2^- complex. Double integration of the signal showed the spin density to correspond to approximately 1.0 electron per functional active center. The complexity of the observed Mo(V)–AsO_2^- spectrum may be interpreted in terms of a number of factors. The 100% natural abundance of ^{75}As, an isotope that possesses a nuclear spin quantum number of $^3/_2$, would be expected, under favorable conditions, to result in a splitting of the Mo(V) spectrum into four lines of equal intensity, centered around the normal Mo(V) absorption position. However, since approximately 25% of the Mo centers of these enzymes are composed of the isotopes ^{95}Mo and ^{97}Mo, both of which have nuclear spin quantum numbers of $^5/_2$, in contrast to ^{92}Mo, ^{94}Mo, ^{96}Mo, ^{98}Mo, and ^{100}Mo, which have spin quantum numbers of $^1/_2$, a significant fraction of the Mo(V)–AsO_2^- signal would be split by an additional six-line hyperfine pattern. The result of these hyperfine interactions is expected to be an extremely complex signal that could only be satisfactorily resolved into its components by use of extensive isotopic substitution and recording the resultant spectra at several microwave frequencies. In addition, a number of Mo(V) signals obtained from XO and other Mo hydroxylases have been shown to be composed of more than one species (Bray, 1980), the composition of which can vary depending upon the conditions used. As a further complication, many of the known Mo(V) spectra have been demonstrated to be complicated by the presence of nuclear hyperfine coupling to both nonexchangeable and exchangeable protons. To probe some of these problems we have examined the EPR spectra of arsenite-treated samples of XDH and AO, as a basis for comparing possible common spectral features with the Mo-

Table I: EPR Spectroscopic Parameters for the Mo(V) Signals from AsO_2^--Treated Xanthine Oxidase, Xanthine Dehydrogenase, and Aldehyde Oxidase

	simulation parameters[a]								
enzyme	g_1	g_2	g_3	A_1	A_2	A_3	Δ_1	Δ_2	Δ_3
XO	2.0062	1.9689	1.9432	1.15	1.7	2.05	0.2	0.2	0.2
XDH	2.0062	1.9691	1.9432	1.15	1.75	2.05	0.2	0.2	0.2
AO	2.0061	1.9702	1.9432	1.15	1.8	2.0	0.2	0.2	0.2

[a] Hyperfine coupling constants (A) and half line widths (Δ) are in milliteslas.

(V)–AsO_2^- spectrum of XO. The resulting spectra, shown in Figure 1, indicate that arsenite inactivation of both of these enzymes yields Mo(V) EPR spectra following reduction, basically similar to that of xanthine oxidase. Close agreement was obtained between the g and A values for the basic Mo(V)–AsO_2^- spectrum, given in Table I, for all three enzymes. However, subtle differences in the ratios and positions of various spectral peaks do indicate that there are some differences. It should be noted that the observed spectra from XDH and AO are complicated by the presence of paramagnetic components in addition to the Mo(V)–AsO_2^- species. In the former protein, this is due to the generation of substantial levels of FADH· free radical signal at approximately g = 2.0036, while in the latter, the presence of a nonfunctional form of the enzyme, referred to as "resting II" [analogous to glycol-inhibited desulfo AO; see Barber et al. (1982)], gives rise to a narrow rhombic signal with g_{av} = 1.97.

The EPR spectrum of Mo(V) ("rapid") in samples of functional XO reduced by dithionite or poised at fixed redox potentials shows evidence of both strongly and weakly coupled protons interacting with the Mo(V) center (Barber & Siegel, 1982a,b).

Samples of XO enriched in deuterium and maintained in D_2O buffer were subjected to AsO_2^- inactivation and then reduced to examine the possibility of coupling of the Mo(V) nucleus to exchangeable protons in the Mo(V)–AsO_2^- complex. Figure 2 shows a comparison of the spectrum obtained in D_2O with that of the normal enzyme in H_2O. No change in either the signal line shape or the line width of any individual peak was detected, indicating the absence of hyperfine interaction between the Mo(V) and either strongly or weakly coupled protons in the arsenite complex.

To determine that changes in the signal line shape could be readily detected, we added excess xanthine anaerobically to AsO_2^--treated xanthine oxidase. While the xanthine failed to reduce the enzyme, poising the protein sample at a controlled potential with dithionite in the presence of redox mediators generated the Mo(V) spectrum shown in Figure 2C. Comparison of this spectrum with that of the arsenite-treated enzyme reduced in the absence of xanthine showed substantial changes in the line shape, in agreement with the earlier work of Johnson & Rajagopalan (1978) on XDH. Addition of xanthine did not abolish the ^{75}As hyperfine interaction.

Comparison of the Mo(V)–AsO_2^- spectra from the three Mo proteins examined indicated that the spectrum of AsO_2^--treated aldehyde oxidase showed the fewest complexities. We thus attempted to simulate the aldehyde oxidase spectrum from samples of both native and arsenite-treated AO, obtained at comparable reduction states. The experimental and simulated spectra are shown in Figure 3. To ascertain the validity of the spectroscopic parameters used in the AO–AsO_2^- simulations, we combined the simulations of the pure Mo(V)–arsenite spectrum with that of the Mo(V) signal of the resting enzyme in various proportions. Addition of 35%

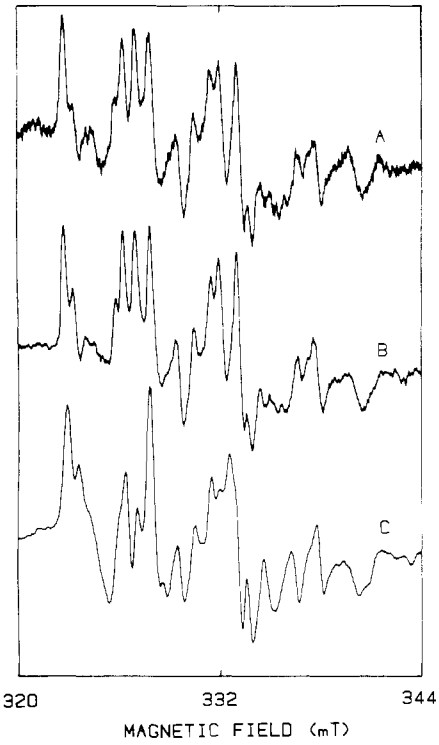

FIGURE 2: Effects of deuterium and xanthine on the EPR spectrum of arsenite-treated xanthine oxidase. (A) Sample conditions were as described for Figure 1A. (B) Xanthine oxidase in D_2O, pH 7.7, was incubated with AsO_2^- as described in Figure 1A and poised at −260 mV. (C) Xanthine oxidase was treated with AsO_2^- as described in Figure 1A. Xanthine (1 mM) was added and poised at −230 mV.

of the simulation of the resting II species to 65% of the AsO_2^- species yielded the computer-derived spectrum shown in Figure 3B, which was judged to be the best fit to the experimentally observed spectrum. The excellent agreement between the two spectra validate the parameters used to simulate the arsenite species. On the basis of this result, simulations using comparable parameters were used to derive the g and A values for the XO and XDH spectra given in Table I.

To examine if modification of the native Mo=S center would result in changes in the arsenite spectra, we incubated samples of glycol-inhibited, formaldehyde-inhibited, and cyanide-treated (desulfo) xanthine oxidase aerobically with AsO_2^- and examined their EPR spectra under appropriate conditions of reduction. While no change in the g values of line shape of the glycol-inhibited Mo(V) spectrum was obtained, small changes in the formaldehyde-inhibited and the slow signal were observed, as shown in Figure 4. It is apparent from these spectra that arsenite can bind to both inhibited and nonfunctional forms of XO. In formaldehyde-treated enzyme, the Mo=S center is presumed to contain an H—C=O ligand at a position normally occupied by substrate (Bray, 1980). The cyanide-treated enzyme contains Mo=O in place of Mo=S (Bordas et al., 1980). Despite this evidence of AsO_2^- binding, however, the dramatic hyperfine coupling of the As nucleus evident in spectra from functional enzyme was not observed when AsO_2^- binds to inhibited or desulfo XO. The changes in the line shape of the formaldehyde and slow signals in fact need involve only minor alterations in the g values of the species or the hyperfine coupling constants of the associated protons.

Arsenite inactivation was found to have a very significant effect on the oxidation–reduction potentials of XO and AO (XDH was not examined). Typical results of a potentiometric titration of AsO_2^--treated XO are shown in Figure 5. Oxidized enzyme, or samples poised at potentials above 0 mV,

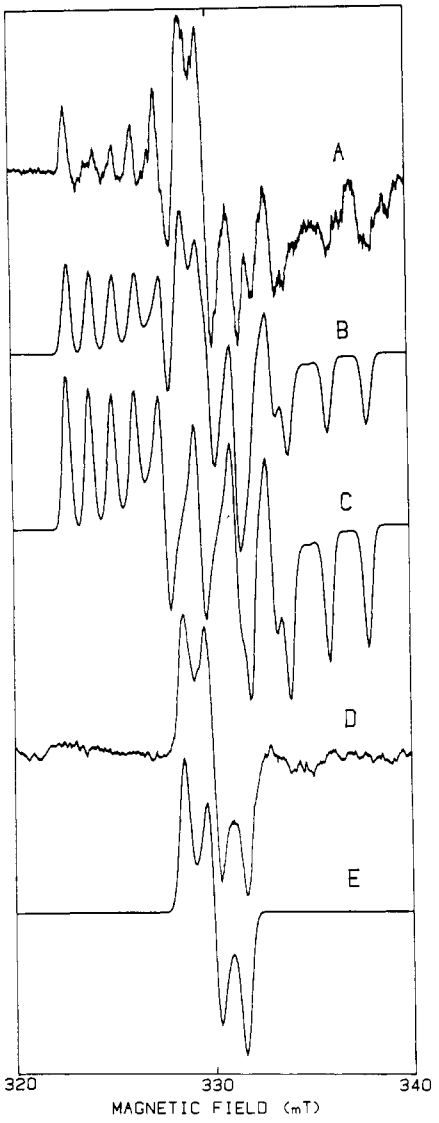

FIGURE 3: Comparison of experimental and computer-simulated EPR spectra of arsenite-inactivated aldehyde oxidase. (A) Sample details are those for spectrum C, Figure 1. (B) Computer-generated spectrum obtained by addition of 65% of the normalized intensity of spectrum C and 35% of spectrum E. (C) Computer simulation of the AsO_2^--inactivated Mo(V) spectrum using the parameters given in Table I. (D) Aldehyde oxidase (12 μM functional sites) in Bicine buffer, pH 7.7, in the presence of dye mediators (11 μM), poised at a potential of −186 mV by using dithionite. (E) Computer simulation of the resting II signal. The following parameters were used: g_1 = 1.9816, g_2 = 1.9727, and g_3 = 1.9632.

showed no detectable Mo(V) EPR signal. However, as the applied potential was decreased, the EPR spectrum characteristic of the arsenite complex was found to appear, reaching a maximum amplitude within the potential range −200 to −350 mV and then decreasing as the potential became more negative. Double integration of the maximum signal amplitude corresponded to a spin density of 1.0 electron per functional Mo center. Midpoint oxidation–reduction potentials for the two phases, corresponding to the transitions Mo(VI)–Mo(V) and Mo(V)–Mo(IV), were found to be −130 and −465 mV, respectively. No change in the EPR signal line shape was detected throughout the course of the titration, although at very negative potentials, the spectrum was complicated by the appearance of slow signal due to nonfunctional enzyme. Preliminary examination of the spectra indicates that there was no significant change in the redox potentials of the slow signal.

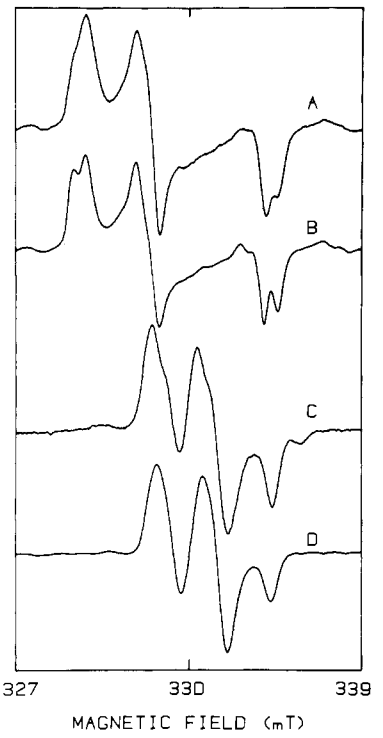

FIGURE 4: Effect of arsenite on the Mo(V) EPR spectra of formaldehyde-inhibited and cyanide-treated xanthine oxidase. (A) Formaldehyde-inhibited xanthine oxidase (100 μM functional centers) was incubated with 2 mM AsO_2^- for 15 min and frozen in liquid nitrogen. (B) Formaldehyde-inhibited enzyme in the absence of AsO_2^-. (C) Cyanide-treated XO (50 μm total centers) was incubated with 2 mM AsO_2^- for 15 min. Methyl viologen (40 μM) was added and the enzyme poised at −400 mV for 30 min prior to freezing. (D) A second sample of desulfo enzyme was reduced as described in (C) in the absence of arsenite.

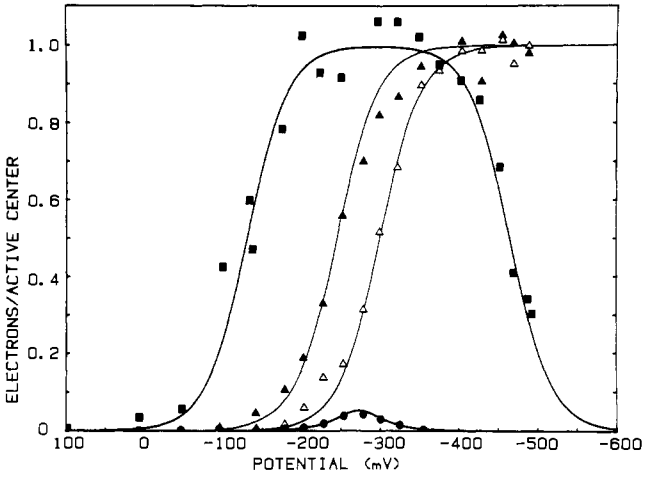

FIGURE 5: Potentiometric titration of arsenite-treated xanthine oxidase. Xanthine oxidase (34 μM functional centers) was incubated with arsenite as previously described. A redox titration was then performed as described by Barber & Siegel (1982a) with mediators at a final concentration of 33 μM. Samples were removed at the indicated potentials and frozen in liquid nitrogen. Mo(V)–AsO_2^- signal (■); FADH· radical signal (●); reduced Fe/S I (△); reduced Fe/S II (▲). EPR spectra were recorded and quantified as described under Materials and Methods. Theoretical Nernst curves were fitted to the data points by computer using the values given in Table II.

Results for the FADH, Fe/S I, and Fe/S II signals are also shown in Figure 5. Midpoint potentials obtained for the FAD/FADH· and FADH·/FADH couples were −330 and −215 mV, respectively; these compare very favorably with those obtained from titrations of native enzyme under similar conditions (Barber & Siegel, 1982a). Potentials obtained for the

Table II: Oxidation-Reduction Potentials for Native and AsO_2^--Treated Xanthine Oxidase and Aldehyde Oxidase

enzyme	pH	oxidation-reduction midpoint potential[a] Mo(VI)/ Mo(V)	Mo(V)/ Mo(IV)	FAD/ FADH·	FADH·/ $FADH_2$	Fe/S $I_{ox/red}$	Fe/S $II_{ox/red}$
XO	7.7	−373	−377	−330	−243	−310	−255
AsO_2^--treated XO	7.7	−130	−465	−330	−215	−300	−245
AO	7.8[b]	−359	−351	−258	−212	−207	−310
AsO_2^--treated AO	7.7	−100	−470	ND[c]	ND	ND	ND

[a] All potentials are given in millivolts with reference to the standard hydrogen electrode. [b] Values obtained in 50 mM KP_i, containing 1 mM EDTA. [c] ND = not determined.

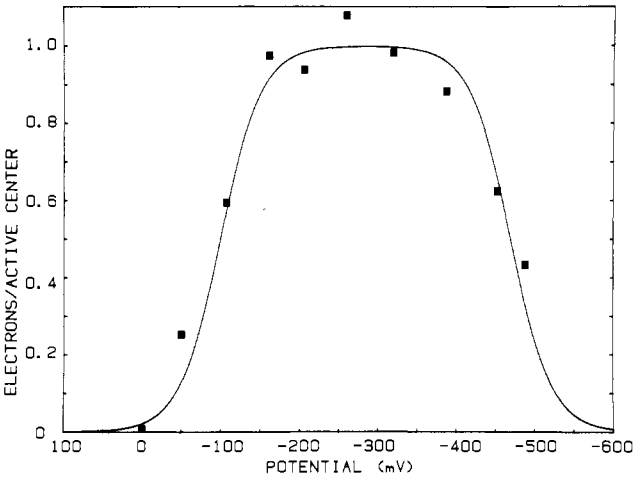

FIGURE 6: Results of potentiometric titration of the Mo center in arsenite-treated aldehyde oxidase. Aldehyde oxidase (12 μm functional sites) was incubated with arsenite as previously described. A potentiometric titration was then performed as outlined in Figure 5.

two Fe/S centers were −300 and −245 mV, respectively, again in good agreement with previous values.

The results of the effects of AsO_2^- treatment on the Mo redox potentials of AO are shown in Figure 6. As for xanthine oxidase, the oxidized enzyme exhibited only the signal referred to as resting II. However, reduction generated the arsenite spectrum, which reached a maximum at approximately −250 mV. Values obtained for the Mo couples in this enzyme and XO are given in Table II along with values obtained from control titrations.

The influence of AsO_2^- inactivation on the strong (dipolar) magnetic interaction between Mo(V) and reduced Fe/S I centers previously found in XO (Lowe & Bray, 1978), XDH, and AO (Barber et al., 1982a,b) was also examined with XO. Mo(V) EPR spectra recorded at 40 K for samples of the AsO_2^--inactivated XO poised at potentials of −227 and −353 mV, respectively, are shown in Figure 7. At these potentials, the calculated distribution of reducing equivalents is such that sample A would be expected to contain 93% Mo(V) and 13% reduced Fe/S I, while in contrast, sample B would contain 100% Mo(V) and 90% reduced Fe/S I. The Mo(V) spectrum obtained from the sample with increased Fe/S I reduction showed significant changes in line shape to that obtained from the high-potential sample, indicating the magnetic interaction between these two centers is not destroyed when the Mo center is complexed by arsenite.

Discussion

The precise mechanism of arsenite inactivation of the molybdenum-containing enzymes XO, XDH, and AO has not been rigorously established. In this work we have confined our investigations to the results of aerobic inactivation of the oxidized enzymes by AsO_2^-, comparing the EPR spectra and

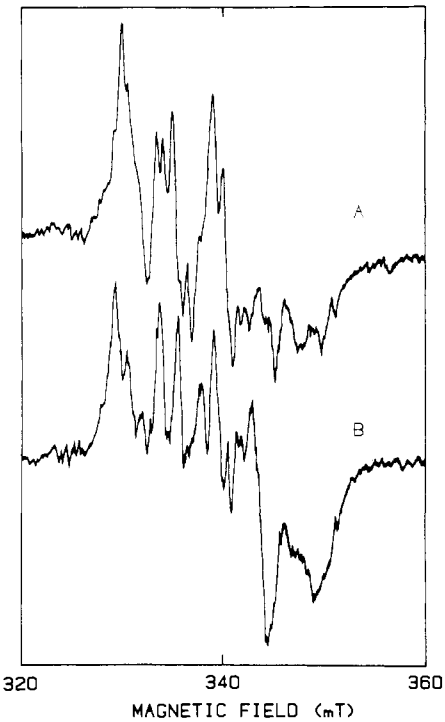

FIGURE 7: Magnetic interaction between Mo(V) and reduced Fe/S I in arsenite-treated xanthine oxidase. Samples were prepared as described in Figure 5. (A) Xanthine oxidase poised at −227 mV. (B) Enzyme poised at −353 mV. EPR spectra were recorded at 40 K by using 0.1-mW microwave power and 1-mT modulation amplitude.

redox potentials of the three enzymes and in addition, for XO, the effects of other modifications of the Mo center on the arsenite reaction product.

The Mo(V) EPR spectra obtained following reduction of the arsenite-inactivated enzymes unequivocally confirm the Mo center as the site of AsO_2^- binding. Although the spectra are broad, a well-resolved hyperfine interaction can be distinguished and separated into a number of four-line components by comparison with computer simulations. The coupling between the Mo(V) and the ^{75}As nucleus results in an essentially anisotropic interaction with coupling constants of the order of 1.1–2.1 mT. These coupling constants are not much greater than those encountered for proton hyperfine coupling in other Mo(V) species. Comparison of the g values of the arsenite signal with those of other species obtained for xanthine oxidase or other Mo-containing enzymes reveals some interesting differences. Apart from the transient species yielding the very rapid signal on limited reduction with xanthine, the magnitude of the separation between the low-field and high-field ($g_1 - g_3$) absorption lines is the largest ($\Delta g = 0.0629$) thus far encountered in these enzymes. This kind of comparison of changes in g-value separation may be conveniently displayed by using a g-value anisotropy diagram as shown in Figure 8. To construct this diagram we have used the data

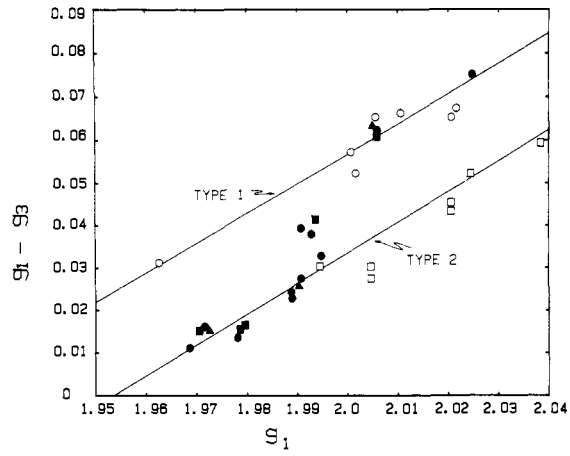

FIGURE 8: EPR *g*-value anisotropy diagram for Mo(V) signals from xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase. *g* values were obtained from the following sources: (●) xanthine oxidase, Bray (1980) and Barber & Siegel (1982b); (▲) xanthine dehydrogenase, Barber et al. (1980); (■) aldehyde oxidase, Barber et al. (1982a,b); (○, □) model complexes, Kikuchi et al. (1982).

for model complexes reported by Kikuchi et al. (1982). However, we have utilized parameters from an increased number of biological Mo(V) species in addition to the most recent *g*-value determinations available for all spectra. By plotting the difference in *g* value between the low- and high-field absorption lines as a function of the g_1 parameter, it is readily apparent that most Mo(V) signals from the enzymes examined thus far can be divided into two main groups. The first has been proposed by Kikuchi et al. (1982) to have octahedral structures and consists of the arsenite and very rapid species while the second, postulated to have square-pyramidal geometry, encompasses the majority of known signals from Mo-containing enzymes. This deviation of the arsenite and very rapid signals indicates a significant change or distortion in the structure of the ligation sphere about the Mo active site in these two complexes compared to the majority of the other biological Mo(V) signals. However, it should be noted that the overall g_{av} remains at 1.97, in good agreement with values obtained for other Mo(V) spectra.

Similar results, in terms of both *g*-value anisotropy and line shape, were obtained for both xanthine dehydrogenase and aldehyde oxidase in the complexed forms although the spectra are not completely identical. It should be noted that the spectra obtained from xanthine dehydrogenase in this investigation did not readily resemble signals previously published for this protein (Johnson & Rajagopalan, 1978).

In contrast to the majority of Mo(V) spectra previously examined, the arsenite-inactivated signal shows no coupling to exchangeable protons, not do the computer simulations require the inclusion of proton hyperfine coupling due to nonexchangeable protons to yield accurate spectra. Thus, binding of the As nucleus to the Mo center seems to involve arsenite binding to, or displacement of, one or more ligands in the native enzyme that are responsible for the proton hyperfine coupling observed in the rapid signal. It is clear that the Mo=S moiety of the native enzymes must be present for Mo–As hyperfine interaction to be detected. Binding of an alternate ligand, such as R—C=O to the Mo=S center prevents formation of the four-line Mo–As-type spectrum, although some type of arsenite–Mo interaction can still be detected in this as well as the Mo=O form of XO. M. J. Barber and L. M. Siegel (unpublished results) have also found that AsO_2^- binds to the Mo center of chicken liver sulfite oxidase, an enzyme with a functional Mo=O center. For this enzyme, shifts in the *g* value are seen, but no strong (four-line) splitting of the Mo(V) spectrum by the As nucleus could be detected.

The subtle changes in both the inhibited and slow signals from XO in the presence of arsenite suggests that prior modification of the Mo center does not preclude arsenite binding, although the hyperfine interaction is not observed. Simulations indicate that the latter spectral changes may be confined to alterations in the proton hyperfine coupling constants. Thus, we may propose that AsO_2^- binding to both these forms may be of the "anion" type that has previously been established to result in minor changes in the slow signal line shape (Gutteridge et al., 1978) as a result of small perturbations in the spatial arrangment of the ligands to Mo.

Modification of the Mo(V)–AsO_2^- spectrum by xanthine establishes that substrate binding to the Mo does not necessarily involve the Mo=S group, though catalytic activity does require this structure. Similar binding of xanthine or uric acid to desulfo enzyme has previously been demonstrated by Pick & Bray (1969).

The results of the potentiometric titrations of native XO and AO, following ligation of the Mo by AsO_2^-, are most significant. Arsenite modification of the Mo center results in a specific stabilization of the Mo(V) oxidation state as compared to the native enzyme. Both the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) potentials are affected, the former being shifted by approximately 250 mV more positive, while the latter is shifted approximately 100 mV more negative in both enzymes. Thus arsenite binds by a factor of 10 more tightly to Mo(V) than to Mo(VI) and by a factor of about 10 more tightly to Mo(V) than to Mo(IV). This is the first demonstration by direct potentiometry of a preferential effect on the Mo(V) oxidation state [although the Mo(V) state is clearly stabilized either kinetically or thermodynamically in the glycol-inhibited forms of XO (Lowe et al., 1976) and AO (Barber et al., 1982)]. However, reaction products such as uric acid (Barber & Siegel, 1982), anions such as borate (Barber & Siegel, 1982b), and the inhibitor, allopurinol (Massey et al., 1970), are known to increase the stability of the Mo(IV) species. Barber & Siegel (1982b) have compared the oxidation–reduction potentials for Mo in various Mo-containing enzymes and have shown them to fall into two main groups. The first, consisting of the Mo=O enzymes, sulfite oxidase and nitrate reductase, exhibits very positive potentials for both the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples, i.e., above 0 mV. It is of interest that arsenite binding to the Mo=O center of chicken liver sulfite oxidase does not result in significant shifts in the midpoint potentials of the Mo center in that enzyme (M. J. Barber and L. M. Siegel, unpublished results). The second, composed of the Mo=S enzymes, XO, XDH, AO, and formate dehydrogenase (the structure of the Mo center of this latter enzyme is presently unknown), exhibit substantially more negative values, around −350 mV. The results of arsenite complexation of XO and AO reported in this work suggests that a third group of potentials may be possible in which values intermediate between these two classifications may be placed.

Registry No. XO, 9002-17-9; XDH, 9054-84-6; AO, 9029-07-6; AsO_2^-, 17306-35-3; Mo(V), 22541-84-0; FADH, 1910-41-4.

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Potentiometric Titrations and Oxidation–Reduction Potentials of Several Iron Superoxide Dismutases†

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ABSTRACT: The iron-containing superoxide dismutases for *Escherichia coli*, *Azotobacter vinelandii*, and *Pseudomonas ovalis* have been titrated with reduced benzylviologen (via in situ coulometric generation) in the presence of a potentiometric monitoring electrode. On the basis of the titration data the midpoint potentials (E_m), the pH dependence of the latter, and the electron stoichiometries for the three proteins have been determined; these results are compared with previous evaluations for Cu/Zn- and Mn-containing superoxide dismutases. At pH 7 the average value of E_m for this group of proteins is +0.26 ± 0.04 V vs. normal hydrogen electrode (NHE). The reduction potential of the Fe-containing protein from *E. coli* decreases by 0.06 V/pH unit from pH 6 to pH 9 but apparently is independent of pH from pH 9.5 to pH 10.8. Hence, the redox reaction for this protein (and, presumably, all of the Fe–proteins) obeys the equilibria

$$E_{ox} + H^+ + e^- \rightleftharpoons HE_{red}$$
$$(E_m)_{pH7} = +0.27 \text{ V vs. NHE} \qquad pH < 9$$
$$E_{ox} + e^- \rightleftharpoons E_{red}$$
$$(E_m)_{pH10} = +0.12 \text{ V vs. NHE} \qquad pH > 9$$

Superoxide dismutases are metalloproteins which catalyze the disproportionation of superoxide ion (McCord & Fridovich, 1969):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

There are three classes of superoxide dismutases as distinguished by the metal ion required for catalysis of reaction 1: Cu, Mn, and Fe. The proteins are isolated as either dimers or tetramers of identical subunits that have molecular weights of 16 000–20 000 with each subunit binding an active metal ion. In general, the Cu–protein, which contains a Zn^{2+} that is not necessary for catalysis (Fee, 1981; Valentine & Pantoliano, 1981), has been isolated from the cytosol of eucaryotic cells while Mn–proteins have been isolated from both pro- and eucaryotic cells. The iron-containing protein has not been observed in mammalian cells and is generally associated with procaryotes, notably anaerobes; however, an iron-containing protein recently has been found in plant tissues (Bridges & Salin, 1981). The distribution, function, and properties of these proteins have been widely discussed (cf. Bannister & Hill, 1980; Bannister & Bannister, 1980).

The iron-containing superoxide dismutases, which normally have two identical 20 000-dalton subunits with one iron per subunit (a four-subunit protein has been reported; Kusonose et al., 1976), exhibit unique spectral and annulation properties (Slykhouse & Fee, 1976; Asada et al., 1975; Fee et al., 1981a). The optical spectrum is characterized by a broad band near 350 nm (ϵ_{Fe} = 1850 M^{-1} cm^{-1}) that is attributed to a ligand-to-metal charge-transfer band. The relatively high energy for this transition indicates that tyrosine is not a ligand to the Fe(III) (Gaber et al., 1974). The EPR spectrum (g_{av} = 4.3) is characteristically rhombic and has been shown to possess unusual magnetic properties (Emptage, 1981). Azide and fluoride act as inhibitors (Misra & Fridovich, 1978; Fee et al., 1981a) of the dismutase activity while cyanide is not an inhibitor nor does it bind to the Fe(III) (Asada et al., 1975; Slykhouse & Fee, 1976). Hydrogen peroxide rapidly destroys the catalytic activity, and this process has been used to dis-

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