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Oxidation–Reduction Potentials of Molybdenum, Flavin and Iron–Sulphur Centres in Milk Xanthine Oxidase

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1. The mid-point reduction potentials of the various groups in xanthine oxidase from bovine milk were determined by potentiometric titration with dithionite in the presence of dye mediators, removing samples for quantification of the reduced species by e.p.r. (electron-paramagnetic-resonance) spectroscopy. The values obtained for the functional enzyme in pyrophosphate buffer, pH 8.2, are: Fe/S centre I, -343 ± 15mV; Fe/S II, -303 ± 15mV; FAD/FADH$^{\cdot}$; -351 ± 20mV; FADH$^{\cdot}$/$FADH_2$, -236 ± 20mV; Mo(VI)/Mo(V) (Rapid), -355 ± 20mV; Mo(V) (Rapid)/Mo(IV), -355 ± 20mV. 2. Behaviour of the functional enzyme is essentially ideal in Tris but less so in pyrophosphate. In Tris, the potential for Mo(VI)/Mo(V) (Rapid) is lowered relative to that in pyrophosphate, but the potential for Fe/S II is raised. The influence of buffer on the potentials was investigated by partial-reduction experiments with six other buffers. 3. Conversion of the enzyme with cyanide into the non-functional form, which gives the Slow molybdenum signal, or alkylation of FAD, has little effect on the mid-point potentials of the other centres. The potentials associated with the Slow signal are: Mo(VI)/Mo(V) (Slow), -440 ± 25 mV; Mo(V) (Slow)/Mo(IV), -480 ± 25mV. This signal exhibits very sluggish equilibration with the mediator system. 4. The deviations from ideal behaviour are discussed in terms of possible binding of buffer ions or anti-co-operative interactions amongst the redox centres.

Xanthine oxidase is a complex enzyme containing molybdenum, FAD and two types of iron–sulphur centre, Fe–S I and Fe–S II. The enzyme molecule is presumed to be a dimer, each half containing one of each type of group. It is now believed that during the enzyme reaction reducing equivalents from a substrate such as xanthine are transferred to the molybdenum, and then become rapidly distributed between all the reducible centres within the half-molecule before being finally transferred to oxygen via the flavin. Thus the relative rates and extents of reduction of the various centres will depend critically on their mid-point reduction potential (Olson *et al.*, 1974; Bray, 1975).

Olson *et al.* (1974) investigated the relative mid-point potentials of iron–sulphur, flavin and molybdenum in xanthine oxidase by reducing samples with increasing amounts of sodium dithionite and measuring the extent of reduction of the centres by means of their optical absorption and e.p.r. (electron-paramagnetic-resonance) spectra. Fe–S I and Fe–S II give rise to e.p.r. signals in their reduced state, which can be distinguished by their *g* values and temperature-dependence (Lowe *et al.*, 1972). Flavin and molybdenum have no e.p.r. spectra in their oxidized [FAD and Mo(VI)] or fully reduced [$FADH_2$ and Mo(IV)] states, but give rise to signals in their half-reduced states [FADH$^{\cdot}$ and Mo(V)] (Bray *et al.*, 1959). By measuring the sizes of these signals together with the optical changes, as a function of the number of reducing equivalents added per enzyme molecule, it was possible to estimate the relative mid-point potentials of the centres, and Olson *et al.* (1974) found that the potentials are clustered fairly close together, within a range of 120mV.

In this paper we report direct measurements of the mid-point potentials of the centres in milk xanthine oxidase, by means of a different method. The enzyme was poised at a series of known redox potentials by reduction in the presence of mediators and a platinum electrode, and samples were taken for e.p.r. measurements. The absolute (rather than relative) values of the potentials that are obtained by this means are necessary for understanding the reaction of the enzyme with different substrates and electron acceptors, and for comparison with similar centres in other systems. The method also permits study of the extent of possible deviations from ideal behaviour, which might for example result from interactions amongst the centres.

In these experiments the molybdenum studied was that which gives rise to the Rapid e.p.r. signal, and which is present in the active enzyme. In

addition, samples not prepared by affinity chromatography (Edmondson *et al.*, 1972) give a Slow molybdenum e.p.r. signal, due to inactive enzyme molecules from which a sulphur atom is missing (desulpho enzyme; see Bray, 1975). Measurements were also made of the potentials of the centres in the enzyme which had been completely inactivated by removal of this sulphur atom, on treatment with cyanide (Massey & Edmondson, 1970), as well as in enzyme in which the flavin had been rendered non-functional by alkylation (Komai & Massey, 1971).

Potentiometric titration of various enzyme systems, by using techniques such as the ones used here, working in the presence of mediator dyes and with e.p.r. detection, have been used for several years in complex membrane-bound electron-transport systems, such as the mitochondrial respiratory chain (e.g. Wilson *et al.*, 1970; Ohnishi, 1973). Since xanthine oxidase is at present better understood than are any of these enzyme systems, we hoped that the work, apart from providing information on xanthine oxidase, would also yield information on the reliability of the method.

Experimental

Preparation of enzyme samples

Xanthine oxidase was purified from bovine milk by the salicylate-denaturation method of Hart *et al.* (1970). Activity/E_{450} ratios at 23.5°C of the samples used varied between 106 and 157. Enzyme concentrations in the potentiometric titrations were 38–75 μM (total enzyme), estimated from E_{450}, taking ε_{mM} = 72 litre·mmol^{-1}·cm^{-1} (Bray, 1975). Total active-centre concentration (functional plus non-functional) was taken as twice the enzyme concentration. The proportion of functional sites was estimated from the activity/E_{450} ratio (23.5°C, 295nm), taking the limiting value of the ratio for fully active enzyme as 197 (McGartoll *et al.*, 1970).

Preparation of desulpho enzyme

Desulpho xanthine oxidase was prepared (Massey & Edmondson, 1970) by treatment of the native enzyme with KCN. Xanthine oxidase (approx. 0.1mM-enzyme) in 55mM-pyrophosphate buffer containing 1mM-EDTA, pH8.2, was placed in a flask, leaving only a small air space. Solid KCN was added to a final concentration of 18–20mM, the flask was stoppered and the solution was stirred occasionally during 2h at about 20°C. The inactivated enzyme was then gel-filtered on a Sephadex G-25 column (2cm×30cm) and concentrated by pressure filtration (PM30 membrane, Amicon Ltd., High Wycombe, Bucks., U.K.). Final activity/E_{450} values were always less than 1.

Preparation of xanthine oxidase containing alkylated flavin

Xanthine oxidase containing alkylated flavin (Komai & Massey, 1971) was prepared by a modification of earlier methods (Bray & Watts, 1966; McGartoll *et al.*, 1970). Native enzyme (0.055mM-enzyme) in 0.1M-sodium phosphate buffer containing 1mM-EDTA and 1mM-salicylate, pH6.2, was placed in a modified Thunberg tube and made anaerobic by repeated evacuation and flushing with high-purity argon. Solid sodium dithionite (final concn. 1.67mM) and iodoacetamide (recrystallized three times from ethanol/water; final concn. 1.26mM) were added from the side arm and were dissolved by gentle shaking. The closed tube was left in the dark at about 20°C for 1.5h. The reaction was stopped by opening the tube and adding cysteine hydrochloride (10mM). The product was finally dialysed at 4°C against several changes of the same phosphate buffer, clarified by centrifuging at 20000rev./min at 4°C for 1h and concentrated by pressure filtration. The degree of alkylation of the enzyme was determined from E_{450} and E_{550} measurements (McGartoll *et al.*, 1970) by assuming the decrease in the ratio E_{450}/E_{550} to be linearly proportional to the percentage alkylation, with 100% alkylation giving 37% decrease (cf. Komai *et al.*, 1969). Alkylation does not affect xanthine dehydrogenase activity (measured with 2,6-dichlorophenol-indophenol by the method of Hart *et al.*, 1970). The percentage of functional active sites was estimated by taking the limiting activity/E_{450} (23.5°C, 600nm) value in this assay to be 152.

Measurement of e.p.r. signals

E.p.r. spectra were recorded on a Varian E9 spectrometer, as described by Lowe *et al.* (1972). Rapid and Slow Mo(V) and FADH· spectra were recorded at 118K with 5mW power, and the reduced Fe–S signals (I and II) were measured together at 25K and 1mW power. When necessary the g_1 feature of the Fe–S II signal was also examined at 25K and 100mW power. In some experiments the g_3 feature of the Fe–S I was measured at 45K and 10mW, conditions under which Fe–S II is too broad to interfere.

Quantification of e.p.r. signals

Double integration of the spectra, correction for differences of g values between the standard and the unknown and correction for failure to integrate all the $I = 5/2$ molybdenum hyperfine lines was as described by Barber *et al.* (1976). When it was necessary to measure both Rapid and Slow Mo(V) signals in the same sample, this was done from measurements of the β and γ peak heights (nomenclature of Palmer *et al.*, 1964), making reference to computed spectra, which were obtained by adding together in various proportions the

experimental pure Rapid and Slow spectra (Swann & Bray, 1972). For measurement of the Fe–S I and Fe–S II signals, two procedures were possible. They could either be measured separately from the amplitudes of the g_1 feature of Fe–S II (at 25K) and that of the g_3 feature of Fe–S I (at 45K). Alternatively, when it was desired to make the measurements from spectra recorded at a single temperature (25K), then the g_1 feature of Fe–S II was again measured, together with the amplitude of the combined g_3 peak resulting from overlap of the two signals in this region. This latter peak was then used to obtain the Fe–S I plus Fe–S II concentration by making reference to computer-added spectra, as for molybdenum. [For details of the calculated pure Fe–S II spectrum, see Lowe (1974) and Bray (1975).] The two procedures usually gave results in satisfactory agreement with one another, though it was noted that the linewidth of the Fe–S signals changed slightly from one buffer to another. Calculation of electrons per active centre detected in any e.p.r. signal was made by dividing the integrated intensity of that signal, calculated as above, by the appropriate active-centre concentration. Fe–S and FADH· signals were expressed relative to total active-centre concentration, Rapid Mo(V) signals relative to functional active-centre concentration and Slow Mo(V) signals relative to non-functional active centres. In most experiments, measurement of the Fe–S signals was simplified by making the assumption (Palmer & Massey, 1969) that full development of the signals, achieved at the end of the titration, corresponded to one electron on Fe–S I and one on Fe–S II.

Buffers

Redox titrations on the native enzyme were carried out in either 55mM-sodium pyrophosphate, pH8.2, or 100mM-Tris/HCl, pH8.2. Both buffers contained 1mM-EDTA and were adjusted with HCl. Buffers for other experiments were: (from Sigma, Kingston-upon-Thames, Surrey KT2 7BH, U.K.) Bicine [*NN*-bis-(2-hydroxyethyl)glycine], Tris, Epps [3-(*N*-2-hydroxyethylpiperazin-*N'*-yl)propanesulphonic acid], Taps {3-[Tris-(hydroxymethyl)methylamino]-propanesulphonic acid}, Caps [3-(cyclohexylamino)-propanesulphonic acid]; (from BDH, Poole, Dorset, U.K.) tetrasodium pyrophosphate, dipotassium hydrogen orthophosphate; (from Fluka A.G., Fluorochem Ltd., Glossop, Derbyshire SK13 9NU, U.K.) morpholine. All were adjusted with either HCl or NaOH.

Redox titrations

Oxidation–reduction potential titrations were carried out in an apparatus similar to that described by Dutton (1971). The sample (1–10ml) was stirred in a vessel maintained at 25°C. The redox potential was measured by a platinum electrode with a calomel reference (Radiometer P101 and K401). These were calibrated by using quinhydrone in standard buffers. The pH was measured in the vessel by a combined glass/calomel electrode (Radiometer GK2301B). The atmosphere was argon, which was purified by passing it slowly through a Nilox apparatus (Jencons Scientific Ltd., Hemel Hempstead, U.K.), containing alkaline sodium dithionite solution, and passed into the apparatus through stainless-steel tubing.

The redox potential of the system was adjusted with small additions of 0.1M-$Na_2S_2O_4$ (in 0.2M-Tris/HCl, pH9.0) or 0.2M-$K_3Fe(CN)_6$ solution. After equilibration for 1min, a sample of the enzyme was forced by gas pressure through a stainless-steel transfer tube into quartz e.p.r. tubes of known internal diameter (approx. 3mm) under Ar atmosphere, and frozen in liquid N_2. Between samples the transfer tube was purged with Ar gas.

The mediators present in the titration were divided into two groups. The first group consisted of: pyocyanine ($E'_0 = -60$mV) (K & K Laboratories, Plainview, NY, U.S.A.); indigotetrasulphonate ($E'_0 = -46$mV), anthraquinone-1,5-disulphonate ($E'_0 = -170$mV), safranine T ($E'_0 = -289$mV) (Ralph N. Emmanuel, Wembley, Middx., U.K.); 2-hydroxy-1,4-naphthoquinone ($E'_0 = -145$mV) (Koch–Light Chemicals, Colnbrook, Bucks., U.K.); anthraquinone-2-sulphonate ($E'_0 = -225$mV) and phenosafranine ($E'_0 = -255$mV) (Hopkin and Williams, Romford, Essex, U.K.). (Note that pyocyanin and safranine T are no longer available from the suppliers indicated.) The second group consisted of: Benzyl Viologen ($E'_0 = -311$mV), Methyl Viologen ($E'_0 = -440$mV) (BDH Chemicals, Poole, Dorset, U.K.); diquat ($E'_0 = -350$mV); triquat ($E'_0 = -540$mV) and *NN'*-dimethyl-3-methyl-4,4'-bipyridylium ($E'_0 = -617$mV). The latter three were kindly supplied by Dr. B. White, ICI Plant Protection Ltd., Bracknell, Berks., U.K.

For titration in the potential range where the flavin free radical was observed, the first group of mediators was added. Titrations with this mediator mixture in the absence of enzyme gave negligible free-radical signals under the conditions of measurement used in the range −100 to −380mV. The remaining mediators, which are Viologen dyes and give rise to free radicals in the reduced state, were added when the potential had been reduced by dithionite additions to below −380mV. The potential was adjusted back to −300mV at this stage, and the titration was then continued by addition of $Na_2S_2O_4$ solution. In some experiments, reversibility of the reduction was checked by titrating in the opposite direction with ferricyanide.

The total time taken for a titration in most cases was 60–90min. Xanthine oxidase is stable at pH8.2

Table 1. *Mid-point reduction potentials for xanthine oxidase*

Values were obtained by computer fitting of the titration data (see the Experimental section). Potentials are expressed in mV relative to the standard hydrogen electrode. The data are compared with the relative values of Olson *et al.* (1974) by assuming a mid-point potential of −303 mV for Fe/S II.

Sample conditions	Potential (mV)					
	Fe/S I	Fe/S II	Mo(VI)/ Mo(V) Rapid	Mo(V) Rapid/ Mo(IV)	FAD/ FADH·	FADH·/ $FADH_2$
Native enzyme in 55 mM-pyrophosphate, pH 8.2	−343	−303	−355	−355	−351	−236
Data of Olson *et al.* (1974)	(−327)	(−303)	(−363)	(−334)	(−363)	(−243)
Native enzyme in 100 mM-Tris/HCl, pH 8.2	−336	−255	−397	−405	−378	−223
Desulpho enzyme (CN^--treated) in 55 mM-pyrophosphate, pH 8.2	−340	−300	−440*	−480*	−344	−237
Deflavo enzyme (alkylated) in 55 mM-pyrophosphate, pH 8.2	−327	−288	−368	−385	—	—

* Slow molybdenum signal.

and assay of the solution in the vessel after titration confirmed that the enzyme retained at least 90% of its activity. Small losses were accounted for partly by dilution by titrant.

Analysis of titration data

The plots of signal intensity against redox potential were displayed on a Nicolet 1020A digital oscilloscope (Nicolet Instrument Corp., Madison, WI, U.S.A.) interfaced to a Hewlett–Packard 9830A calculator (Hewlett–Packard Corp., Palo Alto, CA, U.S.A.). Theoretical curves for the behaviour of singly reduced species and semiquinone forms (Clark, 1960) could be plotted on the display, and the parameters were adjusted to give the best fit to the data, as judged by eye and concentrating on fitting at the peak for Mo(V) and FADH·. Because of deviations of the data from theoretical behaviour, which are discussed later, this procedure was preferred to statistical methods of curve-fitting.

Reduction with salicylaldehyde and calculation of redox potentials from the observed electron distribution

Xanthine oxidase in NaCl solution was diluted with one of a series of buffers, at pH 8.2. Salicylaldehyde was added anaerobically to duplicate samples, which were frozen after 1.5 min, and the e.p.r. spectra were recorded. Final concentrations were: xanthine oxidase (functional active centres), 0.20 mM; salicylaldehyde, 0.19 mM; buffer, 100 mM; NaCl, 100 mM; EDTA, 0.5 mM. Under these conditions, it is expected that only functional enzyme will be reduced and that much of this will be transformed to the two-electron-reduced species. If the redox potentials of the centres are known, the electron distribution in this species may readily be calculated from the equations given by Olson *et al.* (1974). (Note that the equations stated by these workers to apply to the one-electron-reduced species actually apply to the two-electron form.) A computer (PDP 11/10 computer, Digital Equipment Co.) was used to calculate the electron distribution on the two-electron-reduced species, for a series of different assumed potentials, starting from the average values from the redox titrations (Table 1) and varying them until the best fit was obtained for each buffer between the calculated electron distributions and the observed signal intensities.

Results

Mid-point potentials for the active enzyme in pyrophosphate buffer

The intensities of the e.p.r. signals of the various centres are plotted as a function of redox potential in Fig. 1. The buffer used in this case was 55 mM-sodium pyrophosphate containing 1 mM-EDTA, pH 8.2, which has been widely used in e.p.r. studies on xanthine oxidase.

Iron–sulphur centres I and II each showed an apparent single reduction process. The curves through the points (Figs. 1*a* and 1*b*) were calculated for a one-electron reduction ($n = 1$). The radical due to FADH· (Fig. 1*c*) shows an increase corresponding to FAD → FADH· (mid-point potential E_1), then a decrease corresponding to FADH· → $FADH_2$ (mid-point potential E_2). The curve fitted to these points corresponds to two one-electron-reduction processes. The maximal intensity of this curve corresponds to only 5% of the total flavin. The difference between potentials E_1 and E_2 determines the value of this peak (Clark, 1960), and in this case E_2 is more positive than E_1 by about 115 mV. The signal due to Rapid Mo(V) (Fig. 1*d*) shows similar behaviour. The curve plotted in this case assumes that $E_1 = E_2$, corresponding to 33% maximum conversion into Mo(V).

The estimates of the mid-point potentials of the centres used for deriving the curves in Fig. 1 are summarized in Table 1. These values show good agreement, to within experimental error, with the

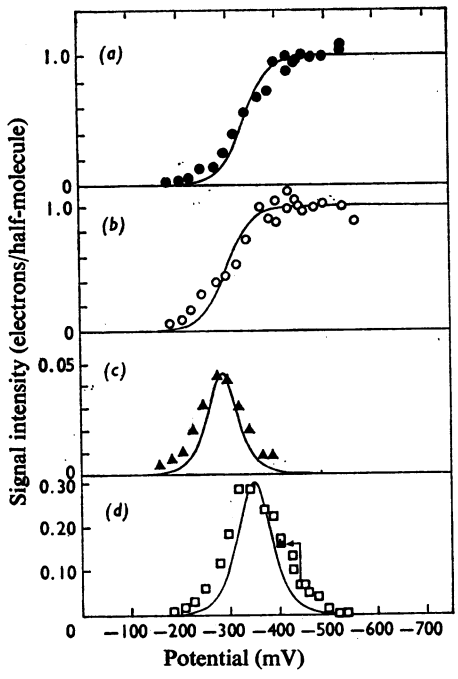

Fig. 1. *E.p.r.-signal intensities versus redox potential for native xanthine oxidase in 55mM-pyrophosphate, 1mM-EDTA at 25°C*

(*a*) Fe–S I; (*b*) Fe–S II; (*c*) FADH·; (*d*) Rapid Mo(V). Samples were prepared and e.p.r.-signal intensities measured as described in the Experimental section. The curves are calculated (*a*) and (*b*) for one-electron-reduction processes and (*c*) and (*d*) for a semiquinone (Clark, 1960). The arrow in (*d*) indicates the partial re-oxidation when the second group of mediators was added (see the Experimental section) and the filled point in the next sample taken.

values of Olson *et al.* (1974) if it is assumed that the mid-point potential of Fe–S II, relative to which all their values were calculated, is −303mV. This provides independent confirmation of the reliability of our method. In this connexion it is particularly important to note that Olson *et al.* (1974) did not use mediator dyes.

The uncertainty in measurements of redox potentials, due to electrode errors, curve-fitting etc. for simple redox curves such as Fe–S I and Fe–S II, is probably about ±15mV, though reproducibility from one experiment to another was generally better than this. There is an additional uncertainty in the error for the semiquinone types of curves from FADH· and Mo(V), since the value of (E_2-E_1) depends on the integrated e.p.r.-signal intensity. An uncertainty of ±15% in the estimation of the maximal signal intensity leads to uncertainty in (E_2-E_1) of ±7mV for FADH· and ±10mV for Mo(V); therefore, although the mid-point potential $E_m=(E_2+E_1)/2$ can be estimated with the same error as the iron–sulphur redox curves, ±15mV, the errors in E_1 and E_2 are of the order of ±20mV.

The experimental points in Fig. 1 seem to follow a somewhat broader line than the theoretical curves. For example the points for Fe–S I and Fe–S II indicate a line of less steep slope, as though the value of n was less than unity. Similarly the points for FADH· and Rapid Mo(V) are spread over a wider range of potentials than the calculated curve. It is not possible in these cases to obtain a better-fitting curve by increasing the value of E_2-E_1; this merely increases the peak height of the curve while producing little effect on its width, even if the height is permitted to go substantially above the experimental value.

This effect, which is clearly a deviation from ideal reduction behaviour, was repeatedly observed with native enzyme, and with desulpho enzyme and alkylated flavin enzyme (see below). Similar results were obtained whether titrating to negative potentials with dithionite or more positive with ferricyanide (see filled point on Fig. 1*d*), showing that it is not due to slow equilibration of the system. However, the effect was less noticeable in Tris buffer than pyrophosphate (see below).

Effect of mediator concentration

It has been reported (Gurtoo & Johns, 1971) that the dye mediator 2,6-dichlorophenol-indophenol can form a strong complex with xanthine oxidase; this dye was not used in the present studies, but it was possible that other dyes might bind to the enzyme under the conditions of titration and thereby modify the mid-point potentials of the centres. The mediator concentration should therefore not be too high, but on the other hand, too low a concentration of mediators might result in incomplete equilibration between the enzyme and the platinum electrode.

Redox titrations were carried out with all mediators at concentrations of 10μM and 100μM The concentration of the enzyme was about 60μM, i.e. the total active-site concentration (functional plus non-functional) was 120μM. The buffer in this experiment was 100mM-Tris/HCl buffer, pH8.2. No significant difference was observed in the behaviour of iron–sulphur, flavin or molybdenum signals at the two mediator concentrations, though, as noted below, behaviour was different from that in pyrophosphate. Fig. 2 shows a comparison of the titration curves for Fe–S I. In all other experiments, unless otherwise stated, the mediators were all used at a concentration of 30μM, so that the concentration of any one mediator was less than that of the enzyme. Further confirmation that there was no reversible binding of the dyes was provided by the finding that

the enzyme could be recovered by gel filtration, without change of visible absorption spectrum, at the end of the titrations.

Studies with additional buffers

The oxidation–reduction titrations of Fig. 1 were repeated in 100mM-Tris/HCl containing 1mM-EDTA, pH8.2. The potentials obtained are summarized in Table 1. Two differences are apparent between the results and those in pyrophosphate buffer (Fig. 1): (i) the potentials, particularly of Fe–S II and Rapid Mo(V), have shifted, the former to more positive and the latter to more negative potentials; (ii) the points appear to fit the shape of the theoretical curves rather more closely. This is illustrated for Fe–S I in Fig. 2 and for FADH• in Fig. 3.

In view of the substantial differences found between the potentials measured in Tris and pyrophosphate buffers, it was decided to carry out additional studies with other buffers. Instead of carrying out complete titrations, however, a series of samples of enzyme were reduced anaerobically with controlled small amounts of the substrate salicylaldehyde. This substrate was selected, since, unlike purine substrates, it does not give rise to a modified Rapid signal as a result of complex-formation between the substrate or product and the reduced enzyme molecules (Bray & Vänngård, 1969; Pick & Bray, 1969). Results are presented in Fig. 4 as histograms showing the relative intensities of the e.p.r. signals with the different buffers. Clearly, the nature of the buffer has a marked effect on the system.

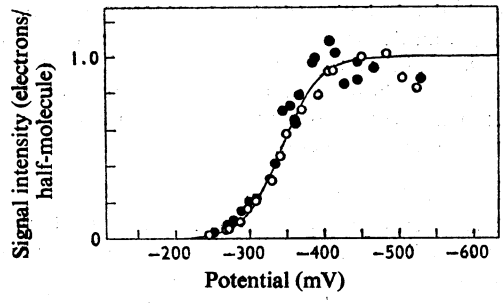

Fig. 2. *Effect of mediator concentration on the reduction of Fe–S I*

Xanthine oxidase was in 100mM-Tris/HCl, pH8.2. Experimental points: ○, mediators all at 10μM concentrations; ●, mediators all at 100μM concentrations.

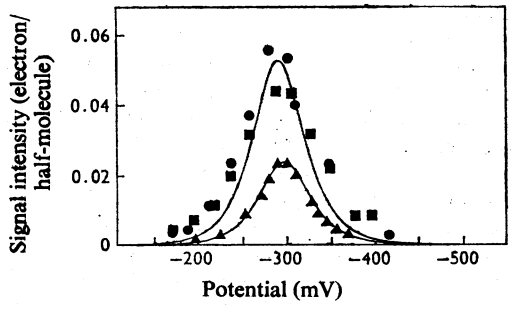

Fig. 3. *Redox behaviour of the FADH• signal*

Experimental points: ■, native xanthine oxidase in 55mM-pyrophosphate/1mM-EDTA, pH8.2; ●, desulpho enzyme in pyrophosphate; ▲, native enzyme in 100mM-Tris/HCl/1mM-EDTA, pH8.2.

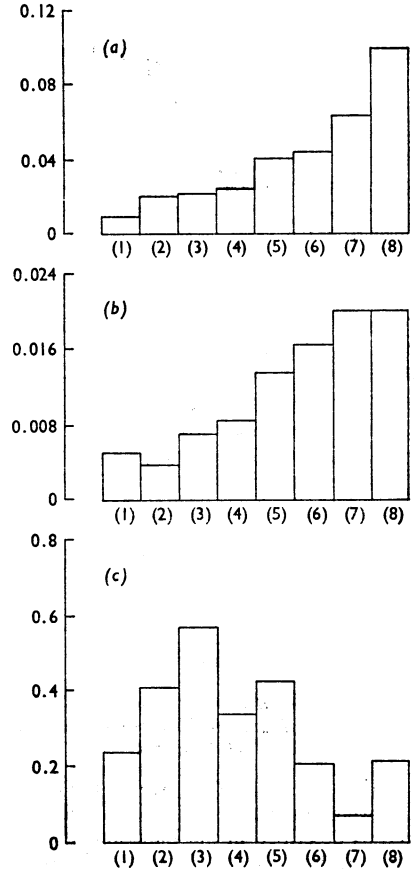

Fig. 4. *E.p.r. signal intensities of xanthine oxidase reduced with salicylaldehyde in various buffers*

Conditions of reduction were as described in the Experimental section. Histograms show the number of electrons per xanthine oxidase half-molecule on (*a*) Rapid Mo(V); (*b*) FADH•; (*c*) Fe–S I plus Fe–S II. The buffers used were: (1) Taps; (2) Tris/HCl; (3) morpholine; (4) Epps; (5) Bicine; (6) Caps; (7) orthophosphate; (8) pyrophosphate.

To compare results obtained with the salicylaldehyde method with those obtained in the potentiometric titrations, it is necessary to remember (cf. Olson *et al.*, 1974) that, when dithionite reduces the enzyme (at least in the presence of mediators), electrons are expected to be distributed on to the different reducible centres in the enzyme molecules according to a pattern determined by the redox potentials. Further, there will also be a distribution, which again is determined by the redox potentials, among xanthine oxidase molecules (or more strictly half-molecules) which have accepted, 1, 2 . . . 6 reducing equivalents. In contrast with this situation, when low amounts of salicylaldehyde are used to reduce the enzyme, only species with even numbers of electrons will be produced. For 1.9 reducing equivalents from salicylaldehyde per enzyme molecules, and assuming the potentials in Table 1, we would expect the distribution in pyrophosphate to be: oxidized enzyme, 24%; $2e^-$-reduced species, 59%; 4^--reduced species, 17%; $6e^-$-reduced species, 0.2%. This may help to explain the very low amounts of FADH· radical detected, since, of all those species, only the $2e^-$-reduced species has substantial amounts of FADH·.

A computer procedure was used (see the Experimental section) to calculate redox potentials in each buffer for the various centres in the enzyme, from the data of the experiment in Fig. 3. It should be remembered that the calculations assume ideal redox behaviour for the centres, which is strictly not the case, for the enzyme in pyrophosphate at least.

The potentials in pyrophosphate and in Tris calculated from the salicylaldehyde data agreed reasonably well with those from the potentiometric titrations. For the other buffers, the calculations suggested (in agreement with the data on Tris and pyrophosphate in Table 1) that the buffers were exerting their effects on electron distribution principally by affecting the molybdenum and Fe–S II potentials.

Effect of modification of molybdenum and FAD

In addition to the results of titrations of the native enzyme in pyrophosphate and Tris buffers, Table 1 also presents results obtained from desulpho xanthine oxidase, from which the active 'persulphide' group in the vicinity of molybdenum has been removed by cyanide treatment. This enzyme can no longer be reduced by substrates such as xanthine, and the molybdenum signals in this case are entirely of the Slow type. The 'alkylated flavin' enzyme is treated with iodoacetate, which modifies the FAD, and converts the enzyme from an oxidase into a dehydrogenase capable of interacting with artificial acceptors only (McGartoll *et al.*, 1970; Komai & Massey, 1971). No FADH· radical signals were observed in this case.

Table 1 shows that modification of the environment of the molybdenum atom does not significantly affect the mid-point potentials of iron–sulphur or flavin; nor does modification of the flavin greatly affect the potentials of molybdenum or iron–sulphur. The centres are presumably at some distance from each other within the enzyme molecule (cf. Bray, 1975).

The Slow molybdenum signal was observed together with the Rapid signal in titration of normal enzyme, and its behaviour appeared to be much less ideal than those of the other centres. In the cyanide-treated enzyme, it was found that equilibration of the Slow molybdenum signal with the mediator system was much slower than reduction of iron–sulphur and flavin groups. After changing the potential of the system, the molybdenum signal intensity continued to change for up to 30min, whereas changes in Fe–S signal size were settled in less than 1min.

Fig. 5 shows the behaviour of the Slow Mo(V) signal of desulpho xanthine oxidase during titration of the cyanide-treated enzyme in pyrophosphate buffer. To ensure that equilibration was complete, a mediator concentration of 100μM was used, and the potential was held constant for 30min before the samples shown were taken. Clearly, lower potentials are required for development of this signal than for Rapid. The Slow intensity deviates from a theoretical titration curve in much the same way as does the Rapid signal. The considerable deviations from ideal behaviour that were observed in previous titrations under our standard conditions were seen as being due to the fact that the molybdenum had not come into equilibrium with the mediator system before samples were taken.

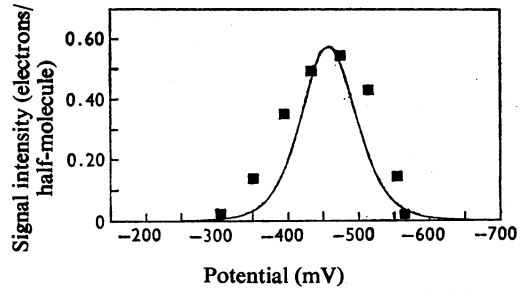

Fig. 5. *Reduction of Slow Mo in desulpho enzyme*

The titration was carried out in the presence of 100μM mediators, and the system was allowed to equilibrate for 30min at each potential before samples were taken.

Discussion

Redox behaviour of the functional enzyme: buffer effects

Behaviour of the four redox-active centres in functional xanthine oxidase in Tris buffer calls for little comment. Behaviour is essentially ideal, within the limits of experimental error.

The effects of different buffer ions on the system are, however, both quite striking and rather complex. Presumably some of these ions must be capable of binding to the enzyme. The complex nature of the results in Fig. 3 implies more than one binding site for them. Both the computing from the salicylaldehyde-reduction data and a comparison of the titrations in Tris and in pyrophosphate show that the molybdenum and Fe–S II centres are most strongly affected. It is possible that buffer ions might bind to molybdenum directly. Many Mo(V) compounds have a weakly bound anion as a ligand in the position *trans* to their molybdenum oxygen, and the presence of such a structure is quite possible in the active centre of xanthine oxidase (Bray, 1975). Selective binding by an anion when the metal was in the quinquivalent state would cause a raising of the Mo(VI) Mo(V) potential and a corresponding increase in the Rapid signal intensity observed on two-electron reduction of the enzyme. It is particularly noteworthy that pyrophosphate buffer, which has been used for most published e.p.r. work on xanthine oxidase (e.g. Palmer *et al.*, 1964; Bray & Vänngard, 1969; Olson *et al.*, 1974), gives the largest Mo(V) signals.

If buffer ions are bound to molybdenum in the e.p.r.-signal-giving form, then some effects of these, if only slight ones, on the form of the e.p.r. spectra would be expected. Although we have not specifically looked for such effects on the Rapid signal in the present work, it has been found (R. T. Pawlik & R. C. Bray, unpublished work) that the form of the Slow Signal observed on dithionite reduction does indeed change slightly on varying the nature of the buffer. Further work (particularly at 35 GHz and in ^2H_2O) will be needed to see if some of the complexities of the Rapid signal (Bray & Vänngard, 1969) are due to a mixture of signal-giving species, with and without bound buffer ions.

There is also the possibility, particularly in relation to Fe–S II, that buffer ions can influence the potentials of the centres by binding to distant sites and causing conformational changes. It has been found (R. Cammack, unpublished work) that pyrophosphate buffer causes a significant shift in the potential of the Fe–S centre in spinach ferredoxin.

In relation to the buffer effects of Fe–S II, it is notable that the potentials of Fe–S I and Fe–S II are much better separated in Tris than in pyrophosphate buffers. This explains why Orme-Johnson & Beinert (1969) were readily able to distinguish the e.p.r. spectra of Fe–S II and Fe–S I by reductive titration of xanthine oxidase by dithionite. On the basis of the experiments of Olson *et al.* (1974), it appears to be difficult to obtain a spectrum of reduced Fe–S II without a more substantial signal from Fe–S I than the one earlier reported. In fact, Orme-Johnson & Beinert (1969) used Tris, whereas Olson *et al.* (1974) used pyrophosphate.

The non-ideal behaviour of the enzyme on titration in pyrophosphate buffer, which led to an apparent value of n of less than 1, calls for some comment. There are at least two possible explanations for this behaviour: (i) there may be heterogeneity in the enzyme molecules, e.g. as a result of binding of buffer molecules, which causes each type of centre to have a range of different mid-point potentials; (ii) negative co-operativity in the reduction of centres, so that reduction of one centre in the molecule makes the potential of other centres more negative. Though the second explanation may play a part, it seems likely that most deviations of the functional enzyme from ideal behaviour are due to buffer binding. It must also be remembered that e.p.r. measurements are made on frozen solutions, and these effects may be accentuated by the higher local concentrations of buffer ions encountered by some of the enzyme molecules during freezing.

Redox behaviour of molybdenum in the desulpho enzyme

Our experiments throw new light on the reduction process of the Slow molybdenum signal of desulpho xanthine oxidase. Olson *et al.* (1974) concluded that this species was Mo(III), on the basis that it required more than six reducing equivalents of dithionite per enzyme molecule before the Slow molybdenum signal was observed. On the other hand the Slow signal is definitely given by one-electron-reduced desulpho xanthine oxidase, irrespective of whether this is obtained by reduction of the oxidized enzyme by using the hydrated electron in pulse radiolysis, or by reoxidation of the fully reduced enzyme by oxygen (Bray *et al.*, 1975; R. C. Bray & M. J. Barber, unpublished work). Hence the Slow signal is clearly due to Mo(V). Moreover, prolonged reduction of desulpho enzyme with dithionite (at a concentration of 28 mM for 10 h in the absence of mediators) causes a further reduction of the molybdenum to a diamagnetic state, and this change can be reversed on reoxidation with oxygen (Bray *et al.*, 1975). The present experiments, in which the reduction is facilitated by the presence of redox mediators, confirm these results and show that the molybdenum giving the Slow signal undergoes reductions at potentials lower than those of the molybdenum giving the Rapid signal.

Unlike the other centres in the enzyme, and the

molybdenum in active system, the 'Slow' molybdenum appears to equilibrate slowly with the mediator system. The same mediators have been used on other systems with apparently satisfactory results, down to potentials lower than those used here (Evans *et al.*, 1974). From the point of view of the application of the technique, this result shows that it is not valid to assume that all centres in an enzyme come into equilibrium with the mediator system equally rapidly.

The slow reduction of the 'Slow' molybdenum under these conditions does not necessarily imply that there cannot be rapid electron interchange between it and the other centres in the same molecule. In the absence of mediators, dithionite reacts with the enzyme predominantly at the flavin site (M. J. Barber & R. C. Bray, unpublished work) (see also Bray *et al.*, 1975). Assuming a similar mechanism when mediators are present, slow reduction of molybdenum would simply be a consequence of its potential being much lower than that of flavin since at low potentials oxidized FAD would only rarely become available for uptake of the final reducing equivalents. It may be noted that Bray *et al.* (1975) obtained evidence for interactions amongst the centres in the desulpho enzyme which are not apparent in the functional enzyme. However, further work on this problem may be required.

Reduction of xanthine oxidase by NADH

Since the redox potential of the system NAD^+/NADH is accurately known (Clark, 1960), partial reduction of xanthine oxidase and of the desulpho enzyme by NADH should provide a valuable check on the potentials determined in the titration studies. NADH reduces the enzyme, though more slowly than does xanthine (Olson *et al.*, 1974). It reacts at the flavin site and therefore reacts equally readily with either the functional enzyme or the desulpho form and mediators are not required. Swann & Bray (1972) gave data on the extent of e.p.r.-signal development on reduction of the enzyme with NADH. With the moderate excess of the reducing agent used by these workers, a potential of around −380mV is expected. Comparison with the present data (Fig. 1) reveals that, at this potential, it would be expected that FAD would be mainly reduced to the $FADH_2$ level and that both Fe–S centres would be essentially fully reduced. The Rapid signal would be expected to be approximately maximally developed, and there would be substantial, but by no means maximal development, of the Slow signal (see Fig. 5). This situation is in fact just what was reported by Swann & Bray (1972). Although these workers used morpholine buffer, their data provide further support for the general reliability of the potentials given in Table 1.

Comparison of the potentials with those in other systems

Though the substantial decrease in the potential of the molybdenum of xanthine oxidase, which accompanies conversion into the desulpho form, is clearly of interest, there seem at the moment to be inadequate data available to compare the potential of molybdenum in this enzyme with that of the metal in other molybdenum-containing enzymes.

The potentials of the two Fe/S systems in the enzyme are within the range of those commonly encountered in other Fe/S proteins, typically −240 to −420mV (Hall *et al.*, 1975). However, the flavin potentials call for more comment. It is interesting to compare the potentials for the two flavin systems (FAD/$FADH^\cdot$ and $FADH^\cdot/FADH_2$) in xanthine oxidase with that of the flavin of free FMN (Draper & Ingraham, 1968), of flavodoxins (Mayhew *et al.*, 1969; Barman & Tollin, 1972) and of turkey liver xanthine dehydrogenase (R. Cammack, M. P. Coughlan, M. J. Barber & R. C. Bray, unpublished work). For addition of the first electron, at pH8.2, xanthine oxidase and dehydrogenase have similar potentials, which are distinctly lower than that of free flavin (about −275mV), whereas flavodoxins have higher potentials. On the other hand, for the second reduction step, xanthine oxidase and free flavin show similar potentials to one another. Xanthine dehydrogenase has about the same potential as the oxidase for the first step, but a low potential, similar to that of flavodoxin from *Peptostreptococcus elsdenii*, for the second. There is no information at the moment as to what structural aspects of the flavin environment in the enzymes determine these potentials.

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