Oxidation–Reduction Potentials of Turkey Liver Xanthine Dehydrogenase and the Origins of Oxidase and Dehydrogenase Behaviour in Molybdenum-Containing Hydroxylases

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(Received 29 September 1976)

Redox potentials for the various centres in the enzyme xanthine dehydrogenase (EC 1.2.1.37) from turkey liver were determined by potentiometric titration in the presence of mediator dyes, with low-temperature electron-paramagnetic-resonance spectroscopy. Values at 25°C in pyrophosphate buffer, pH 8.2, are: Mo(VI)/Mo(V) (Rapid), -350 ± 20 mV; Mo(V) (Rapid)/Mo(IV), -362 ± 20 mV; Fe–S I_{ox.}/Fe–S I_{red.}, -295 ± 15 mV; Fe–S II_{ox.}/Fe–S II_{red.}, -292 ± 15 mV; FAD/FADH•, -359 ± 20 mV; FADH•/FADH₂, -366 ± 20 mV. This value of the FADH•/FADH₂ potential, which is 130 mV lower than the corresponding one for milk xanthine oxidase [Cammack, Barber & Bray (1976) *Biochem. J.* **157**, 469–478], accounts for many of the differences between the two enzymes. When allowance is made for some interference by desulpho enzyme, then differences in the enzymes' behaviour in titration with xanthine [Barber, Bray, Lowe & Coughlan (1976) *Biochem. J.* **153**, 297–307] are accounted for by the potentials. Increases in the molybdenum potentials of the enzymes caused by the binding of uric acid are discussed. Though the potential of uric acid/xanthine (-440 mV) is favourable for full reduction of the dehydrogenase, nevertheless, during turnover, for kinetic reasons, only FADH• and very little FADH₂ is produced from it. Since only FADH₂ is expected to react with O₂, lack of oxidase activity by the dehydrogenase is explained. Reactivity of the two enzymes with NAD⁺ as electron acceptor is discussed in relation to the potentials.

Cammack *et al.* (1976) have reported the redox potentials of the molybdenum, flavin and iron-sulphur centres (Fe–S I and Fe–S II) in milk xanthine oxidase (EC 1.2.3.2). These were measured by using potentiometric titration in the presence of mediator dyes, determining the extent of reduction at each potential by removing samples for low-temperature e.p.r.* spectroscopy. The present paper reports comparable studies on a closely related enzyme having a markedly different acceptor-specificity pattern, namely xanthine dehydrogenase from turkey liver (Cleere & Coughlan, 1975). In earlier work on the turkey liver enzyme (Barber *et al.*, 1976), stoichiometric e.p.r. titration with xanthine had shown much higher conversion of flavin in this enzyme into the FADH• semiquinone than was attained with the milk enzyme. This led us to suggest that the low oxidase activity in the turkey enzyme is due to the

* Abbreviation: e.p.r., electron paramagnetic resonance.

difficulty of reducing its flavin from the semiquinone to the fully reduced state. Similarly, additional, but less-detailed, work by Bray *et al.* (1976) and by Waud & Rajagopalan (1976) has indicated that, for certain molybdenum-containing hydroxylases, the dehydrogenase (type D; NAD⁺-specific) forms yield much more FADH• on reduction by substrates than do the corresponding oxidase (type O) forms into which the enzymes may specifically be converted (Stirpe & Della Corte, 1969; see also Bray, 1975). It was therefore of particular interest to obtain actual redox potentials for the systems FAD/FADH• and FADH•/FADH₂ in turkey liver xanthine dehydrogenase and to compare these with the corresponding values for milk xanthine oxidase, as well as with the redox potentials of the oxidizing and reducing substrates of the two enzymes. It was hoped that such work might help to explain the origins of acceptor-specificity differences among the molybdenum-containing hydroxylases.

Materials and Methods

Potentiometric titrations

Turkey liver xanthine dehydrogenase was purified and assayed by a modification of the method of Cleere & Coughlan (1975). Potentiometric titrations were carried out as described by Cammack *et al.* (1976) and other procedures were as described by Barber *et al.* (1976). In order to observe the FADH⁺ e.p.r. signal in the titrations, addition of Viologen dyes was delayed until the potential had been lowered to -430 mV. After their addition, the potential was again raised, to about -370 mV, to check that equilibrium had been reached for the other signals. All titrations were in 50 mM-sodium pyrophosphate buffer containing 1 mM-EDTA and adjusted to pH 8.2 with HCl. The concentration of enzyme in the titrations was about 30 μ M (total enzyme) and the concentration of each of the mediators was 30 μ M. The percentage of functional active sites varied from 23 to 44. The remainder of the enzyme is expected to be almost exclusively in the desulpho form. Quantification of the various e.p.r. signals was as described by Cammack *et al.* (1976), except that the Fe-S I signal was generally measured from the g_2 feature at 25 K. There is little interference from Fe-S II signal in this region. Additional measurements at 50 K confirmed the validity of this procedure.

Redox potential for uric acid/xanthine

Equimolar quantities of deoxygenated solutions of xanthine and uric acid in the usual pyrophosphate buffer, pH 8.2, together with Methyl Viologen, diquat and triquat [see Cammack *et al.* (1976) for suppliers] as mediators, were added to the potentiometric titration cell and the system was made anaerobic by purging with Ar. A small amount of milk xanthine oxidase was added. The potential stabilized after a few minutes and was then measured. The quantity of enzyme used was such that significant conversion of xanthine into uric acid was not expected to occur. The observed potential is therefore the redox potential for uric acid/xanthine. The experiment was repeated with xanthine dehydrogenase from *Veillonella alcalescens* (Dalton *et al.*, 1976).

Calculation of electron distribution in reduced enzyme species and calculation of theoretical titration curves for xanthine reduction

Electron distributions on to the various centres in reduced species of xanthine oxidase and xanthine dehydrogenase which had accepted different numbers of reducing equivalents per half-enzyme molecule (denoted by the symbols XO_{1e} , XO_{2e} , ..., XO_{6e} and XD_{1e} , XD_{2e} , ..., XD_{6e}) were calculated from the redox potentials by using the procedures and

equations of Olson *et al.* (1974). Macroscopic electron-affinity constants and the corresponding macroscopic redox potentials were also calculated similarly for the oxidized enzyme and for each of the reduced molecular species.

The procedure for simulating the titrations with xanthine, in which enzyme species containing odd numbers of electrons are not expected to participate, was as follows. A series of applied potentials were assumed. Then, for instance, at a potential of E mV, it may readily be shown that the fractional conversion of xanthine oxidase into form XO_{4e} is equal to $1/(pq+q+1+1/r)$, where $p = k^2/L_1L_2$, $q = k^2/L_3L_4$ and $r = k^2/L_5L_6$. In these expressions, L values are the macroscopic affinity constants of Olson *et al.* (1974) and k is given by $k = \text{antilog}(E/60)$. [Note that for some comparative purposes Olson *et al.* (1974) divided L values by statistical factors, but this was not appropriate here.] By using the above and similar expressions, the fractional conversion of the enzymes into the forms XO_0 , XO_{2e} , XO_{4e} , XO_{6e} (and similarly for xanthine dehydrogenase) could be calculated for any chosen value of the applied potential, E . The uric acid/xanthine ratio at each value of E could also be calculated, from the known redox potential (see below) of this system. Then to obtain the total conversion, for example, of molybdenum in the enzyme into Mo(V) at the potential E , it was simply necessary to add together the amounts of Mo(V) contributed by the fractions of forms XO_{2e} and XO_{4e} known to be present at this potential [forms XO_0 and XO_{6e} do not contain Mo(V)]. Similarly, the total number of electrons taken up by the enzyme on reduction to this potential was given by 2 times the concentration of form XO_{2e} plus 4 times the concentration of XO_{4e} plus 6 times the concentration of XO_{6e} . This total must equal twice the concentration of uric acid formed, since xanthine is a two-electron reducing agent. By combining it with the uric acid/xanthine ratio, the relative concentration of free unchanged xanthine was obtainable. The amount of xanthine that would have to be added in the titration (expressed relative to the half-enzyme concentration) to give the potential, E , was then equal to the uric acid concentration plus the free xanthine concentration. The calculation was carried out on a PDP 11/10 computer (Digital Equipment Co., Galway, Ireland) and was repeated for a series of values of the applied potential, E , to cover the entire titration.

Results

Redox potentials of turkey liver xanthine dehydrogenase

Redox potentials for the various systems in turkey liver xanthine dehydrogenase, determined by potentiometric titration in the presence of mediators,

Table 1. Oxidation-reduction potentials measured for turkey liver xanthine dehydrogenase at 25°C and pH 8.2, in 50 mM-pyrophosphate buffer

Measurements of potentials, which are expressed relative to the hydrogen electrode, were carried out as described in the Materials and Methods section.

System	Potential (mV)
Mo(VI)/Mo(V) (Rapid)	-350 ± 20
Mo(V) (Rapid)/Mo(IV)	-362 ± 20
Fe-S I _{ox.} /Fe-S I _{red.}	-295 ± 15
Fe-S II _{ox.} /Fe-S II _{red.}	-292 ± 15
FAD/FADH•	-359 ± 20
FADH•/FADH ₂	-366 ± 20

system FADH•/FADH₂ is about 130 mV lower than it is in the oxidase. A smaller, though significant, difference is that the potential for Fe-S I system is about 50 mV higher in the dehydrogenase than in the oxidase. Thus, in contrast with the situation in the oxidase, the two iron-sulphur systems have similar potentials in the dehydrogenase. Potentials of the remaining systems in the dehydrogenase are all within about 10 mV of the corresponding ones in the oxidase.

We did not carry out experiments on CN⁻-treated turkey liver xanthine dehydrogenase. However, the Slow Mo(V) signal from the non-functional desulpho form of the enzyme was detected along with the Rapid signal during our titrations. Though we did not wait sufficiently long for the Slow species to equilibrate fully, this signal behaved very similarly to the corresponding one from the milk enzyme (Cammack *et al.*, 1976). Potentials associated with its appearance and disappearance were clearly low, and probably quite similar to the corresponding ones for the desulpho milk enzyme. Our enzyme samples contained more desulpho than functional enzyme (see the Materials and Methods section). However,

Fig. 1. Comparison of the conversion of FAD into FADH• during potentiometric titration of xanthine oxidase and xanthine dehydrogenase

Intensity of the FADH• e.p.r. signal (expressed as unpaired electrons in the signal per total enzyme half-molecule) is plotted as a function of redox potential. Δ , Milk xanthine oxidase; $\blacktriangle$, turkey liver xanthine dehydrogenase. Theoretical curves through the experimental points were calculated by using the following values of the potentials [in mV; from Table 1 and from Cammack *et al.* (1976)]: FAD/FADH•: oxidase, -351; dehydrogenase, -359; FADH•/FADH₂: oxidase, -236; dehydrogenase, -366. The titrations were carried out at 25°C in pyrophosphate buffer, pH 8.2.

are summarized in Table 1 and the titrations are illustrated in Figs. 1 and 2. The midpoint potentials may be compared with the corresponding values reported for milk xanthine oxidase by Cammack *et al.* (1976). In Fig. 1, intensity of the FADH• (flavin radical) signal of milk xanthine oxidase, plotted as a function of the potential, is compared with corresponding data for turkey liver xanthine dehydrogenase. Conversion into the flavin radical is clearly very much higher for the dehydrogenase than it is for the oxidase at all potentials below about -260 mV. In terms of midpoint redox potentials, the most striking difference between the two enzymes is that, in the turkey dehydrogenase, the potential for the

Fig. 2. Behaviour of the Mo(V) (Rapid) (a), Fe-S I (b) and Fe-S II (c) signals of turkey liver xanthine dehydrogenase in potentiometric titrations

Signal intensity is plotted as a function of the potential. The experiment was carried out at 25°C in pyrophosphate buffer, pH 8.2. Intensity of the Rapid signal is expressed relative to the concentration of functional active centres and the Fe-S signals are relative to total active centres. The lines through the experimental points were calculated from the values of the potentials given in Table 1.

by analogy with the milk enzyme (Cammack *et al.*, 1976), we assume that potentials, other than those of molybdenum, are the same in the desulpho and the functional turkey dehydrogenase.

Redox potential of uric acid/xanthine

Early work by Green (1934) gave a potential of -441 mV for the system uric acid/xanthine at pH 8.07. Modern electrochemical work (e.g. Dryhurst, 1972) so far seems to have failed to produce a value of the potential. The method used by Green (1934) was simply to measure the potential generated at a platinum electrode by mixtures of uric acid and xanthine in the presence of mediator dyes, with small amounts of xanthine oxidase added. We repeated this measurement with milk xanthine oxidase, in our pyrophosphate buffer, and found a value of -440 mV for the uric acid/xanthine potential, which is close to that expected from the data of Green (1934). Another molybdenum-containing hydroxylase, for which the back reaction is much more readily demonstrated than it is for the milk enzyme, is the xanthine dehydrogenase from *Veillonella alcalescens*. We also repeated the potential determination with this enzyme in place of xanthine oxidase. A slightly higher value was obtained, which was, however, not very steady, possibly owing to significant dismutation (cf. Smith *et al.*, 1967) of xanthine to give hypoxanthine and uric acid.

Titration of xanthine dehydrogenase with xanthine

Barber *et al.* (1976) reported results of an e.p.r. titration in which xanthine dehydrogenase was reduced with xanthine, and compared them with a similar titration of the milk enzyme. Their data are reproduced in Fig. 4(a) and 4(b). As in the potentiometric titrations discussed above, the chief difference between the two enzymes concerns the behaviour of the $\text{FADH}^\bullet$ free radical. $\text{FADH}^\bullet$ signals are much stronger for the dehydrogenase than for the oxidase, and for the former they do not disappear even in the presence of a substantial excess of xanthine. It was suggested that this is because the $\text{FADH}^\bullet/\text{FADH}_2$ potential is sufficiently low that xanthine is incapable of reducing the flavin completely.

Accepting the value of -440 mV for the redox potential of uric acid/xanthine, we note from Table 1 that, though the $\text{FADH}^\bullet/\text{FADH}_2$ potential is the lowest in xanthine dehydrogenase, nevertheless its value is more than 70 mV higher than that of the reducing system. Therefore the failure of Barber *et al.* (1976) to obtain complete reduction of the flavin of the enzyme appeared surprising, and further investigation was called for.

We first consider the simulation of titrations of milk xanthine oxidase with xanthine. Reductive titrations of this enzyme by dithionite have been successfully simulated from relative values of the

redox potential by Olson *et al.* (1974). With dithionite, when a sufficiently long reaction time is allowed, the situation is relatively simple in that both functional and desulpho enzyme are reduced and that all the possible species, namely XO_0 , XO_{1e} , ..., XO_{6e} attain equilibrium with one another. However, when xanthine is the reducing agent, an important difference is that, at least at relatively short reaction times, only enzyme species which have accepted two, four or six electrons per active centre are expected to be present, since xanthine is capable of transferring two electrons only to the enzyme. Further, desulpho enzyme is not expected to be reduced by xanthine at short reaction times. The procedure for calculating the titration curves from the redox potentials, for reduction by xanthine, is described in the Materials and Methods section. It is necessarily more complicated than that given by Olson *et al.* (1974) for reduction by dithionite. Further, it does not assume that the reducing agent has a very low potential relative to those of the enzyme.

Experimental titrations of the milk enzyme with xanthine have been reported by, among others, Bray *et al.* (1961) and Barber *et al.* (1976). Dithionite titrations were described by Beinert (1971) and by Olson *et al.* (1974). Comparison reveals an important difference between the two reducing agents. Whereas with xanthine the Mo(V) and $\text{FADH}^\bullet$ peaks occur at essentially the same xanthine concentration, on the other hand with dithionite the flavin peak is distinctly before, i.e. at a lower dithionite concentration than, the molybdenum peak. An attempt to simulate the xanthine titration of the milk enzyme by using the potentials determined in potentiometric titrations by Cammack *et al.* (1976) failed to reproduce this feature, which cannot therefore be due to the difference between a two- and a one-electron reducing agent. The discrepancy is presumably due to the formation of complexes between reduced xanthine oxidase and substrate molecules (Michaelis complexes) or products. There is abundant e.p.r. evidence for the existence of such complexes (Pick & Bray, 1969; Olson *et al.*, 1974; Barber, 1976; M. J. Barber, D. J. Lowe & R. C. Bray, unpublished work). The expected effect of complex formation would be to raise the redox potentials for molybdenum of the enzyme.

To confirm that this effect could be observed experimentally in potentiometric titrations, we titrated milk xanthine oxidase in the presence of uric acid (results not shown). E.p.r. data on changes in the form of the Rapid signal (M. J. Barber & R. C. Bray, unpublished work; Barber, 1976) indicate that the reduced enzyme forms a complex with this purine molecule having a dissociation constant of about 0.3 mM. Titration of the enzyme in pyrophosphate buffer in the presence of 0.67 mM-uric acid gave the following changes in the midpoint potentials [in mV,

expressed relative to the potentials given by Cammack *et al.* (1976) for the enzyme without any added purine]: Mo(VI)/Mo(V) (Rapid), +17; Mo(V)/Mo(IV), +73; FAD/FADH[•], -11; FADH[•]/FADH₂, -2. Thus the main effects were on the molybdenum potentials, and they were in the expected direction.

Such results are not directly applicable to the xanthine titrations, since here, of course, the uric acid concentration varies. To simulate these, we therefore tried the effect of using a series of different potentials. We found (Fig. 3) that the best fit to titrations of the milk enzyme could be obtained by using the values of Cammack *et al.* (1976), but with about 60 mV

Fig. 3. Calculated titration of xanthine oxidase with xanthine: effect of complex-formation by molybdenum on the peak positions for the Mo(V) (Rapid) and FADH[•] signals

Theoretical titration curves for reduction of functional xanthine oxidase by xanthine were calculated from the redox potentials as described in the Materials and Methods section. Only reduced functional enzyme half-molecules that had accepted two, four or six electrons were considered in the calculation; the potentials used for the enzyme (0 mV added) were those of Cammack *et al.* (1976), with uric acid/xanthine as -440 mV. The formation of complexes by the molybdenum is expected to raise the redox potentials for Mo(VI)/Mo(V) and for Mo(V)/Mo(IV) (see the text) and the main effect of this on the theoretical curves is to shift the position of the maximum of the Mo(V) (Rapid) signal towards lower xanthine concentrations. A series of simulated curves was obtained for various assumed molybdenum potentials, other potentials being held constant. The calculated relative positions of the FADH[•] and Mo(V) peaks (expressed as the ratio of the number of molecules of xanthine required per half-enzyme molecule for maximum development of each signal) is plotted as a function of the number of millivolts added to the two molybdenum potentials. The arrow denotes the experimental value of the ratio for titration of milk xanthine oxidase by xanthine (from Fig. 4a).

added both to the Mo(VI)/Mo(V) potential and also to the Mo(V)/Mo(IV) potential. When this was done the main features of titration of the milk enzyme with xanthine (Fig. 4a) were accurately reproduced in the simulations (Fig. 4c). The only serious discrepancy between the simulated curves and the experimental ones is that the maximum observed FADH[•] signal is some 3 times more intense than is the predicted one. Presumably this, like the shift in the molybdenum peak, is somehow due to substrate or product effects on the enzyme. In any case, the discrepancy between calculation and experiment was found to disappear if the FAD/FADH[•] potential was raised by 30 mV, and at the same time the FADH[•]/FADH₂ potential was lowered by the same amount; these shifts are only just outside the experimental errors quoted by Cammack *et al.* (1976).

We then turned our attention to simulating the xanthine titration of turkey liver xanthine dehydrogenase. We started from the potentials of Table 1, with 55 mV added to each molybdenum potential, by analogy with the milk enzyme as discussed above, and as above we assumed that there was no reduction of the desulpho enzyme. The theoretical curves calculated in this way (Fig. 4d) may again be compared with the experimental curves (Fig. 4b). The behaviour of molybdenum and of the Fe-S system is adequately reproduced in the simulations. Further, and most important, the simulations agree with experiment in predicting that the dehydrogenase should yield maximally 3 to 4 times more radical than does the oxidase. However, as with the oxidase, and presumably for similar reasons, the absolute amount of radical predicted for the dehydrogenase is rather too low. (Flavin potentials had to be shifted by only about one-half as much as in the oxidase to improve the simulation very greatly in this respect.) There are further discrepancies, however. These concern the position of the experimental FADH[•] peak (Fig. 4b), which was at a rather higher xanthine concentration than in the theoretical curve (Fig. 4d). Also, whereas experimentally, in the presence of excess of xanthine, the radical signal remained high (about one-half its maximum intensity), in contrast, in the theoretical curve, it decreased to a negligible size when only slightly more than 6 mol of xanthine/mol of active centre had been added. As considered below, we believe that these serious discrepancies are due to interference in the experimental titration by the desulpho form of turkey liver xanthine dehydrogenase.

Table 2 summarizes calculated electron distributions expected in the presence of xanthine for functional milk xanthine oxidase and for functional turkey liver xanthine dehydrogenase, for species that have accepted two, four or six electrons per active centre. Effects of uric acid on molybdenum potentials only have been allowed for.

Fig. 4. Experimental (a and b) and calculated (c and d) titrations of xanthine oxidase (a and c) and xanthine dehydrogenase (b and d) with xanthine

The fractional extents of conversion in the functional enzymes to the species indicated is plotted as a function of mol of xanthine added per mol of functional active centre. Experimental data points are replotted from Barber *et al.* (1976). The symbols are: total reduced iron-sulphur (Fe-S I + Fe-S II), ○ and ●, —; Mo(V) (Rapid), △ and ▲, ----; FADH₂, □ and ■, ····; the lines through the points in (a) and (b) have no theoretical significance. Open and closed symbols refer to different enzyme samples. For the milk enzyme both samples were 80% functional; for the turkey enzyme they were 23% (closed symbols) and 39% (open symbols) functional respectively. The calculation of the theoretical curves (c and d) is described in the Materials and Methods section. As in Fig. 3, only reduced functional half-enzyme molecules that had accepted two, four or six electrons were considered. The potentials used were as in Table 2 with uric acid/xanthine as -440 mV.

Table 2. Calculated electron distributions on functional xanthine oxidase and xanthine dehydrogenase half-molecules which have accepted, relative to the fully oxidized state, two, four or six electrons from xanthine

Fractions of each redox-active grouping (or pair of groupings) present in the reduced state in each reduced form of the two enzymes were calculated from the individual redox potentials as outlined by Olson *et al.* (1974) (see the Materials and Methods section). Potentials used in the calculation for the turkey dehydrogenase were basically those from the potentiometric titration (Table 1), and for milk xanthine oxidase were those of Cammack *et al.* (1976) for pyrophosphate buffer. However, allowance had to be made for the effects of xanthine and uric acid on the molybdenum potentials (see the text) and this was done by adding 55 mV to each molybdenum potential, for each enzyme.

No. of electrons accepted	Enzyme	Fraction present as:					
		Mo(V)	Mo(IV)	FADH [•]	FADH ₂	Fe-S I _{red.} + Fe-S II _{red.}	
2	Oxidase	0.292	0.238	0.070	0.393	0.188	
2	Dehydrogenase	0.532	0.152	0.065	0.001	0.548	
4	Oxidase	0.445	0.485	0.044	0.914	0.356	
4	Dehydrogenase	0.115	0.878	0.244	0.023	0.929	
6	Oxidase	0	1.000	0	1.000	1.000	
6	Dehydrogenase	0	1.000	0	1.000	1.000	

Kinetics of reduction of turkey liver xanthine dehydrogenase by xanthine

To seek an explanation for the anomalous behaviour of the FADH[•] free-radical signal at high xanthine concentrations in the titrations, we carried out investigations on the kinetics of reduction of preparations of the enzyme by this substrate. Some results are summarized in Fig. 5. Though we have not made a very detailed study, nevertheless our results are adequate to show both similarities and differences between the milk and turkey enzymes.

Stopped-flow experiments reported by Barber *et al.* (1976) implied that the early stages of reduction of the turkey enzyme were similar to those occurring with the milk enzyme. However, from rapid-freezing experiments these workers reported a difference between the two enzymes at a slightly later stage of the reduction, in that, whereas with the milk enzyme the rather small FADH[•] signal decreased in the time-interval 0.18–0.8 s, on the other hand with the turkey enzyme this signal was much larger at both reaction times and did not decrease significantly between them. This point is also illustrated in Fig. 5 and is readily explicable from the electron distributions given in Table 2. For both enzymes, disappearance of the FADH[•] signal must correspond to reaction with the third and last xanthine molecule per active centre, i.e. to conversion of the four-electron-reduced species into the six-electron-reduced species. For the milk enzyme the half-time for this process, from the data of Olson *et al.* (1974), seems to be about 0.5 s. According to these workers reduction of form XO_{2e} to XO_{4e} is slower than reduction of form XO₀ to XO_{2e}, because form XO_{2e} contains less Mo(VI) than does form XO₀. From Table 2, it appears that, whereas form XO_{4e} still has about 7% of its molybdenum in the hexavalent state, on the other hand for

Fig. 5. Time-course of changes in intensity of the FADH[•] signal during anaerobic reaction of turkey liver xanthine dehydrogenase with xanthine at pH 8.2

Signal intensity, expressed as unpaired electrons per functional active centre, is plotted against time, on a logarithmic scale. The xanthine concentration was 0.2 mM and the functional active-centre concentration 0.02 mM, with 38% of the enzyme in the functional state. The temperature was about 20°C and rapid freezing was used up to 1 s, with manual mixing for longer reaction times. E.p.r. spectra were recorded at 120 K and 5 mW and integrations were corrected for saturation. The arrow denotes the reaction time used by Barber *et al.* (1976) for xanthine titrations. The dashed part of the curve was calculated by analogy with the milk enzyme and represents a theoretical curve for disappearance of FADH[•] (owing to reduction of XD_{4e} to XD_{6e}; rate constant 0.14 s⁻¹), followed by its reappearance at higher intensity (phase III; owing to one-electron transfer from XD_{6e} to oxidized desulpho enzyme; rate constant 0.0017 s⁻¹).

form XD_{4e} the corresponding value is 10 times less, namely 0.7% of Mo(VI). We would therefore

expect, on this basis, reduction of the turkey enzyme to the six-electron-reduced species to have a half-time of around 5s. The electron distributions of Table 2 therefore explain the persistence of the $\text{FADH}^\bullet$ signals at 0.8s reaction time and they also lead to the very important prediction that the six-electron-reduced form of the dehydrogenase, since it is formed so slowly, could not play any significant part in enzyme turnover.

Fig. 5 shows that, when the reaction time is increased to a few minutes, the $\text{FADH}^\bullet$ signal from the turkey enzyme increases above the values achieved in rapid-freezing experiments and thereafter remains high for many hours. For the milk enzyme a corresponding phenomenon, owing to reduction of non-functional desulpho enzyme by reduced functional enzyme molecules, was studied in some detail by Swann & Bray (1972). According to Olson *et al.* (1974), the initial stage of this process [Phase III of Swann & Bray (1972)] corresponds to reaction of form XO_{6e} with oxidized desulpho enzyme, to give form XO_{5e} and one-electron-reduced desulpho enzyme. For the milk enzyme, the most striking feature of Phase III reduction is reappearance of the Rapid molybdenum signal from the functional enzyme as form XO_{5e} . However, comparison of the calculated electron distributions on functional and desulpho forms of milk and turkey enzymes shows that, for the dehydrogenase, the main feature of the Phase III reaction, defined as above, would rather be an increase in the $\text{FADH}^\bullet$ signal. Since we have no experimental points in the region of 20s reaction time it is not clear whether $\text{FADH}^\bullet$ -signal intensity decreased in this region (----, Fig. 5) or remained steady (—, Fig. 5). Although a decrease would be predicted by analogy with the milk enzyme, it is possible that for the turkey enzyme, reaction with desulpho enzyme (Phase III) follows so rapidly on reaction with the third xanthine molecule that no significant minimum is to be expected.

In any case, the data suggest very strongly that persistence of the $\text{FADH}^\bullet$ signal at high xanthine concentrations with a reaction time of 1.5 min [experiments of Barber *et al.* (1976); see arrow in Fig. 5] depends on interference in the titration by the desulpho enzyme. This conclusion is not at variance with the finding by Barber *et al.* (1976) (see Fig. 4b) that two enzyme samples, one containing 23 and the other 39% of functional enzyme, were indistinguishable on titration. This is because, with desulpho enzyme in excess, the $\text{FADH}^\bullet$ signal appearing in Phase III is due almost entirely not to the desulpho enzyme, which is reduced, but to the functional enzyme, which is reoxidized. Finally, the conclusion that the titration of Barber *et al.* (1976) was significantly distorted by interference by desulpho enzyme is further supported by the rather high iron-sulphur-signal intensities that these workers reported

(fractional conversion into $\text{Fe-S I}_{\text{red.}} + \text{Fe-S II}_{\text{red.}}$ equals 1.25 at high xanthine concentrations; see Fig. 4).

Discussion

Values of the redox potentials for turkey liver xanthine dehydrogenase

The values of the redox potentials for the flavin, molybdenum and iron-sulphur centres of functional turkey liver xanthine dehydrogenase are quite like those in the milk enzyme (Cammack *et al.*, 1976), apart from $\text{FADH}^\bullet/\text{FADH}_2$, which has a low potential in the dehydrogenase, comparable with that in flavodoxin from *Peptostreptococcus elsdenii* (Mayhew *et al.*, 1969): a smaller difference between the enzymes relates to the relatively high potential of the Fe-S I system in the dehydrogenase.

Redox potentials and reduction of the enzyme

Contamination with the desulpho forms, which are not readily separated from the functional enzymes, has greatly complicated work on the molybdenum-containing hydroxylases (Bray, 1975). The potentials for the turkey enzyme, together with the electron distributions calculated from them, seem capable of accounting for all available data on reduction of the enzyme by xanthine, when proper allowance is made both for complex-formation with uric acid and for interference by the desulpho enzyme, which is known to be present (Cleere *et al.*, 1974; Barber *et al.*, 1976). Though the potential for $\text{FADH}^\bullet/\text{FADH}_2$ in the enzyme is such that uric acid/xanthine ought to be capable of reducing the flavin completely, nevertheless the $\text{FADH}^\bullet$ e.p.r. signal persists for extended periods. This situation is quite analogous to the similar persistence of the Rapid Mo(V) signal from the milk enzyme (Swann & Bray, 1972), and its origin must be kinetic rather than thermodynamic in nature. As discussed above, the slow phase of reduction no doubt involves reactions of the type shown in eqns. (1)–(4) [where $\text{XD}(\text{ds})$ represents a half-molecule of the desulpho enzyme].

Persistence of $\text{FADH}^\bullet$ from XD_{5e} should be due to slowness of the intermolecular electron-transfer reactions between protein molecules (eqns. 1, 2 and 4). The calculated macroscopic redox potentials for the functional enzyme (Table 3) confirm that even for the reaction of eqn. (3), the uric acid/xanthine potential remains favourable.

In contrast with xanthine, dithionite, which is

expected to reduce both the functional and the desulpho enzyme directly, by a one-electron mechanism (Bray *et al.*, 1975), readily reduced the flavin of the enzyme fully (Barber *et al.*, 1976; see their Fig. 1c). The potential for dithionite is not certain, but may be around -500mV at pH 8.2 (Mayhew & Ludwig, 1975), i.e. considerably lower than that for uric acid/xanthine.

Although the kinetics of reduction of the enzyme by NADH have not been reported in detail (but see Barber *et al.*, 1976), the electron distribution in Table 2 and the potentials in Table 3 enable certain predictions to be made. It is expected that both functional and desulpho enzyme would be reduced directly by this substrate, acting at the FAD site. Olson *et al.* (1974) suggested that slowness of the later stages of reduction of xanthine oxidase by NADH was due to there being very little oxidized FAD available in form XO_{4e} for the substrate to react with. Evidently, this will not be a serious limitation to reduction of the dehydrogenase, according to the electron distribution in Table 2 (distributions are slightly different in the absence of xanthine from those given; nevertheless this does not alter the conclusion). On the other hand, and again in contrast with the oxidase, relative to NAD^+/NADH , the macroscopic potentials of the dehydrogenase (Table 4) are such that reaction with the third NADH molecule could approach completion only with a substantial excess of substrate. Though the binding

of NADH or of NAD^+ might modify the potentials, it will be interesting to see if this prediction can be confirmed experimentally for the turkey enzyme.

Our results may be compared with very limited e.p.r. data reported by Waud & Rajagopalan (1976) on the D-form of rat liver xanthine dehydrogenase. They found that the strong $\text{FADH}^{\bullet}$ signal, produced either by NADH or by xanthine, was 'stable', i.e. that it was present at 80s reaction time. Assuming the rat enzyme to be similar to the turkey enzyme with regard both to potentials and to contamination with desulpho enzyme, then it may be concluded that the 'stability' is of thermodynamic origin when NADH is used, but of kinetic origin, dependent on the presence of contaminating desulpho enzyme, when xanthine is used.

Finally we may note, though with due caution, the possible evolutionary significance of the difference between the potentials of the Fe-S I centres in milk xanthine oxidase and turkey liver xanthine dehydrogenase. Olson *et al.* (1974) proposed that the iron-sulphur centres of the oxidase serve as electron reservoirs, helping to maintain molybdenum in the oxidized state, to facilitate reduction of the enzyme by xanthine. That this applies also to the dehydrogenase is suggested by calculation of the electron distributions in a hypothetical hybrid enzyme, having flavin as in the dehydrogenase, but having an Fe-S I centre as in the oxidase. Because of the low proportion of Mo(VI), reduction by xanthine from the

Table 3. *Oxidation-reduction potentials, at pH 8.2, relating to reactions of xanthine oxidase and xanthine dehydrogenase with xanthine, O_2 and NAD^+*

Potentials for the two-electron reduction are the average of the potentials for the two one-electron steps. Macroscopic potentials for reduction of enzyme molecules by xanthine were calculated as described in the Materials and Methods section, with the potentials of the centres as in Table 2. Enz denotes the enzyme half-molecule and the subscripts indicate the number of electrons taken up. The sources for the values in this Table are: (a) Cammack *et al.* (1976); (b) Table 1 of the present paper; (c) Wood (1974); (d) Clark (1960); (e) Green (1934) and the present paper.

No. of electrons in reaction	System	Potentials (mV)	
		Oxidase	Dehydrogenase
1	$\text{FAD}/\text{FADH}^{\bullet}$	$-351^{(a)}$	$-359^{(b)}$
1	$\text{FADH}^{\bullet}/\text{FADH}_2$	$-236^{(a)}$	$-366^{(b)}$
2	FAD/FADH_2	-294	-363
2	$\text{Enz}_0/\text{Enz}_{2e}$	-281	-277
2	$\text{Enz}_{2e}/\text{Enz}_{4e}$	-300	-314
2	$\text{Enz}_{4e}/\text{Enz}_{6e}$	-335	-366
1	$\text{O}_2/\text{O}_2^{\bullet-}$	-160	$(-330)^{(c)*}$
2	$\text{O}_2/\text{H}_2\text{O}_2$	$+305^{(c)\dagger}$	
2	NAD^+/NADH	$-350^{(d)}$	
2	Uric acid/xanthine	$-440^{(e)}$	

* The value given corresponds to the potential for 1M-O_2 and $1\text{M-O}_2^{\bullet-}$. This seems more meaningful for the present purposes than the value in parentheses, which is for a partial pressure of O_2 of 1 atmosphere (about 1.3mm-O_2 in water) and $1\text{M-O}_2^{\bullet-}$.

† At pH 7.0.

two-electron-reduced to the four-electron-reduced species for such an enzyme is predicted to be some 4 times slower than it is for the real dehydrogenase.

Redox potentials and differences in acceptor specificity between xanthine dehydrogenase and xanthine oxidase

We now consider to what extent the redox potentials are capable of explaining the striking differences in acceptor specificity between milk xanthine oxidase and turkey liver xanthine dehydrogenase. The turnover number (catalytic centre activity) at 23.5°C for fully functional milk xanthine oxidase with xanthine as reducing substrate and oxygen as the oxidizing substrate is 16.2 s^{-1} (both substrates at infinite concentration). The corresponding value for turkey liver xanthine dehydrogenase, with its main oxidizing substrate, NAD^+ , is 26 s^{-1} . Apart from the main activities, the oxidase reacts at a low, but significant, rate with NAD^+ (0.6 s^{-1}), as does the dehydrogenase with O_2 (0.4 s^{-1}) (Krenitsky *et al.*, 1974; Bray, 1975; Coughlan & Cleere, 1976). It seems well established that both these acceptors interact with the enzymes at the flavin site (see Bray, 1975).

Table 3 summarizes redox potentials relevant to a consideration of the acceptor specificity of the enzymes. Potentials of $\text{FADH}^\cdot$ and FADH_2 in both are favourable for either one- or two-electron reduction of O_2 . On the other hand, for two-electron NAD^+ reduction by FADH_2 , the potential is favourable in the dehydrogenase, but unfavourable in the oxidase. The potentials, being thermodynamic quantities, cannot themselves, of course, tell us anything about reaction rates. However, if we accept that intramolecular electron transfers are quite rapid, then the relative amounts of the various reduced species will be determined by the potentials, and these might in turn govern the probabilities of the various reaction pathways. For reasons discussed above, form XD_{6e} is not expected to participate in turnover with xanthine as substrate. Table 2 therefore makes it clear that, during turnover, reduced flavin in the dehydrogenase will be present almost exclusively as $\text{FADH}^\cdot$, whereas in the oxidase flavin will be present in both reduced forms, but with FADH_2 predominating.

In flavoproteins generally, those in which the flavin gives rise to the 'red' type of semiquinone are good oxidases, whereas those which give the 'blue' semiquinone tend to be dehydrogenases (Massey *et al.*, 1969). In milk xanthine oxidase and in turkey liver xanthine dehydrogenase (and probably also in all other molybdenum-containing hydroxylases) the flavin radical is of the 'blue' type (Bray, 1975). Hence oxidase activity in these enzymes may be something of an anomaly. A further point is that 'blue' flavoprotein semiquinones seem to be much

less reactive to O_2 than are their fully reduced forms. An extreme case is flavodoxin from *Peptostreptococcus elsdenii*, for which the semiquinone reacts some 10^5 times more slowly with O_2 than does the fully reduced form (Mayhew & Ludwig, 1975). More pertinently, Olson *et al.* (1974) were able to simulate the reactions of reduced xanthine oxidase with O_2 on the basis of its $\text{FADH}^\cdot$ reacting much more slowly with O_2 than did its FADH_2 .

The present studies are thus fully in agreement with the main overall conclusion of Barber *et al.* (1976), if not with its detailed derivation. This conclusion was that lack of FADH_2 in the turkey enzyme, owing to a low potential for its $\text{FADH}^\cdot/\text{FADH}_2$ system, is the cause of low oxidase activity in this enzyme. Quantitatively, though the exact agreement may be fortuitous, if we assume that the enzymes cycle mainly between the two-electron- and the four-electron-reduced forms in turnover, then according to Table 2 the oxidase has 40 times more FADH_2 available than does the dehydrogenase, and in agreement with this its turnover rate with O_2 is 41 times higher than that of the dehydrogenase (see above).

Regarding reaction of the enzymes with NAD^+ , our data are rather less definitive. One-electron reduction of NAD^+ to yield a radical is not expected (Land & Swallow, 1968), but on the other hand in the dehydrogenase, as noted above, flavin is reduced by xanthine to the semiquinone rather than to FADH_2 . The most obvious interpretation would be that $\text{FADH}^\cdot$ reacts with the coenzyme to form a bound NAD radical and is thereupon re-reduced rapidly and intramolecularly by another centre in the enzyme, without dissociation of the product. These reactions would then be followed by a second electron transfer from $\text{FADH}^\cdot$ to the bound NAD radical, to yield NADH . On the basis of such a scheme, one might attempt to relate proportions of $\text{FADH}^\cdot$ in the reduced enzymes with their relative activities to NAD^+ as acceptor. In qualitative agreement with this, from Table 2, form XD_{4e} contains 5 times as much $\text{FADH}^\cdot$ as does XO_{4e} , and the dehydrogenase turns over 43 times faster (see above).

An alternative mechanism whereby the reaction with NAD^+ might occur [as originally mentioned by Waud & Rajagopalan (1976)], would be that, when the coenzyme is bound to the enzyme, the potential of its flavin is shifted so as to facilitate reduction to FADH_2 , so making possible direct two-electron reaction of flavin with NAD^+ . The potential shift would be analogous to the effects of xanthine and uric acid on the potentials of molybdenum. On this basis, the essential features of the dehydrogenase would be a binding site for NAD^+ . Elimination of such a site during conversion of the rat enzyme from the D- into the O-form may perhaps be suggested by NADH -inhibition data of Della Corte & Stirpe (1970).

However, further work would be required to distinguish between the two mechanisms.

Molecular basis of differences in the flavin redox potentials

The means whereby binding to a specific protein changes the redox potential of flavins is not fully understood [for a review relating particularly to flavodoxins, see Mayhew & Ludwig, (1975)]. What group or groups in the vicinity of the flavin of the turkey liver xanthine dehydrogenase are responsible for the low potential of its FADH[•]/FADH₂ is not certain. This low potential is responsible for the low oxidase activity of the enzyme and clearly also has an important bearing on the NAD⁺ dehydrogenase activity. In those molybdenum-containing hydroxylases which, unlike the turkey enzyme, may be interconverted between D- and O-forms, the flavin in the D-forms presumably has a low potential also. Here, this may be due to the presence of a nearby thiol group (Della Corte & Stirpe, 1972; Battelli *et al.*, 1973; Bray, 1975; Bray *et al.*, 1976; Waud & Rajagopalan, 1976).

We thank Dr. D. J. Lowe for much help with the e.p.r. and computing facilities. The work was supported by the Medical Research Council.

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