The effect of pH on the exchangeability with deuterium of protons coupled to molybdenum(V) in the active and the desulpho forms of xanthine oxidase

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The effect of pH variation on the exchangeability with deuterium of protons strongly coupled to Mo(V) in the active and desulpho forms of xanthine oxidase was studied by e.p.r. and rapid freezing, in extension of the work of Gutteridge, Tanner & Bray [*Biochem. J.* (1978) 175, 887–897]. Above neutrality, exchange rates increased with increasing pH. Detailed studies were made on the desulpho enzyme under a variety of conditions, and exchange rate constants at 22°C ranged from 0.16 s⁻¹ at pH 6.6 to 1.6 s⁻¹ at pH 11.3. The mechanism of proton exchange in the enzyme is discussed. The interpretation by the above workers that the strongly coupled proton of the active enzyme is on sulphur and that of the desulpho enzyme is on oxygen remains valid (and is in agreement with other work), as do their proposals for the structures of the protonated and deprotonated species. However, pK values cannot be calculated from the exchange data. It is likely that the relatively low rates of exchange observed are due to the difference of structure between the protonated and the deprotonated forms. In the case of the desulpho enzyme, an exchange mechanism, which involves the proton exchanging both as such and along with oxygen in the form of a hydroxyl ion, is discussed.

E.p.r. spectroscopy has yielded much information on the structures of molybdenum centres in enzymes (Bray, 1980b). Gutteridge *et al.* (1978b) made comparative studies on the Mo(V) e.p.r. signals from the functional (giving the signal known as Rapid) and the desulpho (giving the signal known as Slow) forms of xanthine oxidase. Their conclusions are summarized in Scheme 1. Though rigorous proof of the correctness of Scheme 1 may still be lacking, it has since been strongly supported by extended-X-ray-absorption-fine-structure studies (Bordas *et al.*, 1980; Cramer *et al.*, 1981) as well as by other work (Wahl & Rajagopalan, 1981; Bray & Gutteridge, 1982; George & Bray, 1983).

A key piece of evidence used by Gutteridge *et al.* (1978b) in deducing these structures concerned the exchangeability with water of protons, strongly coupled to molybdenum(V), which are apparent in the e.p.r. spectra. These protons were reported to exchange with rate constants of 85 s⁻¹ and 0.4 s⁻¹ in the active and desulpho forms respectively. It was presumed that these exchanging protons were those shown in Scheme 1, though at the time such strong

coupling of protons to molybdenum had not been observed in low-molecular-mass molybdenum(V) compounds. [More recently a Mo(V)–OH structure, showing proton coupling comparable with that in the enzyme, has been described in irradiated crystals by Yamase *et al.* (1981) and Yamase (1982).] Thus, according to Gutteridge *et al.* (1978b), the presumed more acidic character of the proton on the sulphur atom in the active enzyme, in comparison with that on oxygen in the desulpho form, accounted for the more rapid proton exchange in the active enzyme form.

Active enzyme:

Desulpho enzyme:

Scheme 1. *Reduction of active and of desulpho xanthine oxidase, as proposed by Gutteridge et al. (1978b)*

The protons shown are those strongly coupled to Mo(V) whose exchangeability has been studied. [Active enzyme may be converted into the desulpho form by treatment with cyanide (Massey & Edmondson, 1970).]

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In the case of the active enzyme, the nature of the group bearing the proton is of particular interest. This is because, during enzyme turnover, the substrate proton, replaced by a hydroxy group in the catalytic process, has been shown to be transferred transiently to this site in the enzyme (Bray & Knowles, 1968; Gutteridge *et al.*, 1978a; R. Lange & R. C. Bray, unpublished work). Thus the terminal sulphur ligand of molybdenum in the oxidized enzyme is believed to be a proton acceptor in the catalytic reaction.

Gutteridge *et al.* (1978b; see also Bray, 1980a) attempted to deduce pK values for the coupled protons in active and desulpho xanthine oxidase from their exchange data. However, as is considered below in the Discussion section, their calculations were not valid. We now present here a study of the effects of pH on the exchange rates of the protons, carried out with a view to understanding the nature of the exchange reactions.

Materials and methods

Xanthine oxidase was prepared by the salicylate denaturation method (Hart *et al.*, 1970; Bray, 1982). Concentrations of functional and non-functional active centres were calculated from measurements of activity and A_{450} (Bray, 1975). Desulpho enzyme was obtained from the functional enzyme by treatment with cyanide (Massey & Edmondson, 1970). Samples in $^2\text{H}_2\text{O}$ were prepared by dialysis. Contamination of such samples by $^1\text{H}_2\text{O}$ (checked in some cases by n.m.r.) was about 2%, and, where appropriate, corrections were applied for this.

Rapid freezing was carried out as described by Gutteridge *et al.* (1978a). Rapid signals from the functional enzyme and Slow signals from the desulpho enzyme were generated by reduction with $\text{Na}_2\text{S}_2\text{O}_4$ under appropriate conditions, as detailed in the captions to the Figures (cf. Bray *et al.*, 1975). Experiments involving dilution with $^2\text{H}_2\text{O}$ were performed generally as described by Gutteridge *et al.* (1978b). pH values were measured on solutions from the syringes from the rapid-freezing apparatus. pH values in $^2\text{H}_2\text{O}$ correspond to uncorrected readings of the pH-meter standardized in $^1\text{H}_2\text{O}$.

E.p.r. spectra were recorded on a Varian E9 spectrometer linked to a computer and visual-display system (Bray *et al.*, 1978). The extent of proton exchange in each sample was obtained by analysing the e.p.r. spectra with the computer, essentially as described by Bray & Gutteridge (1982). After scaling of the spectra to equal integrated intensities, the ^1H form present in each spectrum was exactly subtracted out, and thus the fraction of it present in the spectrum was estimated. Semi-logarithmic plots of the exchange time-course data were linear for at least 80–90% of the reaction. Data consisting of six

to nine points were fitted to a first-order exponential by using a derivative-free non-linear regression program (BMDPAR; Department of Biomathematics, University of California, Los Angeles, CA, U.S.A.).

Results

Exchange of the strongly coupled proton of the Rapid signal

We followed the procedure, involving dilution with $^2\text{H}_2\text{O}$, employed by Gutteridge *et al.* (1978b) to study exchange of the strongly coupled proton of the Rapid type 1 signal. Typical results from experiments at a number of pH values are shown in Fig. 1. We worked in the presence of nitrate to ensure that the signal was of the Rapid type 1 form. The form of the spectrum was essentially independent of pH over a wide range, and that in $^1\text{H}_2\text{O}$ is illustrated in Fig. 1(a). On dilution with $^2\text{H}_2\text{O}$ at pH 9.4, the spectrum indicated that ^2H had exchanged to replace ^1H , apparently completely, at the shortest reaction time employed (Figs. 1b and 1c). On the other hand, if the experiment was performed at pH 6.9, then the exchange was markedly slower (Figs. 1d and 1e).

Our results are thus consistent with those reported by Gutteridge *et al.* (1978b), and indicate, further, that exchange rates increase with increasing pH in the range studied. Gutteridge *et al.* (1978b) assumed that a 'dead time' of 3 ms should be added to reaction times in their rapid-freezing experiments, in order to allow for non-instantaneous quenching of the reaction. In fact, hydrogen/deuterium exchange in normal acids is a process that is likely to be particularly difficult to stop by cooling. In agreement with this, close inspection of the data of Gutteridge *et al.* (1978b), and of similar experiments in the present work involving proton exchange in the Rapid signal, suggests (on the assumption of a first-order rate process) that the dead time may have been underestimated by perhaps 10 ms. In view of these problems and of the rapidity of the exchange process, particularly at high pH values, we did not attempt to quantify proton-exchange rate constants for this signal.

Exchange of the protons of the Slow signal

In comparison with the protons of the Rapid signal from active xanthine oxidase, the strongly coupled proton of the Slow signal from the desulpho enzyme exchanges only slowly, and this process is therefore more amenable to quantitative study. We performed $^2\text{H}_2\text{O}$ dilution experiments on the Slow signal under a variety of conditions. Typical e.p.r. traces are illustrated in Fig. 2. Quantitative analysis yielded linear semi-logarithmic plots (Fig. 3), indicating accurately first-order proton-exchange kinetics. Results of all our experiments are sum-

Fig. 1. Spectra of the Rapid signal at pH 9.4 or 6.9, after dilution with $^2\text{H}_2\text{O}$

(a) Corresponds to the spectrum in $^1\text{H}_2\text{O}$ at pH 9.4; (b) and (c) are spectra of correspondingly prepared samples, frozen 3 and 65 ms respectively, at 12°C , after addition of $^2\text{H}_2\text{O}$ to a final concentration of 77%. (d) and (e) are spectra from a similar experiment at pH 6.9 in which the final $^2\text{H}_2\text{O}$ concentration was 71%, and the reaction times were respectively 11 and 90 ms. In all cases the signal was generated by reduction of active xanthine oxidase for 2 s with 5 mM-dithionite. The reaction medium contained 0.45 M- NaNO_3 , 4 mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine] and 0.1 M-Ches [2-(cyclohexylamino)ethanesulphonic acid]/NaOH or 0.1 M-Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid}/NaOH. Spectra were recorded under the following conditions: temperature, about 120 K; microwave frequency, 9.3 GHz; microwave power, 10 mW; modulation amplitude, 0.16 mT. The arrow here and in Fig. 2 corresponds to $g = 2.0037$.

Fig. 2. Spectra of the Slow signal at pH 6.6 (a-c) or at 9.7 (d-f) after dilution with $^2\text{H}_2\text{O}$

The signal was generated by reducing desulpho xanthine oxidase with 5 mM-dithionite for 1-4 h. Spectra (a)-(f) correspond to samples frozen at the following times at 22°C after addition of $^2\text{H}_2\text{O}$ to a final concentration of 64%: 0.026, 4.2, 36.2, 0.026, 0.50 and 6.00 s. The buffer was 0.1 M-Bes/NaOH in (a)-(c) and 0.1 M-Ches/NaOH in (d)-(f), and the spectra were recorded as described in the caption to Fig. 1.

marized in Fig. 4, which shows a plot of the first-order exchange rate constant shown on a logarithmic scale against pH, extending up to pH 11.3. [Note that the molybdenum centre of xanthine oxidase is stable up to high pH values; see, e.g., Lowe & Bray (1978).] Rates in Bicine buffer

(Fig. 4) are in fair agreement with the results obtained by Gutteridge *et al.* (1978b).

The main conclusions from these studies are considered in the Discussion section. Clearly, as for the Rapid signal, the exchange of the strongly coupled proton of the Slow signal occurs more rapidly at higher pH values. To obtain further information we performed two experiments in the converse manner to those described above, that is starting from enzyme in $^2\text{H}_2\text{O}$ and diluting with

Fig. 3. Time course of exchange of ^2H into the Slow signal at pH 6.6 (●) and at pH 9.7 (▲) at 22°C

The experiments are the ones described in Fig. 2. $(X_\infty - X_t)$, where X is the percentage of the ^2H form present in the spectrum at times t and infinity, estimated as described in the Materials and methods section, is plotted on a logarithmic scale against the time, t (in s), from dilution with $^2\text{H}_2\text{O}$. The lines through the experimental points were computed and correspond to first-order rate constants at pH 6.6 and 9.7 of 0.166 ± 0.008 (S.E.M.) s^{-1} and 1.141 ± 0.085 (S.E.M.) s^{-1} .

$^1\text{H}_2\text{O}$. In 0.1 M-Ches buffer, k_{1n}/k_{2n} was found to be about 0.6 at pH 9.0 and about 0.9 at pH 9.8 (see Fig. 4).

A number of further points require comment. The exact form of the Slow signal is known (Gutteridge *et al.*, 1978b) to be particularly sensitive to buffers and anions. Thus it is not surprising that the spectrum in $^1\text{H}_2\text{O}$ at pH 6.6 (Fig. 2a) is not quite identical with that at pH 9.7 (Fig. 2d). Such changes of form are undoubtedly indicative of the presence of more than one species, and may well indicate (S. Gutteridge & R. C. Bray, unpublished work) the presence of 'type 2' (with two strongly coupled protons) as well as 'type 1' Slow signals (with one only) (cf. Bray, 1980b). Nevertheless it seems that our observed spectra are dominated by a single species, and the finding of linear semi-logarithmic plots is consistent with this.

The Rapid type 1 signal shows not only the strongly coupled proton, whose exchange we have

Fig. 4. Effect of pH and of buffer on the first-order rate constant for exchange of ^2H or ^1H into the Slow signal at 22°C

Values of the rate constant, k , measured as illustrated in Figs. 2 and 3, are plotted on a logarithmic scale against the pH value. Error bars correspond to ± 2 (S.E.M.); they are shown with continuous lines for experiments involving dilution of enzyme in $^1\text{H}_2\text{O}$ with $^2\text{H}_2\text{O}$ and with broken lines for those involving dilution of enzyme in $^2\text{H}_2\text{O}$ with $^1\text{H}_2\text{O}$. Buffers were as follows: Δ , 0.1 M-Bes/NaOH; $\circ$, 0.1 M-Bicine/NaOH; $\bullet$, 0.2 M-Bicine/NaOH; $\square$, 0.1 M-Ches/NaOH; $\blacksquare$, 0.5 M-Ches/NaOH; ∇ , 0.1 M-Caps [3-(cyclohexylamino)propanesulphonic acid]/NaOH. The straight line drawn through the experimental points between pH 8.5 and pH 9.5 corresponds to a 10-fold increase of k for an increase of 1 pH unit (see the text).

studied, but has also a second, relatively weakly coupled proton, which exchanges fast (Gutteridge *et al.*, 1978b). These workers obtained evidence, in the Slow signal also, for a second extremely weakly coupled proton, which again exchanged fast with the solvent. This conclusion was further supported in the present work. Thus in all experiments, a very slight sharpening, for example of the g_3 features of the Slow spectrum, was on close inspection apparent within 25 ms of dilution with $^2\text{H}_2\text{O}$, i.e. before significant exchange of the strongly coupled proton had taken place.

Discussion

We first consider possible mechanisms for proton exchange in the enzyme system. An acid, XH

(which might include, e.g., either MoSH or MoOH), can ionize according to either eqn. (1) or eqn. (2):

From the work of Eigen (1964) it is known that, except for special types of acids, the values of the rate constants k_{-1} and k_{+2} (involving reactions with H_3O^+ and OH^- respectively) are near to the diffusion-controlled limiting value of about $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$. It follows from this that the rate of exchange with the solvent of the proton of a normally ionizing acid shows a minimum value, which occurs in the region of neutrality. The minimum rate constant for proton exchange in such a system is about 10^4 s^{-1} at 25°C (see, e.g., Cornish-Bowden, 1976). At high and at low pH values, proton-exchange rate constants are expected to increase from this value, 10-fold for each unit of pH change.

For an MoOH group, exchange of oxygen (cf. Bray & Gutteridge, 1982; Gutteridge & Bray, 1980) and of hydrogen simultaneously have also to be considered, by mechanisms such as those of eqns. (3) and (4):

For these, k_{+3} and k_{-4} would, by analogy with k_{-1} and k_{+2} of eqns. (1) and (2), be in general expected to have rate constants of around $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$. Hydrogen-exchange studies alone will therefore not distinguish proton exchange from hydroxyl-ion exchange.

Clearly the rate constants for proton exchange reported by Gutteridge *et al.* (1978b) and confirmed and extended in the present work are much too small to be compatible with normally ionizing acids. [In fact, pK values calculated by these workers and by Bray (1980b) were obtained by ignoring eqn. (2) above and are therefore not valid.]

In our work, exchange rates are presumed in all cases to be dominated by proton (or hydroxyl-ion) dissociation or abstraction rates. This is because there is no evidence in e.p.r. work on xanthine oxidase for the existence of deprotonated species complementary to the Rapid- and Slow-signal-giving species. [The enzyme differs in this respect from sulphite oxidase Cohen *et al.*, 1971; Bray *et al.*, 1983) and from nitrate reductase (Vincent & Bray,

1978).] Because of this, we conclude that, for whichever of eqns. (1)–(4) are relevant, the equilibria must be substantially in favour of the left-hand side, so that the ‘off’ rates will always be limiting.

Factors that may account in particular cases for reactions such as those controlled by k_{+2} or k_{+3} occurring at less than the diffusion-controlled limit have been discussed by Eigen (1964). The most relevant of these to the present case is the situation where recombination of the proton or hydroxyl ion is associated with a structural modification, as, e.g., in reactions of CO_2 with OH^- or of HCO_3^- with H_3O^+ . These reactions involve substantial changes both in molecular geometry and in carbon–oxygen bond lengths, and they are known (Eigen, 1964) to occur with rate constants 10^5 – 10^6 times lower than the diffusion-controlled limit.

For xanthine oxidase proton uptake at the molybdenum centre is known to be intimately associated (Stiefel, 1973; Barber & Siegel, 1982; Porras & Palmer, 1982) with reduction of the metal, which in turn is known (Cramer *et al.*, 1981) to involve substantial changes in ligand environment (including lengthening of a molybdenum–sulphur bond for active enzyme and of a molybdenum–oxygen bond for the desulpho form). Thus protonation and deprotonation are fully expected to involve co-ordination changes at the molybdenum atom (cf. Scheme 1). It seems that these structural changes could readily account (cf. Eigen, 1964) for the low exchange rates observed by Gutteridge *et al.* (1978b) and confirmed and extended in the present work.

The observed increases in exchange rate, with increasing pH value above neutrality, are qualitatively in accordance with expectations, though detailed analysis is not at present possible. Because of the relatively high rate constants, we were unable to obtain quantitative values for the Rapid signal. For the Slow signal, in 0.1 M buffers, pH appears to have little effect on the exchange rate constant (Fig. 4) in the range from pH 6.6 to 8.0–8.5, suggesting that k_{+1} or k_{+4} is rate-limiting under these conditions. It is of course well known that buffer ions may participate in proton exchange, and it is therefore not surprising that rather higher rates were observed (Fig. 4) on increasing the concentration of Bicine, though not of Ches, buffer. In the range from about pH 8.5 to 10 the exchange rate constant increases with increasing pH. This could arise if exchange were subject to base catalysis or if it were inhibited by a protonated group in the enzyme. For either mechanism, k_{+2} should predominate at high pH values. For exchange controlled solely by k_{+2} , the rate of exchange should increase by a factor of 10^3 -fold between pH 8 and 11. Since only a 10-fold increase in rate constant is observed over this range (Fig. 4), exchange cannot

be determined solely by k_{+2} , and a more complex mechanism must be operating. The exchange rate might be dependent on reaction of two hydroxyl ions with the enzyme rather than of one (to give a slope of 0.5 on Fig. 4). Though there are known to be several exchanging oxygen atoms (Bray & Gutteridge, 1982), this would obviously be difficult to reconcile with a single slowly exchanging proton in the Slow signal (Gutteridge *et al.*, 1978b). The most likely explanation is that at high pH values exchange is inhibited (cf. Vaughan *et al.*, 1970) by deprotonation of a nearby group, which might, for example, be the active-site lysine (Greenlee & Handler, 1964a,b; Nishino *et al.*, 1982), reported to ionize with a pK of 10.7.

Though the possible errors in our rate constants determined in $^2\text{H}_2\text{O}$ at pH 9.0 and 9.8 were relatively large (Fig. 4), the data appear to exclude a significant positive isotope effect. Thus, in contrast with the process of oxygen exchange in $[\text{MoO}_4]^{2-}$, studied by von Felten *et al.* (1978), for which $k_{\text{H}}/k_{\text{D}}$ was reported to be about 3, exchange involving rate-limiting proton transfer cannot be involved in the exchange processes that we studied.

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