Recent studies on xanthine oxidase and related enzymes


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Xanthine oxidase, xanthine dehydrogenase and aldehyde oxidases are closely related flavoprotein enzymes, sometimes known as the molybdenum-containing hydroxylases [1–5]. They all have two identical subunits each of M, about 150 000. In addition to an FAD, each subunit contains a molybdenum as the pterin molybdenum co-factor and two iron-sulphur centres, FeSI and FeSII. The catalytic cycle involves reducing substrates such as xanthine reacting at the molybdenum centre, reducing the metal from Mo(VI) to Mo(IV), with reoxidation occurring via the flavin. The authors' laboratories is considered. Topics discussed include the relationship between xanthine dehydrogenase and aldehyde oxidase, the location of the NAD+–binding site, the properties of certain xanthine dehydrogenase variants from rosyl mutant strains of Drosophila melanogaster and ENDOR (electron-nuclear double resonance) studies of Mo(V) species, which provide new information bearing on the catalytic reaction at the molybdenum centre, and show that this involves participation of a Mo–C bond in the cycle.

Structural information

Thoenes et al. [8] reported that the amino acid sequence of aldehyde oxidoreductase from Desulfovibrio gigas shows considerable similarity to those of the xanthine dehydrogenases [4,9]. As in the latter enzymes, the N-terminal domain is associated with the Fe–S centres and the large C-terminal domain with the pterin molybdenum co-factor. However, this protein lacks the central flavin domain of the xanthine dehydrogenases. Salient features from the refined high-resolution structure of the D. gigas enzyme as recently described by Romão and Huber [6] and Romão
et al. [7] are as follows. The protein is folded into four domains, with the first resembling the plant ferredoxins, the second representing a novel ferredoxin and the third and fourth involved in molybdenum–cofactor binding. This cofactor is in the form of molybdopterin cytosine dinucleotide. In this, the enzyme contrasts with other members of the group, which contain the simple molybdopterin. The structure of the pterin derivative is not precisely that proposed by Rajagopalan et al. [10], but involves a tricyclic system in which the —OH group on the side chain in the pterin 6 position has ring closed with the 7 position to give an additional pyran ring. This structure is analogous to that of a recently reported tungsten dimolybdopterin cofactor [11]. Although amino acid residues in the vicinity of the molybdopterin atom are clearly identified in the D. gigas enzyme structure, ligation of the metal has not so far been fully defined. In particular, since it was the inactive desulpho form of the enzyme [12] that was studied, the location of the sulphido molybdenum ligand, known to be catalytically important in these enzymes, has not so far been unambiguously established.

**Regions of the flavin domain associated with oxidase/dehydrogenase differences**

Some xanthine dehydrogenases are NAD⁺-dependent enzymes, not able to use oxygen as oxidizing substrate, whereas others, although apparently normally existing in vivo in this state ('D-forms') may become converted, often reversibly, to oxidase ('O-forms') that react with oxygen and are no longer capable of reacting with NAD⁺ [13]. Thus, the most extensively studied of these enzymes, bovine milk xanthine oxidase, although traditionally studied as an oxidase, may also be isolated [14] in a dehydrogenase form. However, the precise structural differences, in the flavin region of the enzymes, between the D- and the O-forms remain elusive.

The amino acid sequences of eight xanthine dehydrogenases have so far been published (see [9] for references; [15]) and are very similar to one another. We have now shown [16] by partial protein sequencing of rabbit liver aldehyde oxidase that the additional sequence of Wright et al. [17] is not, as originally claimed, that of human xanthine dehydrogenase, but that of an aldehyde oxidase. Thus, for the first time, xanthine dehydrogenase/aldehyde oxidase sequence comparison has become possible. Comparison may be made, for example, between the sequences of human aldehyde oxidase [17] and human xanthine dehydrogenase [18,19]. Such comparisons should be particularly significant in relation to the origin of differences between the oxidase and dehydrogenase forms, since we confirmed [16] that rabbit aldehyde oxidase, unlike the xanthine dehydrogenases, is a permanent oxidase. As such, it might be hypothesized to lack an NAD⁺-binding site.

Of the flavin domain of *Drosophila melanogaster* xanthine dehydrogenase (the first of these enzymes to be sequenced), 22% of the sequence is fully conserved in seven xanthine dehydrogenases, but this figure falls to 15% if human aldehyde oxidase is included. Most noticeable [16] in the differences between the aldehyde oxidase and xanthine dehydrogenase flavin domains is the sequence region between residues 387 and 396 (*Drosophila melanogaster* sequence numbering is used throughout). Significantly, this region includes a residue (Tyr-395) shown [20] to be labelled in affinity labelling with an NAD⁺ analogue. Another nearby sequence region, where there is a quite substantial difference between aldehyde oxidase and xanthine dehydrogenase, is that between residues 343 and 365. In earlier work [21], we drew attention to some similarity of the sequence, within this region, to a motif identified by Karplus et al. [22] as being involved in NADP⁺ binding in ferredoxin:NADP⁺-reductase, and predicted that this part of the xanthine dehydrogenase sequence might be involved in NAD⁺ binding. Although the similarities are small, that this region is indeed implicated in NAD⁺ binding was recently established (W. A. Doyle, J. F. Burke, A. Chovnick, J. R. S. Whittle and R. C. Bray, unpublished work; see below), from studies of xanthine dehydrogenase variants from *rosy* mutant strains of *Drosophila melanogaster*. Specific mutations within this region were shown to reduce selectively activity to NAD⁺ and to increase Kₘ values for NAD⁺ and NADH. Activity to dyes as oxidizing substrates was not affected, while oxidase activity remained undetectable. The latter finding emphasizes the point (cf. [15]) that loss of activity to NAD⁺ in these enzymes is not obligatorily associated with the development of activity to oxygen.

In summary, therefore, this work implicates both the 387–396 and the 343–365 sequence regions in the NAD⁺-binding site.
Xanthine dehydrogenase variants from rosy mutant strains of Drosophila melanogaster

The work quoted above formed part of a larger study (W. A. Doyle, J. F. Burke, A. Chovnick, L. F. Dutton, J. R. S. Whittle and R. C. Bray, unpublished work) of xanthine dehydrogenase variants from rosy mutant strains of Drosophila melanogaster. This gene encodes xanthine dehydrogenase in this organism, and its expression affects the eye colour of the flies. More than 200 rosy mutant strains are available, of which at least 23 are known [23] to involve specific amino acid changes in the protein sequence. We have now in part characterized a number of these xanthine dehydrogenase variants, including two, as discussed above, in which the mutation is in the flavin domain, and one in which it is in the molybdenum domain (G1011E). The latter is the subject of a separate communication [24] and is considered further below. Additionally, we have prepared a further enzyme variant (see separate communication [25]) by site-directed mutagenesis and expression in Drosophila, yielding an 'engineered' rosy mutant strain. Note that earlier related work from this laboratory [21,26] on D. melanogaster xanthine dehydrogenases, and in particular the claim that the E89K xanthine dehydrogenase lacks Fe-S centres, has been withdrawn [27,28] (apart from the sequence comparisons [21], which remain valid). Our latest work establishes unambiguously that the E89K enzyme is indistinguishable from the wild-type enzyme, but expressed at about one-quarter of its level.

Information relating to the molybdenum domain and to substrate-binding

Understanding the relationship between aldehyde oxidase and xanthine oxidase/dehydrogenase must involve consideration not only of the flavin, but also of the molybdenum domains. Sequence comparisons [16] show that, for the molybdenum domain, rather more of the xanthine dehydrogenase sequence is conserved in aldehyde oxidase than for the flavin domain. At the substrate specificity level, it has long been known that the enzymes have low but overlapping specificities for reducing substrates. Our recent work [16] included both a comparison of Mo(V) EPR signals from milk xanthine oxidase and rabbit aldehyde oxidase and a comparative study of sulphide incorporation into the desulpho forms of these enzymes to give the functional forms. This reaction involves replacement of an o xo molybdenum ligand (Mo=O) by sulphido (Mo=S). Mo(V) EPR signals may be thought of as providing a series of fingerprints for structure in the vicinity of the molybdenum atom. By this criterion, examination [16] of the so-called Slow signals from the desulpho forms of the enzymes reveals the extreme similarity of their molybdenum centres, notwithstanding the substrate specificity differences. Results from the sulphide incorporation study are presented in Figure 1. Under the conditions of the experiments, sulphide incorporation was 20-fold lower for aldehyde oxidase than for xanthine oxidase. Interestingly, the pH optimum for this process was 2 pH units lower for the former enzyme than for the latter. This implies a difference in the pK„ values for charged groups in the vicinity of molybdenum that affect the approach of the sulphide ion. It seems likely that these charged groups are also the ones responsible for known differences in the pH optima of the enzymes, for the oxidation of positively charged substrates. Finally, although interpretation of EPR of the Rapid Mo(V) signals from the functional forms of the enzyme is complicated, the data point strongly [16] to identical molybdenum environments in the two enzymes, with only the substrate-binding regions differing in charged groups and probably otherwise also.

Figure 1

Effect of pH on the extent of resulphuration of the desulpho forms of xanthine oxidase and aldehyde oxidase

Samples of the desulpho forms of bovine milk xanthine oxidase (XO) or of rabbit liver aldehyde oxidase (AO) were treated aerobically with Na2S at the pH values indicated, then assayed for enzymic activity; complete reaction would give 100% functionality. The data are replotted from [29] and [16].
Although, because of the ready availability of the water. In the oxidized state the molybdenum variant showed it to have the unique property of the pterin. The catalytic reaction at molybdenum is formally of the type:

\[ \text{RH} + \text{H}_2\text{O} \rightarrow \text{ROH} \]

The oxygen atom that is incorporated into the substrate, RH (e.g. xanthine), to yield the product, ROH (e.g. uric acid), is known from isotopic studies to be derived ultimately from water. In the oxidized state the molybdenum atom of the enzyme was clearly shown (see [3] for references) some years ago by extended X-ray absorption fine structure (EXAFS) spectroscopy to bear an oxo as well as a sulphido ligand. This oxo ligand has been widely assumed [3,5,32-37] to be transferred in the catalytic cycle to the substrate and regenerated from water. Thus, xanthine oxidase has frequently been referred to as an 'oxo transfer' enzyme. However, our latest studies [37a] show that, on the contrary, the oxo group does not participate directly in the reaction. Furthermore, they reveal that there is transient formation in the catalytic cycle of a Mo-C bond, involving the xanthine C-8 position. This, together with our preliminary work [38], constitutes the first evidence for such a bond in a biological system. However, such bonds presumably also arise during reduction of acetylene and cyanide by nitrogenase. Cobalamines represent virtually the only other known example in biology of transition metal-carbon bonds.

Figure 2 shows the reaction mechanism at the molybdenum centre as we now propose it [37a]. Evidence favouring this mechanism will be summarized briefly. This mechanism, like earlier ones, is based in part on information concerning the structure of the enzyme species giving rise to the Mo(V) EPR signal known as Very Rapid. This signal corresponds to a kinetically competent, transient intermediate in enzyme turnover. Information on the structure of the signal-giving species has been based [3] on analysis of its EPR parameters, and in particular of the hyperfine coupling to various ligand atoms. Our new work involves, specifically, the analysis of \(^{13}\text{C}\)-hyperfine coupling of the C-8 carbon of the bound xanthine residue to Mo in the Very Rapid species. We used ENDOR spectroscopy rather than EPR, since this permits more detailed analysis of the hyperfine tensor and, in particular, facilitates extraction of the anisotropic component of the coupling, \(A_{\text{ano}}\). From this, in turn, after various corrections have been applied, the dipolar component can be deduced, and from it the molybdenum-carbon distance. Our best estimate of the Mo-C distance in the Very Rapid species from such measurements and calculations is about 2.3 Å [37a]. This is towards the upper limit for the length expected for such a bond. However, confidence in the reality of a Mo-C bond in this species is increased by two further findings. First, back-calculation of the expected value of \(A_{\text{ano}}\) from the structure usually assumed for the Very Rapid species, which involves a Mo-O-C linkage, indicates values that seem incompatible with the experimental data. Second, in another Mo(V) species from the enzyme, not on the catalytic pathway and known as Inhibited, a still shorter Mo-C distance of about 1.9 Å has been established [37a,38] by similar techniques. Such a distance is compatible only with a Mo-C bond.

Note that, in Figure 2, the Mo-C bond in the Very Rapid species is shown as part of a three-membered ring with oxygen. This is a known type of structure in molybdenum chemistry, referred to by organometallic chemists as 'side-on' or 'η^2' bonding. Figure 3 shows an example of a molybdenum complex having such a structure.

Evidence concerning the origin in the enzyme (see Figure 2) of the oxygen that appears in the uric acid comes from hyperfine coupling studies.
Figure 2
The proposed catalytic cycle for xanthine oxidation to uric acid by xanthine oxidase

The initial step involves formal addition of the elements of the substrate molecule across the Mo=S double bond, yielding a Mo(VI) species. This is followed by attack by a 'buried' water molecule (perhaps reacting as a Mo ligand), giving a reduced Mo species. Transfer of an electron to Fe-S and FAD gives the transient intermediate that gives the Very Rapid EPR signal. The cycle is completed by transfer of the second electron and dissociation of the uric acid product. Amino acid residues participating in the reaction are not included.

with $^{17}O$. ENDOR work using this isotope [37a], both on the Very Rapid species and on a structurally analogous species obtained with the inhibitor alloxanthine, was consistent with earlier EPR data [32,33,40] in providing no support for mechanisms that involve participation of the terminal oxo group. Such mechanisms require two coupled oxygens in the Very Rapid species, one as the oxygen destined to become that of the uric acid and the other as the terminal oxo group, regenerated by the action of water. By analogy, the corresponding species from alloxanthine should have one coupled oxygen. In fact, only one coupled oxygen is detected in the former species and none in the latter. These findings are consistent with the mechanism of Figure 2, which is closely related to one put forward earlier [41] on the basis of reactions of model compounds, but not consistent with an oxo transfer mechanism. The scheme of Figure 2 involves reaction not of the oxo group, but of a water molecule (buried in the protein to explain its relatively slow exchange in the oxidized enzyme). This must be in the vicinity of molybdenum, and could react as a ligand of the metal. These mechanistic conclusions are supported by new kinetic studies [37a].
The structure of the complex \([\text{Mo}^{n+} \text{C}_2\text{H}_2(\text{en})\text{H}_2\text{O}]\)

The structure is reproduced with permission from Gambarotta et al. [39]. The CO group of formaldehyde is bonded side-on to Mo, with the metal in a lower oxidation state. Copyright 1985 American Chemical Society.

of oxygen exchange and transfer in xanthine oxidase, using EPR and mass spectrometry.

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Molecular evolution and substrate specificity of acyl-CoA dehydrogenases: chimaeric ‘medium/long’ chain-specific enzyme from medium-chain acyl-CoA dehydrogenase

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Acyl-CoA dehydrogenases (ADHs) from mammalian mitochondria catalyse the \( \alpha, \beta \)-dehydrogenation of fatty chain acyl-CoA conjugates, the first and rate-limiting step in the \( \beta \)-oxidation cycle:

\[
\begin{align*}
\text{R-CH}_2\text{-CH}_2\text{-COS-CoA} + E, & \rightarrow \\
\text{R-CH}=\text{CH-COS-CoA} + E,\text{dH}_2
\end{align*}
\]

They are members of a superfamily to which belong also peroxisomal acyl-CoA oxidases (AOX) [1,2] and enzymes involved in the catalysis of branched-chain amino acids. Related enzymes from lower organisms, which are assumed to function by the same basic chemical mechanism, can also be appended to this family. The acyl-CoA dehydrogenases differ in their specificity for the configuration of the substrate acyl chain. The three-dimensional structure of medium-chain acyl-CoA dehydrogenase (MCADH) [3–5], probably the best studied member of the family, and that of a bacterial butyryl-CoA dehydrogenase [6] have recently been resolved. That of the long-chain enzyme (LCADH) has been modelled on the structure of MCADH [7]. The sequences of approx. three dozen enzymes belonging to the superfamily have been reported and are available from public databases [8].

While the chemical mechanism of the \( \alpha, \beta \)-dehydrogenation is quite clear [9–11], we are only at the beginning of understanding the various modes by which the protein activates the substrate(s) and brings about catalysis [11,12]. However, this is only one main topic of current research with ADHs. A second point of interest deals with the molecular mechanisms that bring about chain length specificity of ADHs. This point will be addressed to some extent in this contribution.

Functional groups of catalytic importance

Substrate \( \alpha, \beta \)-dehydrogenation is initiated by abstraction of the substrate \( \alpha \)-H as \( \text{H}^+ \) by a base, which has turned out to be a glutamate-\( \text{COO}^- \) in all enzymes studied to date. Most interestingly, the position of this Glu in the sequence is not


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