Human xanthine oxidoreductase: in search of a function
R. Harrison
School of Biology and Biochemistry, University of Bath, Bath BA2 7AY, U.K.

The molybdoenzyme, xanthine oxidoreductase (XOR), has been much studied, largely because of its ready availability on a large scale from cows’ milk [1]. It catalyses the oxidation of a wide range of substrates, most notably hypoxanthine and xanthine, generating xanthine and uric acid respectively, in the process of purine catabolism. The enzyme is a homodimer of 150 kDa subunits and exists in two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204) and xanthine oxidase (XO; EC 1.1.3.22). XDH preferentially reduces NAD', whereas XO does not reduce NAD', preferring molecular oxygen. Reduction of oxygen by either form of the enzyme yields O_2^- and H_2O_2, and it is the capacity to generate such reactive oxygen species (ROS) that has led to a great deal of interest in XOR as a pathogenic factor in many instances of ischaemia–reperfusion injury [2]. More recently, an increasing body of evidence suggests a role for ROS in normal signal transduction [3–5].

The commonly cited mechanism involving XOR in ischaemia–reperfusion injury [6,7] can be summarized as follows. During ischaemia the cell’s energy charge falls and transmembrane ion gradients are dissipated, leading to elevated cytosolic concentrations of Ca^{2+}. This in turn activates a protease that irreversibly converts XDH, predominant in vivo, into XO. Concurrently, depletion of cellular ATP leads to AMP, which is catabolized successively to adenosine, inosine and finally hypoxanthine. Hypoxanthine accumulates and serves as reducing substrate for the newly converted XO, which, on readmission of molecular oxygen to the tissues, catalyses the generation of O_2^- and H_2O_2. This sequence of events is summarized in Figure 1.

The above pathogenic mechanism is based on the properties of the well-characterized bovine milk [1], rat [8] and chicken [9] liver enzymes, and, although results obtained in experimental animal systems are commonly extrapolated, at least implicitly, to humans, relatively little is known about the human enzyme.

We have purified human XOR from breast milk and found it to have surprising properties. In a preparation procedure [10] refined from that originally reported [11], frozen breast milk is thawed and the separated cream is washed to remove membrane-associated XOR. The enzyme is solubilized by addition of butanol and subjected to (NH_4)_2SO_4 fractionation before sequential heparin and ion-exchange chromatography. By these means, 10–20 mg of human XOR are obtained from 1500 ml of breast milk. These yields are comparable with those obtained by similar procedures from fresh cows’ milk [10].

The resulting human XOR is highly purified. On SDS/PAGE it runs as a single band, corresponding to approx. 150 kDa. The UV/visible spectrum is very similar to that of pure bovine milk enzyme [1] and, like pure enzyme from other sources, shows a protein (A_280) to flavin (A_450) ratio of 5.0–5.5 [1]. Unexpectedly, the activity towards most reducing substrates is very low, representing in the case of xanthine only 2–3% that of purified bovine milk enzyme.
Xanthine Oxidase: Enzymology and Pathophysiology

Mechanism for production of ROS during ischaemia-reperfusion

Adapted with permission from reference [7].

[10]. This low catalytic activity results primarily from an exceptionally low molybdenum content of the human enzyme [12]. XOR is known to contain three major redox sites, comprising a molybdenum, an FAD and two iron-sulphur centres, and it is directly to the molybdenum centre that most reducing substrates donate their electrons. XOR characterized from other sources, such as bovine milk or rat liver, often contains some 30–40% 'demolybdo' enzyme, lacking the molybdenum atom. Thus, the molybdenum content of a typical purified preparation of bovine milk XOR examined recently was approx. 65% of that predicted on the basis of one Mo per 150 kDa subunit. The corresponding value for purified human milk XOR is 2–5% [12]. The situation is further complicated by the occurrence of a second inactive form of XOR, 'desulpho' enzyme, in which an Mo=S grouping, essential for activity, is replaced by Mo=O. Desulpho XOR often makes up 30–40% or more of the molybdenum-containing bovine milk enzyme and, according to our preliminary EPR data [12], accounts for at least this percentage of molybdenum-containing human milk XOR. In summary then, purified human milk XOR appears to contain only 2–5% of the 'theoretical' Mo (cf. e.g. 65% for bovine milk XOR) and about 1% of 'theoretical' activity towards xanthine (cf. approx. 40% for bovine XOR).

What about XOR in other human tissues? Levels of XOR enzymic activity have been reported to be very low in most human tissues [13–15]. Indeed, immunoaffinity purification of XOR from human heart yielded a protein with specific activity towards xanthine similar to that of purified human milk XOR [16,17]. Liver and intestine, on the other hand, may differ from most other human tissues in that they show relatively high xanthine oxidase activity [13,15], and enzyme purified from liver showed specific activity very similar to those of bovine milk and rat liver enzymes [18]. It appears possible therefore that human XOR might be tissue-specific, with 'high-activity' enzyme in a limited number of tissues (e.g. liver and intestine) and 'low-activity' enzyme, similar to that in breast milk, in the rest.

The question clearly arises as to the function of low-activity human XOR, predominant in breast milk and most probably in other tissues. It is conceivable that the enzyme might be subjected to some forms of post-translational activation. Desulpho–sulpho enzyme conversion of bovine milk XOR, with resultant activation, has been effected in vitro by incubation of reduced enzyme with sulphide ion [19], and in vivo enzymic activation in response to increased protein diet has been attributed to desulpho–sulpho conversion in chickens [20] and rats [21]. We ourselves have evidence of similar post-translational up-regulation of XOR in the human epithelial cell line, HB4a [22]. A number of cytokines, including interleukin 1, interleukin 6, tumour
necrosis factor α and interferon γ, stimulate XOR activity in HB4a cells. In the case of interferon γ, activity towards pterin (which acts at the Mo site) is increased by 8–10-fold. Under the same conditions, XOR-specific mRNA is increased 2–3-fold, as is XOR protein, (determined by ELISA). This clearly suggests the involvement of post-translational activation of XOR by 2–3-fold, a size of increase that could plausibly be explained by desulpho–sulpho conversion.

We have found further evidence of in vivo post-translational activation that, in this case, is too great to be readily explained on the basis of desulpho–sulpho conversion. XOR enzymic activity (to hypoxanthine or pterin) was determined in serial samples of breast milk obtained from 14 mothers during the first few weeks post partum [23]. In all cases, enzyme activity reached a peak at some point during the first 15 days and fell thereafter, in some cases by as much as 98%, to basal levels that were subsequently largely maintained. Corresponding changes in XOR protein levels were not observed and consequently the specific activity of XOR followed the above pattern, with differences between peak and basal levels of up to 50-fold. On the basis of the properties of human milk XOR, post-translational changes of such magnitude are unlikely to be explained by desulpho–sulpho interconversions alone, and the possibility of another means of regulating human XOR activity in vivo, with implications for control of ROS production, is intriguing.

Apart from the latter example of in vivo up-regulation, the specific activity of our human XOR with conventional substrates is generally very low, a fact that poses problems in the context of its role as a source of ROS in many human tissues. We were accordingly prompted to investigate the capacity of human XOR to oxidize the alternative substrate, NADH [10]. NADH oxidase activity is possessed by XORs from other sources [24–26], but is not generally recognized and has been relatively little studied. NADH differs from other reducing substrates in donating its electrons directly to the FAD site, rather than to Mo [1,27], and so might be expected to be unaffected by variations in the latter site. Indeed, the NADH oxidase activity of purified human milk XOR was found to be generally very similar to that of the bovine enzyme. Both enzymes, particularly in their dehydrogenase forms, XDH, are capable of oxidizing NADH to form O₂⁻ in the presence of molecular oxygen, at appreciable rates. O₂⁻ production by human (Figure 2) and bovine XDH shows apparent substrate inhibition, which can be explained in terms of a model involving two-stage recycling of oxidized enzyme [10]. For either enzyme, the maximal rate of O₂⁻ production is approx. 0.2 μmol/min per mg. In the case of human XOR, this value is some four times greater than that (0.05 μmol/min per mg) for the oxidase form (XO) in the presence of xanthine, the rate that is relevant to ROS production according to the hypothesis outlined in Figure 1. These data suggest an alternative mechanism for generation of ROS during ischaemia–reperfusion in at least some human tissues. According to such a mechanism (Figure 3), ischaemia leads to a build up of NADH, and it is the accumulation of reducing substrate that serves to trigger ROS production when oxygen returns in reperfusion. Levels of NADH in the cytosol of normal rat hepatocytes have been estimated to be approx. 1 μM [28] and, in the perfused rat heart, to rise some 5–6-fold in ischaemia [29], approximately the range over which the rate of O₂⁻ production rises steeply (Figure 2). These changes refer to constant oxygen levels and it is worth noting that the dependence of O₂⁻ production on oxygen concentration is linear over the range that would be expected in transitions from ischaemia to normoxic tissue [10]. A further factor is pH. All the rates quoted above refer to pH 7.4. At pH 6.5, possibly more relevant to ischaemia tissues [30], the preference of human XOR for oxidation of

![Figure 2](image-url)

**Figure 2**

O₂⁻ production by human XDH

Adapted with permission from reference [10]. The fitted curve is based on a model involving two-stage recycling of oxidized enzyme [10].

![Graph](graph-url)

[NADH] (μM)

Activity [μmol min⁻¹ mg⁻¹]
NADH as opposed to xanthine is further enhanced. There are clearly dangers in over extrapolation from these data, which will certainly be complicated by inhibition resulting from high levels of NAD⁺ in the cytosol, under both normoxic and ischaemia conditions [28,31,32]. Nevertheless, the mechanism of Figure 3 appears to offer an alternative means of ROS production and has the advantage of not requiring XDH into XO conversion, both the extent and time scale of which are controversial [33-37].

Although the scheme of Figure 3 is particularly attractive for some human tissues, the situation is less clear for other mammals and possibly also for human liver and intestine, where activities towards xanthine are much higher. Thus in our studies [10], the maximal rate of O₂⁻ production from bovine XO in the presence of xanthine is 1.7 μmol/min per mg (cf. 0.05 μmol/min for human XOR), some 8-fold higher than from bovine XDH in the presence of NADH. There are, nevertheless, many complicating factors, some of which are mentioned above, and it may well be that both mechanisms, outlined in Figures 1 and 3, contribute more or less to ROS generation in ischaemia–reperfusion depending on the system.

There is growing evidence for the involvement of ROS, not only in ischaemia–reperfusion injury and other disease states [2,38,39] but also in normal signal transduction [3-5]. In most of these instances, the source of ROS is uncertain, although XOR is commonly cited as a candidate. The enzymic activity considered is invariably oxidation of hypoxanthine or xanthine and its involvement is examined in terms of the effects of inhibitors, such as allopurinol or oxypurinol [40], although other inhibitors such as amfutizol [41] or BOF 4272 [42] have also been used. All these inhibitors block the molybdenum centre of XOR and do not affect NADH oxidation [10]. Similar considerations apply to studies based on tungsten incorporation [43]. Accordingly, it is likely that ROS generation resulting from NADH oxidase activity of XOR would not have been detected in most systems studied, and this source of ROS may be more widespread than generally appreciated. There is currently no specific inhibitor of this activity. It is strongly blocked by diphenyleneiodonium [10], which is, however, a relatively non-specific inhibitor of flavoenzymes, including NADPH oxidase [44,45]. It is worth noting that the effects of adding diphenyleneiodonium to an experimental system might result from inhibition of XOR-associated NADH oxidase activity, a fact not commonly considered.

In summary, the low specific activity with conventional reducing substrates of XOR from breast milk and possibly from other human
tissues raises questions about the enzyme’s function in general and particularly as a source of ROS. Evidence is presented for post-translational activation of the human enzyme, both \textit{in vitro} and \textit{in vivo}, a potential for regulation that is attractive in the context of a source of ROS. Our attention has also been focused on the NADH oxidase activity, which is common to XORs of all species studied and is ‘unimpaired’ in the case of the human milk enzyme. Steady-state kinetic analysis suggests that resulting rates of O$_2$ production could be a significant factor in ischaemia–reperfusion injury and could also serve to generate ROS under normal physiological conditions. Such NADH oxidase activity in experimental systems may well have been overlooked in the past because of its independence of the molybdenum redox site, itself the target of most XOR inhibitors.

Much of the work described has been supported by the Medical Research Council in a project grant to the author together with Dr. R. Eisenthal and Dr. A. Wolstenholme (Bath University) and Professor R. C. Bray (Sussex University). Further support has been obtained from the BBSRC, The Wellcome Trust and the Arthritis and Rheumatism Research Council. The latter funding was held jointly with Professor D. Blake and Dr. C. Stevens of the Royal London Hospital.

1 Bray, R. C. (1975) Enzymes 3rd Ed. 12, 299–419
Xanthine oxidoreductase gene: structure and regulation
M. Terao, M. Kurosaki, S. Zanotta and E. Garattini*
Molecular Biology Unit, Centro Catullo e Daniela Borgomanierio, Istituto di Ricerche Farmacologiche ‘Mario Negri’, via Eritrea, 62. 20157 Milano, Italy

Introduction
Xanthine oxidoreductase (XOR) is the key enzyme in the catabolism of purines, catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. XOR exists in two interconvertible forms known as xanthine dehydrogenase (XDH) and xanthine oxidase (XO) [1,2]. XDH transfers the reducing equivalents generated by the oxidation of the substrates to NAD⁺, whereas XO transfers them to molecular oxygen producing O₂⁻ anions. The enzyme is a cytosolic molybdoflavoprotein consisting of two identical subunits of approx. 150 kDa each [3,4]. The monomeric subunit contains two Fe–S redox centres, an NAD⁺ and a molybdenum cofactor-binding site. The last feature places XOR into the small family of mammalian molybdenum-containing proteins along with aldehyde oxidase (AO) [5,6] and sulphite oxidase.

In this article, we review some of the data obtained in our laboratory in the last few years on XOR structure and regulation. In the first part, we present data on the sequence similarity between XOR and AO, using primary structural data obtained for the two bovine enzymes. We also discuss the differences in tissue and cell distribution between the two enzymes. In the following sections, we briefly describe the structure of the XOR gene and its regulatory sequences. Finally, we provide evidence that XOR is a highly regulated enzyme, taking XOR gene expression in the mammary gland as an example.

Similarity of XOR to AO
The level of amino acid identity among XOR species is very high [7], indicating a remarkable level of conservation. A thorough discussion of the topic is beyond the scope of this article. Sufficient to say that the similarity among XORs extends throughout the length of the protein, although hot spots of amino acid identity are located in the N-terminal region, where the two Fe–S centres are located, in the molybdenum cofactor and in the substrate-recognition domains.

The only other protein with which mammalian XORs show a remarkable level of similarity is AO, another molybdoflavoprotein, the complete amino acid sequence of which has been recently elucidated in the cow [6]. Interestingly, AO has substrate specificity, subunit composition and redox centre distribution similar to that of XOR [8,9]. To perform a sequence alignment between XOR and AO in the same animal species, we cloned the full-length XOR cDNA from bovine liver. Except for eleven substitutions (residue 200 K/E; residue 367 V/M; residue 553 D/H; residue 959 K/R; residue 977 E/Q; residue 1040 E/G; residue 1244 V/L; residue 1268 P/A; residue 1279 A/R; residue 1280 R/A; residue 1281 G/A), one insertion (residue 189 Q) and one