

## STRUCTURE AND FUNCTION OF XANTHINE OXIDOREDUCTASE: WHERE ARE WE NOW?

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**Abstract**—Xanthine oxidoreductase (XOR) is a complex molybdoflavoenzyme, present in milk and many other tissues, which has been studied for over 100 years. While it is generally recognized as a key enzyme in purine catabolism, its structural complexity and specialized tissue distribution suggest other functions that have never been fully identified. The publication, just over 20 years ago, of a hypothesis implicating XOR in ischemia-reperfusion injury focused research attention on the enzyme and its ability to generate reactive oxygen species (ROS). Since that time a great deal more information has been obtained concerning the tissue distribution, structure, and enzymology of XOR, particularly the human enzyme. XOR is subject to both pre- and post-translational control by a range of mechanisms in response to hormones, cytokines, and oxygen tension. Of special interest has been the finding that XOR can catalyze the reduction of nitrates and nitrites to nitric oxide (NO), acting as a source of both NO and peroxynitrite. The concept of a widely distributed and highly regulated enzyme capable of generating both ROS and NO is intriguing in both physiological and pathological contexts. The details of these recent findings, their pathophysiological implications, and the requirements for future research are addressed in this review. © 2002 Elsevier Science Inc.

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### INTRODUCTION

Xanthine oxidoreductase (XOR) is a complex molybdo-flavoenzyme that is readily available from cows' milk, where it forms a major component of the milk fat globule membrane (MFGM) [1,2]. Largely because of this availability, XOR has been known for 100 years and studied in its essentially pure form for over 60 years [3]. The enzyme has consequently become a model for structural and mechanistic studies of molybdoenzymes in general, involving the application of electron spin resonance, x-ray absorption fine structure, and many other physico-

chemical techniques [4,5]. The last few years have seen a dramatic increase in molecular information, with determination of its cDNA and gene sequences and publication of its three-dimensional structure.

XOR is generally recognized as the terminal enzyme of purine catabolism in man, catalyzing the hydroxylation of hypoxanthine to xanthine and of xanthine to urate. Until recently, little was known of the human enzyme, although inherited XOR deficiency, xanthinuria, has long been recognized, and, puzzlingly, known to be asymptomatic [6,7]. Some 20 years ago, attention was directed toward the physiological and pathological significance of XOR by Granger, McCord, and colleagues, who proposed a role for XOR-derived reactive oxygen species (ROS) in ischemia-reperfusion (IR) injury [8–10]. Their hypothesis, which generated several hundred publications, undoubtedly helped to stimulate interest in XOR as a source of ROS, not only in many pathological states, but also in signal transduction generally. As recognition of the pathophysiological involvement of XOR increases, so does the need to understand its enzymology, kinetics, and control. As noted above, our structural knowledge of XOR has markedly increased in recent years. Moreover, a great deal of new information has

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become available concerning species and tissue variations of the enzyme, its substrate specificity, and regulation. For these reasons, it seems to be appropriate to review recent developments and to attempt to inter-relate them.

In general, the present survey focuses on works published in the last 10 years, and this is reflected in the reference list, which, wherever possible, covers earlier work by quoting more recent, especially review, articles. In view of the potential clinical relevance of much of the work described, emphasis is given to studies using mammalian, particularly human, enzyme and tissues.

## DISTRIBUTION

### *Assays in tissues*

XOR activity has been detected in all species examined, including bacteria [11,12]. In mammalian tissues, activity is widely distributed, with highest levels being found in liver and intestine [12]. There is, however, considerable species variation, as exemplified by the wide range of levels in blood [12] and heart [13,14]. In humans, apart from liver and intestine, most tissues show little XOR activity [12–17]. XOR mRNA levels were also found to be highest in liver and intestine of mouse [18] and man [17], with low but detectable levels in most other organs examined. In mice, XOR protein, determined by immunoblotting, followed the same pattern [18].

### *Histochemical studies*

Jarasch and coworkers [19] were the first to address in detail the cellular distribution of XOR. In immunolocalization studies, using rabbit anti-bovine MFGM antibodies, they showed the presence of the enzyme in epithelial and capillary endothelial cells of mammary gland and in capillary endothelial cells of a range of other bovine tissues. Endothelia of larger blood vessels were not stained, nor were intestinal epithelial cells or hepatocytes. The same group [20] subsequently confirmed the above localizations in both bovine and human tissues by using naturally occurring human anti-XOR antibodies (see “Circulating XOR”).

Subsequent histochemical studies in other species have failed to confirm many aspects of this early work. Thus, XOR activity [21,22] and protein [23] have been detected in epithelial cells of a wide range of rat tissues, particularly those of the alimentary and respiratory tracts. Moreover, the presence of XOR in hepatocytes of rat [21,23–25] and chicken [26] has been demonstrated by both immunochemical and activity stains.

Human tissue has been less studied, but again, results vary. Moriwaki et al. [27] used polyclonal anti-(human

liver XOR) antibodies to stain post-mortem tissue slices and detected XOR in a wide range of cells throughout the digestive tract, in the liver, and in many other tissues. On the other hand, Linder and colleagues [28], using polyclonal anti-(human milk XOR) antibodies and biopsied tissue, found a more limited distribution. XOR was detected mainly in hepatocytes and Kupfer cells of the liver, in enterocytes and goblet cells of jejunum, and in the mammary gland. Additionally, the enzyme was present in capillary endothelial cells of jejunum, skeletal muscle, and kidney. These latter findings are essentially in accord with those from activity staining of biopsy material, which reported XOR only in liver and intestine [29]. They also agree with Hellsten-Westing's [30] report that, in human skeletal muscle, the enzyme is confined to capillary endothelium. Hellsten-Westing [30] detected XOR also in human macrophages and mast cells; the former location being subsequently confirmed by DeJong et al. [31].

We ourselves have used a mouse monoclonal antibody raised against purified human milk XOR to study biopsied tissue from human liver and intestine (H. Martin, D. Tosh, and R. Harrison, unpublished data). Hepatocytes were found to be variably stained, most strongly in the periportal region, while sinusoidal endothelial and Kupfer cells were not stained. Most interestingly, bile duct epithelial cells were strongly stained, particularly toward the apical region, implying secretion of XOR into the bile. In the intestine, surface epithelial cells were found to be strongly positive for XOR, consistent with previous studies. The enzyme was also detected in the tight junctions between cultured human gut epithelial cells [32].

The presence and role of XOR in plasma and serum is dealt with separately in “Circulating XOR.”

Histochemical localization of XOR has recently been reviewed usefully [33,34].

### *Subcellular localization*

Subcellular localization of XOR has also been subject to debate. Jarasch and coworkers [19] reported the enzyme to be exclusively cytosolic in endothelial cells of bovine tissue.

Subsequent ultrastructural studies showed XOR enzymic activity in peroxisomes of rat hepatocytes [35,36], but these findings were questioned by Ichikawa and coworkers [24], whose immunogold labeling showed enzyme protein to be present in the cytosol only.

More recently, confocal microscopic studies of cultured human endothelial cells, using fluorescent affinity-purified polyclonal anti-human milk XOR antibodies, showed XOR to be present not only throughout the cytoplasm but also on the outer surface of the cell mem-

brane [37]. The enzyme was assymmetrically located on the cell surface, showing, in many cases, a higher intensity on those faces apposed by closely neighboring cells. XOR has also been detected on the outer surface of cultured bovine and porcine [38] endothelial cells and at the luminal surface of rat liver sinusoidal endothelial cells [39].

In the confocal studies noted above [37], cytoplasmic XOR was seen to be concentrated in the perinuclear region and consistently showed punctate staining suggestive of a vesicular location. Subsequent use of mouse monoclonal antibodies, in a range of human endothelial and epithelial cells, has confirmed this apparent localization of XOR to intracellular vesicles [32]. While their function is unknown, it may be speculated that they serve to store XOR prior to its export from the cell

#### Possible reasons for discrepancies

Clearly many of the above discrepancies reflect differences in labeling procedures. Particularly in human tissue, studies based on XOR activity often correlate poorly with those based on enzyme protein, most probably because of the occurrence of XOR in inactive forms, such as desulfo- or demolybdo-XOR (see "Properties of Purified XOR"). It is possible that proportions of inactive forms will depend not only on the tissue, but also on the exposure of that tissue to factors such as ischemia, inflammation or infection (see "Regulation"). With regard to immunolocalization, differing specificities and avidities of antibodies, both polyclonal and monoclonal, need to be considered. Clare and Lecce [40] have made the useful observation that immunoglobulins can purify with XOR, with consequent complications in the use of antisera raised against contaminated enzyme. It is also important to ensure that anti-XOR antibodies do not cross react with the closely related enzyme aldehyde oxidase [23]. In view of the wide distribution of XOR mRNA in both mouse [18] and human [17] tissues, it is likely that the expression of not only enzymic activity but also protein will be influenced by a range of factors affecting the tissue chosen for experimentation (see "Regulation").

Reasons for discrepancies in the literature concerning localization of XOR, particularly those involving activity staining procedures, have been reviewed by Kooij [41].

### PROPERTIES OF PURIFIED XOR

#### Mammalian and avian XOR

The best-characterized form of XOR is undoubtedly that purified from bovine milk [1,3]. XOR has also been purified from rat [42,43], chicken [44,45], and turkey

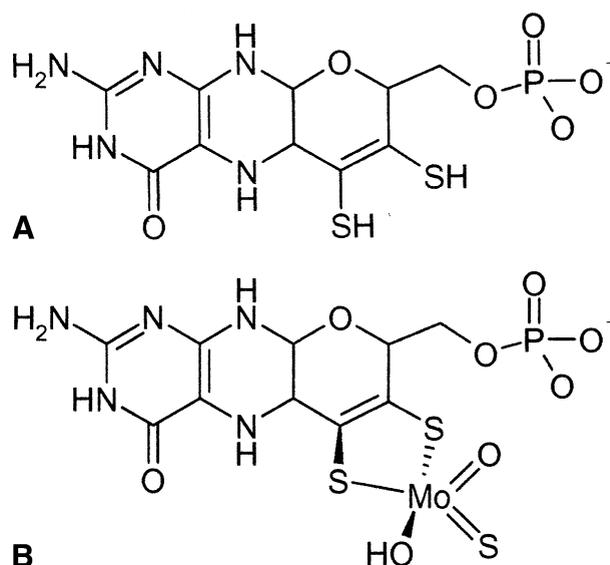


Fig. 1. (A) Molybdopterin (B) The molybdenum cofactor, Mo-co, comprising Mo ligated to molybdopterin through the sulfur atoms of the dithiolene group. The active, sulfo-form of the cofactor is shown. In the inactive, desulfo-form the Mo=S grouping is replaced by Mo=O.

[46] livers and from mouse mammary gland [47]. While these preparations are less well characterized than that from bovine milk, their properties are generally similar to those of the latter. In all cases, XOR occurs as a homodimer of approximately 300 kDa; each subunit contains four redox centers; *viz.* a molybdenum cofactor (Mo-co), one FAD and two  $\text{Fe}_2\text{S}_2$  sites [1,5]. The Mo-co comprises an organic pterin derivative (molybdopterin), containing a cyclized dithiolene side chain (Fig. 1A), together with one Mo atom. The Mo is pentacoordinated by the two dithiolene sulfur atoms of molybdopterin, by a further sulfur and two oxygen atoms (Fig. 1B).

The mammalian enzyme exists in two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204), which predominates *in vivo*, and xanthine oxidase (XO; 1.1.3.22). These forms can be interconverted reversibly by sulphide reagents or irreversibly (XDH to XO) by proteolysis [48]. The avian enzymes, in contrast, occur only in the XDH form. XDH preferentially reduces  $\text{NAD}^+$ , whereas XO cannot reduce  $\text{NAD}^+$ , preferring molecular oxygen. Reduction of molecular oxygen by either form of the enzyme yields superoxide and hydrogen peroxide and it is the capacity of XOR to generate such ROS that is of major interest in clinically related studies.

In addition to hypoxanthine and xanthine, XOR catalyzes the hydroxylation of a wide range of N-heterocyclic and aldehyde substrates. It can also act as an NADH oxidase [1]. As shown schematically in Fig. 2, most reducing substrates act at the Mo site although, uniquely, NADH donates its electrons to FAD [1,49]. Following

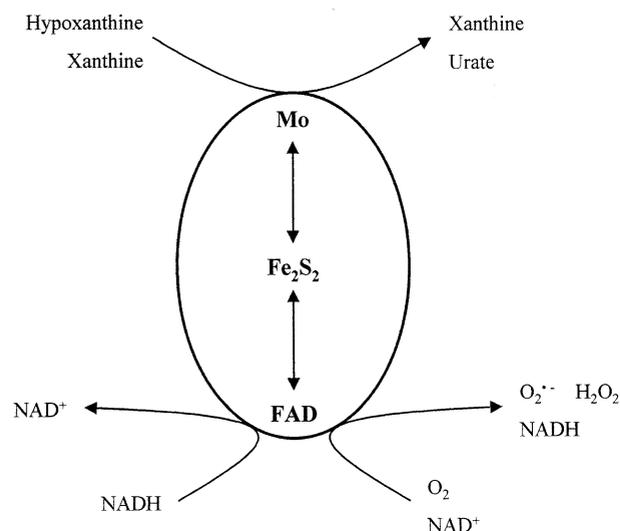


Fig. 2. Schematic diagram showing XOR-catalyzed oxidation of xanthine and hypoxanthine (typifying most reducing substrates) at the Mo site, and of NADH at the FAD site. Reduction of  $\text{NAD}^+$  or molecular oxygen takes place at FAD.

rapid equilibration between the redox centers, electrons are usually passed on, at the FAD center, either to  $\text{NAD}^+$  or to molecular oxygen (Fig. 2). As for reducing substrates, specificity is low for oxidizing substrates, which include dyes such as methylene blue and 2,6-dichlorophenolindophenol, ferricyanide, and many quinones [1]. Compared with  $\text{NAD}^+$  and oxygen, however, these have been little studied.

The catalytic mechanisms of XOR have been reviewed comprehensively [5].

Very recently, XOR has been shown to catalyze the reduction of nitrates and nitrites to nitrites and nitric oxide (NO), respectively. Inorganic nitrates and nitrites have been shown to accept electrons from the Mo site, in contrast to organic nitrates, which are reduced at the FAD site (see "XOR-Catalyzed Generation of NO").

As much as 60% of purified bovine milk XOR is inactive toward conventional reducing substrates such as xanthine [1,50,51]. This "inactive" enzyme is made up of two forms. Demolybdo-XOR lacks Mo (possibly also

molybdopterin) and commonly constitutes about 40% of total enzyme, while some 30–40% of the remaining Mo-containing enzyme is desulfo-XOR, in which the  $\text{Mo}=\text{S}$  grouping (Fig. 1B), essential for catalytic activity, is replaced by  $\text{Mo}=\text{O}$  [52,53]. There is evidence for the presence of desulfo-XOR also in rat [43,54], chicken [55], and turkey [56] livers (see "Regulation"). Deflavo-XOR lacks FAD and has not been reported to occur naturally. Information concerning inactive forms of XOR is summarized in Table 1.

Expression of active recombinant XOR has proved to be difficult. Nishino and colleagues have extensively studied the expression of rat liver XOR in the baculovirus-insect cell system. While the native and recombinant proteins were identical in size, the latter was largely inactive, comprising a mixture of demolybdo- and other inactive forms [63,64]. Transient expression of human XOR has been briefly reported in COS-1 cells, lysates of which showed enzyme activity. There was, however, no indication of the content of inactive forms [65]. Perhaps most promising are the recent reports of the expression, in *Aspergillus nidulans*, of *Drosophila* XOR with enzyme activity indistinguishable from that of the native enzyme purified from fruit flies [66,67].

### Human XOR

Most discussions of the pathophysiological roles of XOR are based on the properties of the bovine milk or rat liver enzymes and results obtained with experimental animals are commonly extrapolated to humans.

Until relatively recently, the only reported purification of human XOR was that of Krenitsky et al. [68], who described a preparation from post-mortem liver with xanthine oxidase activity (1800 nmol urate/min/mg) and other properties very similar to those of the bovine milk enzyme. XOR from breast milk has since been characterized [69,70] and shown to have surprisingly low activity toward "conventional" reducing substrates. Thus, activity to xanthine is only about 5% that of purified bovine milk XOR; a fact that can be largely explained in terms of an exceptionally low Mo content of the human

Table 1. Occurrence and Interconversion of XOR Forms

Form of XOR	Occurrence in vivo	Interconversion in vitro
XDH	approx. 80% XOR [57,58]	$\text{XDH} \rightarrow \text{XO}$ , proteolysis (irreversible) sulfhydryl reagents (reversible) [48]
XO	approx. 20% XOR [57,58]	$\text{XO} \rightarrow \text{XDH}$ , sulfhydryl reagents [48]
Demolybdo	0?–95% [43,51,59]	Incorporation of Mo not generally possible, but see [60]
Desulfo	approx. 40% of molybdo form [43,51,56]	Sulfuration [53], desulfuration [61]
Deflavo	0%	Flavo $\rightarrow$ deflavo [62]

enzyme. Purified human milk XOR contains at least 95% demolybdo-enzyme, compared with 30–40% for the bovine milk enzyme [50,51,71]. Interestingly, lack of Mo in human milk XOR is accompanied by deficiency in  $\text{Fe}_2\text{S}_2$  clusters, primarily Fe/S I (see “Structure”), which is, to some extent, replaced by a new cluster type, Fe/S III [71], first observed by Rajogopalan and coworkers in bovine milk XOR [72]. It seems likely that Mo deficiency in human milk XOR results immediately from lack of incorporation of molybdopterin. Absence of cofactor might be expected to lead to major changes in conformation of the apoprotein and consequently in the nature of the neighboring  $\text{Fe}_2\text{S}_2$  groups. The possible significance of variable molybdopterin incorporation will be discussed in “Regulation.”

The extent to which XOR activity is tissue-dependent is of interest [73]. XOR activity is known to be relatively high in liver and intestine and low in most other human tissues [12,16,29], a fact that could reflect differences in XOR protein levels and/or specific activity. That the latter is at least a factor was suggested by the, respectively, high (1800 nmol urate/min/mg) and low (50–100 nmol urate/min/mg) specific activities of XOR purified from human liver [68] and milk [51]. This idea was reinforced by evidence of low activity XOR in human heart [74] and cultured human mammary epithelial cells [75]. Recent findings, however, suggest that the “bovine-level” activity reported for purified human liver XOR [68] may not reflect the situation in vivo. Thus, immunoaffinity purification of XOR from human liver [76] gave enzyme with much lower specific activity (200 nmol/min/mg). The purification of human liver XOR described by Krenitsky et al. [68] depends on affinity-chromatography using a guanine analogue, which may be expected to bind only active enzyme. It is, accordingly, possible that the lower activity immunoaffinity-purified enzyme is more truly representative of the situation in liver tissue.

On the other hand, by comparing XOR protein (enzyme-linked immunosorbant assay [ELISA]) and activity levels in human tissue homogenates, Sarnesto et al. [16] obtained true (molecular) specific activities of 2700–3000 nmol/min/mg (liver and intestine) and 600 nmol/min/mg (breast milk). While these data clearly support the idea that the true specific activity of human XOR is tissue-dependent, the values appear to be high; those for liver and intestine being high even by bovine standards, and that for breast milk being some 6-fold higher than that found in many samples of purified enzyme [51]. Similar approaches have, in our hands, also led to questionably high values for specific activities of a range of XOR preparations, suggesting that ELISA may tend to underestimate XOR protein [76]. Unfortunately, activities of enzymes purified by immunoaffinity proce-

dures are also subject to some doubt, in view of possible inactivation during sometimes stringent elution conditions [76]. On balance, it seems that the true specific activities of human XOR, while highest in liver and intestine, are generally much lower than in other mammals. This issue, nevertheless, deserves further study.

## STRUCTURE

The structure of the genetic loci coding for human [77] and mouse [78] XOR have been elucidated and the respective genes have been assigned to chromosomes 2p22 [79] and 17 [78]. These mammalian genes contain 36 exons and the exon-intron structure is highly conserved, differing greatly from those of the *Drosophila* or *Calliphora* genes, which are more compact, with only four or five exons [80].

Of mammalian and avian XORs, cDNA sequences have been reported for rat [81], mouse [82], chicken [83], cow [84], cat [85], and man [86–89]. The sequence of chicken XOR corresponds to 1358 amino acids, and is slightly longer than those of the mammalian enzymes, which range from 1330–1335 and show approximately 90% homology among themselves.

Despite initial apparent discrepancies, a single sequence has now been agreed upon for XOR of human liver and small intestine. The cDNA sequence (Genbank D11456) originally reported for human liver XOR [86] differed from that (Genbank U39487) subsequently cited for human small intestine XOR [89] at 5 bases. However, D11456 has been updated (2000) and is now identified to U39487. XOR of human milk also has very recently been shown to share the same sequence [90].

Nishino and coworkers showed that proteolytic cleavage of rat and chicken XOR led to three major domains of approximately 20, 40, and 85 kDa, associated with the  $\text{Fe}_2\text{S}_2$ , FAD and molybdenum cofactors, respectively [81,83,91].

A major advance in our understanding of XOR came with the elucidation of the crystal structure of the enzyme, aldehyde oxidoreductase (MOP), from the Gram-negative bacterium, *Desulfovibrio gigas* [92]. A number of enzymes, including XOR, contain Mo-co (Fig. 1), which in prokaryotes usually occurs as a dinucleotide with guanine, cytosine, adenine, or hypoxanthine. Such enzymes are currently divided into four different families [93]. One of these families is represented by XOR and includes MOP, a homodimer of 907 amino acid residues per monomer, each of which contains Mo-co (as the cytosine dinucleotide) and, like XOR, two different  $\text{Fe}_2\text{S}_2$  centers (Fig. 3Ai, generated, like Figs. 3Aii and 3Aiii, from MOLSCRIPT [94]). Unlike XOR, however, MOP does not contain FAD. The MOP monomer is organized into four domains. The first two, N-terminal,

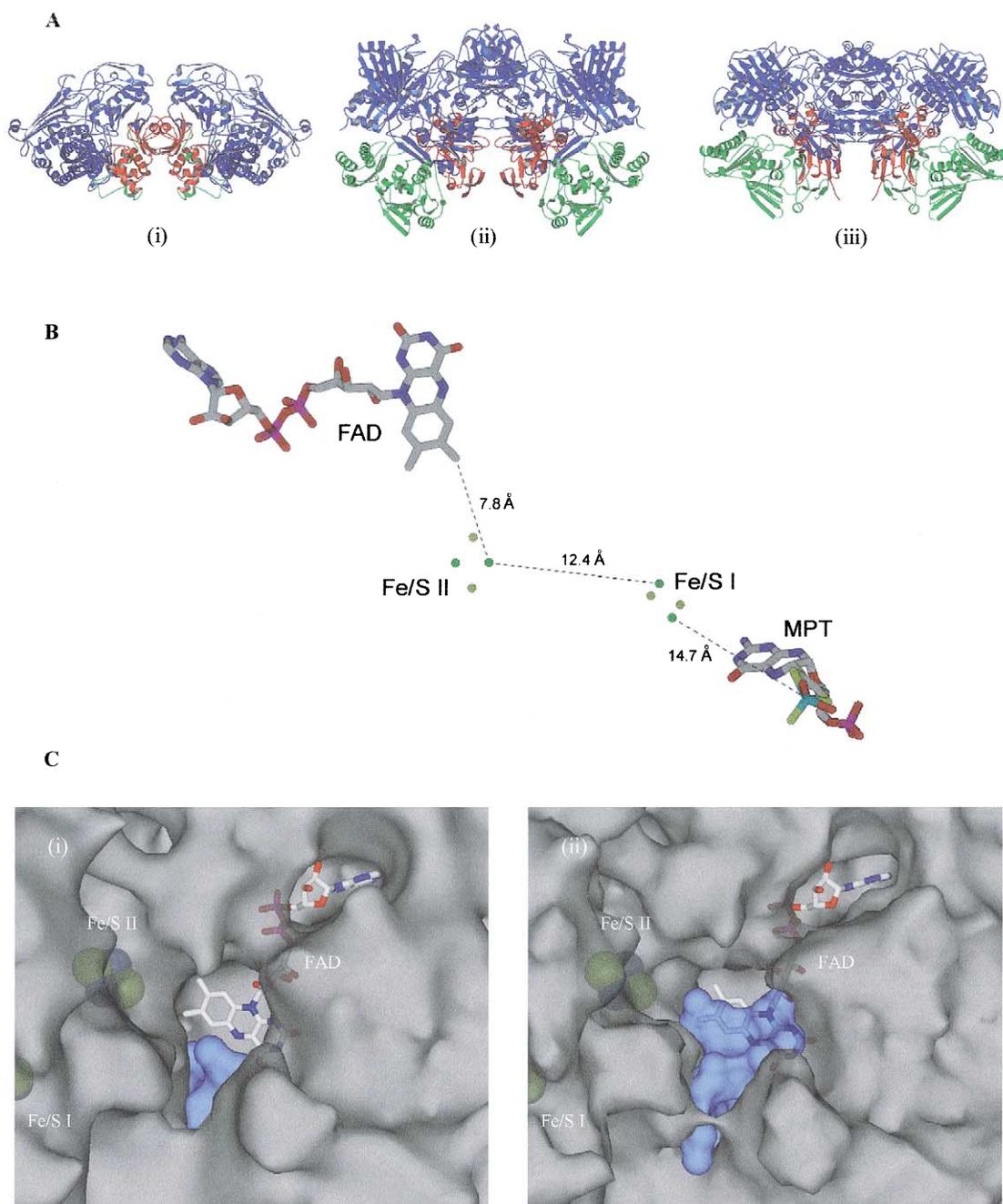


Fig. 3. (A) Ribbon diagrams of the structures of three members of the XOR family. The Fe<sub>2</sub>S<sub>2</sub>, FAD [or connecting peptide in A(i)] and Mo domains are shown in red, green, and blue, respectively. A(i) *D. gigas* aldehyde oxidoreductase (MOP). A(ii) *O. carboxidovorans* CODH. A(iii) *B. taurus* XOR. Images were generated using MOLSCRIPT [94], from the data of Huber and colleagues [92,95], Dobbek et al. [97], and Enroth et al. [100], respectively. (B) Relative orientations of the cofactors in *B. taurus* XOR. Atoms are color-coded as follows: C, white; N, blue; O, red; S, yellow; Fe, green; P, purple; Mo, cyan. Intercofactor distances are given in angstroms and the measured distances indicated by the dotted lines. Image generated using SPOCK [<http://quorum.tamu.edu/spock/>], from the data of Enroth et al. [100]. (C) Semi-transparent surface representation of the NAD binding cleft of *B. taurus* XDH (i) and XO (ii). FAD, Fe/S I, and Fe/S II are shown for purposes of orientation. The surface of the loop 423–433 is shown in blue and is clearly seen to occlude the NAD binding cleft in XO but not in XDH. Images generated using SPOCK from the data of Enroth et al. [100].

domains each contain one Fe<sub>2</sub>S<sub>2</sub> group, while the third and fourth domains both bind to Mo-co, which is deeply buried in a tunnel between them. Mo was seen to be

pentacoordinated, with two dithiolene sulfur ligands from the Mo-co and three oxygens, one of which was proposed to be replaced by sulfur in the sulfo-form of the

enzyme (Fig. 1B). Higher resolution studies [95] allowed formulation of a detailed mechanism for MOP-catalyzed hydroxylation of aldehydes (to carboxylic acids), which serves as a model for XOR-catalyzed hydroxylation of purines and related substrates. More recently, the structure of a closely related aldehyde oxidoreductase (MOD), from *Desulfovibrio desulfuricans*, has been elucidated and it confirms the molecular features that are essential for the function of these enzymes [96].

Carbon monoxide dehydrogenase (CODH), from the aerobic bacterium *Oligotropha carboxidovorans* is another XOR-related enzyme for which a crystal structure has been reported [97]. This enzyme serves as a model for XOR because it not only contains Mo-co and two  $\text{Fe}_2\text{S}_2$  centers but, unlike MOP and MOD, also has an FAD cofactor (Fig. 3Aii). CODH differs from XOR, however, in that Mo, the two iron centers and FAD occupy three separate subunits of a heterotrimer, rather than three domains of a single monomer.

The N-terminal domains of MOP, MOD, and XOR, and the corresponding subunit of CODH, all contain eight strictly conserved cysteine residues, which have been shown to serve as ligands to the two  $\text{Fe}_2\text{S}_2$  groups [92]. The four N-terminal cysteines form the canonical  $\text{Fe}_2\text{S}_2$  cluster-binding motif of the regular plant type ferredoxins and combine to bind one of the  $\text{Fe}_2\text{S}_2$  groups. The second such group is bound by the remaining four cysteines, which are arranged in an unusual protein fold, unique to these enzymes. The two  $\text{Fe}_2\text{S}_2$  centers, while indistinguishable by visible absorption spectra, give very different electron paramagnetic resonance (EPR) signals and have long been referred to as Fe/S I and Fe/S II on the basis of these signals [5]. Mutagenesis studies [98] have now allowed assignment of the Fe/S II and Fe/S I signals to the N-terminal and C-terminal clusters, respectively. Crystal structures of MOP, MOD and CODH show that Fe/S I lies relatively close to Mo (approx. 15 Å), while Fe/S II is further removed (approximately 12 Å from Fe/S I.) (Fig. 3B). A more systematic nomenclature, accordingly, refers to Fe/S I as proximal and Fe/S II as distal [96,97]. The spatial arrangement of redox centers is compatible with the proposed flow of electrons from Mo to Fe/S I to Fe/S II. The latter, distal center is located near to the surface of the molecule and is well positioned to pass electrons on to the physiological electron acceptor (in the case of MOP or MOD, which lack FAD) or to FAD, which for CODH is only 8.7 Å away.

Despite the fact that crystals of XOR were reported as far back as 1954 [99], the structures of the oxidase and dehydrogenase forms of the bovine milk enzyme have only very recently been solved [100]. Consistent with the earlier proteolytic data, noted above, XOR was seen to comprise three domains (Fig. 3Aiii). The N-terminal domain (1–165) contains both  $\text{Fe}_2\text{S}_2$  clusters, followed

by a long connecting peptide (166–225), which leads into the FAD domain (26–531). Another, partially disordered segment (532–589) links the FAD domain with the large C-terminal Mo-co domain (590–1332). As for the related enzymes, discussed above, Mo, Fe/S I, Fe/S II, and FAD are suitably located to allow electron transport in the quoted sequence, with respective distances between nearest neighbors of 14.7 Å, 12.4 Å, and 7.8 Å [100]. The relative dispositions of the cofactors are shown in Fig. 3B. The redox potentials of the centers are such that the electron transfer is thermodynamically favorable. Regarding the mechanisms of such transfer, apart from the first and longest step, no obvious “through-bond” pathways are evident and “tunneling” is proposed as the most probable means [100].

While MOP provided a wealth of information concerning the Mo and  $\text{Fe}_2\text{S}_2$  centers of XOR, it lacks an FAD domain. Corresponding detail of the FAD domain of XOR had, accordingly, to await the crystal structure of CODH, particularly as XOR shows no sequence homology to most known flavoproteins. In fact, the FAD domain of CODH [97] and (as subsequently shown [100]) of XOR itself are characteristic of an emerging structural family of FAD-binding proteins, which share considerable structural homology but have very low sequence identity. Two other members of this family have been structurally characterized *viz.*, vanillyl-alcohol oxidase and UDP-N-acetylenolpyruvyl-glucosamine reductase (MurB), an enzyme involved in bacterial cell wall biosynthesis [101].

From the crystal structure of XDH, it has been concluded that FAD binds to the protein in a deep cleft, with the *si* face of its isoalloxazine ring accessible to  $\text{NAD}^+$  [100]. As noted in the previous section, while XDH readily reduces  $\text{NAD}^+$ , XO cannot, and the structural reasons for this are clearly of interest. It might be anticipated that the greatest changes accompanying XDH to XO conversion would be seen at the FAD active site, and this was indeed found to be the case [100]. The details of these changes were less predictable. The XO molecules used for crystal study were generated by proteolysis using pancreatin, which cleaves the enzyme after Leu 219 and Lys 569 of XDH. These residues are some distance from the FAD, making direct influence on its binding site improbable. In XDH, however, the peptide chain around Phe 549 interacts with the side chain of Arg 427. Removal of this interaction following proteolysis apparently triggers a major structural rearrangement of a highly charged loop (Gln 423-Lys 433) on the *si* face of the flavin ring, whereby several residues move by as much as 20 Å. An important consequence of this rearrangement is that access of  $\text{NAD}^+$  to the *si* face of the FAD alloxazine ring is blocked (Fig. 3C). The *re* side of FAD, where molecular oxygen might be expected to

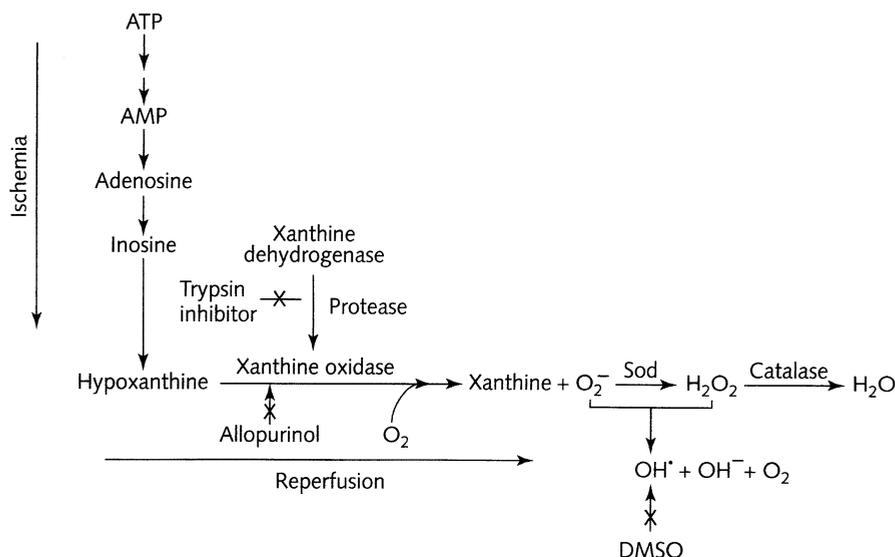


Fig. 4. Mechanism of generation of ROS in IR as proposed by Granger et al. [10], reproduced with permission.

bind, is little affected. A further effect of the shift in the peptide loop, accompanying XDH to XO conversion, is to make the flavin environment more positively charged. The side chain of Asp 429 is removed from its close contact with the flavin ring and replaced by Arg 426 [100]. In fact, changes of this nature had been detected previously by the use of a flavin active-site probe [102].

Whereas proteolysis irreversibly converts XDH to XO, reversible conversion can be achieved by the use of sulfhydryl-reactive reagents. Specifically, Cys 535 and Cys 992 have been identified as central to this process, which is speculated to involve formation of disulfide bonds [103]. These cysteine residues in XDH are sufficiently far apart that disulfide bond formation would necessitate conformational change [100]. Clarification of the nature of such changes awaits diffraction-quality crystals of the cysteine-modified enzyme.

#### ISCHEMIA-REPERFUSION INJURY

Granger and colleagues [8–10] focused attention on XOR by proposing a key role for the enzyme in the pathogenesis of ischemia-reperfusion (IR) injury. Their hypothetical mechanism, outlined in Fig. 4, can be briefly summarized as follows. In the course of ischemia, transmembrane ion gradients are dissipated, allowing elevated cytosolic concentrations of calcium. This, in turn, activates a protease that irreversibly converts XDH, predominant *in vivo*, into XO. Concurrently, cellular ATP is catabolized to hypoxanthine, which accumulates. On reperfusion, readmitted oxygen, hypoxanthine, and XO combine to generate superoxide and hydrogen peroxide. These reactive oxygen species can interact to yield a range of cytotoxic agents, including hydroxyl radicals.

The publication of this hypothesis stimulated many hundreds of papers investigating its relevance in a range of tissues and species. Many studies, particularly the earlier ones, confirmed the proposed role of XOR in IR damage in intestine [104], liver [105], and kidney [106]. Supportive evidence most commonly involved attenuation of damage following inactivation of XOR by specific inhibitors such as allopurinol or oxypurinol [107], or by administration of a tungsten-rich, molybdenum-deficient diet to experimental animals, which leads to inactive enzyme [104]. As is often the case with such high-profile hypotheses, that of Granger and colleagues has been examined increasingly critically with the passage of time. At relatively high concentrations (>500  $\mu\text{M}$ ), allopurinol and oxypurinol have been shown to act as powerful scavengers of hydroxyl radicals *in vitro* [108] and the possibility that the beneficial effects of these inhibitors could result from such scavenging has been examined. Allopurinol is also known to aid in preservation of the nucleotide pool and the potential involvement of this activity has also been addressed [104].

Perhaps most controversial has been the extent and time scale of XDH to XO conversion in ischemic tissues, particularly liver. Despite vigorous debate [109,110], the consensus is probably that, while this conversion occurs, it is too slow to play a major role in IR-induced tissue damage [57,58,105,110–115]. In fact, it can be argued that XDH to XO conversion is not essential to involvement of XOR in IR. Upregulation of overall XOR activity as a consequence of ischemia and/or of substrate level changes could equally well lead to increases in ROS [73,116] (see also “Regulation”).

The role of XOR in IR injury of the heart has been especially contentious, largely because of the very low activities of XOR detected in rabbit, pig, and, particularly, human hearts [13,14,117]. The brain also has been much discussed in this context, with evidence both for [118,119] and against [120,121] a major involvement of XOR in IR injury. It is worth bearing in mind that, because XOR is most probably localized to certain cell types (e.g., those of the vasculature), it is levels and changes of the enzyme within these cells that have relevance to IR. Such information is not, in general, available from studies on whole tissue. A further point is that circulating XOR, derived from high-activity tissues, such as liver, could be concentrated in the vasculature of lower activity tissues, such as heart, and there effect injury (see "Circulating XOR").

In fact, in recent years, research into IR injury has tended to focus on the microvasculature, particularly on the involvement of neutrophils, which are known to infiltrate postischemic tissue [104,122–124]. In this context, interactions between neutrophils and the endothelium are of primary importance. Such interactions are relevant not only to IR injury but also to a range of vascular pathologies [125,126] and will be discussed in the section, "Pathological Roles of XOR in the Vasculature."

#### CIRCULATING XOR

The presence of circulating XOR in mammals has long been recognized, and many different assays have been developed for its determination, particularly in humans, in which levels are particularly low. Most of these assays measure enzymic activity, and involve quantification of product by a range of means, including radioactivity (urate or xanthine) [127–129], fluorescence (isoxanthopterin) [130–132], chemiluminescence (superoxide) [133] and UV-visible spectroscopy (urate) [112, 134–139]. Given the variety of techniques employed, it is perhaps not surprising that reported concentrations of XOR in healthy human subjects range widely, from 0–4200 mU/l [140] (1 mU = 1 nmol urate or isoxanthopterin/min). In fact, the consensus, particularly from the more sensitive assay procedures, is that concentrations in normal human serum are very low. Thus, the assay of Shamma'a and colleagues [127], using radiolabeled xanthine, led to a normal range of 0–0.5 mU/l, while that of Yamamoto et al. [132], based on high-performance liquid chromatography separation and fluorescence determination of isoxanthopterin (oxidation product of pterin), similarly gave a normal range of  $0.21 \pm 0.1$  mU/l. It is worth noting that circulating XOR is almost certainly all in the oxidase form, as a result of XDH to XO conversion by serum proteases [115].

Alternatively, XOR protein can be assayed by ELISA [16,75,141,142], which has the advantage, particularly relevant to humans, of being able to detect inactive forms of the enzyme (see "Properties of Purified XOR"). Batelli et al. [142] have described a competitive ELISA based on purified human milk XOR and rabbit anti-XOR antibodies. The minimum detectable concentration of enzyme was reported to be 32 ng/ml and the mean titer, in 11 normal serum samples, was  $462 \pm 443$  ng/ml. Using a sandwich ELISA with rabbit anti-human XOR antibodies [75,141] (minimum detectable concentration 0.2 ng/ml) we find considerably lower levels of circulating XOR in normal controls. Thus, mean values in 600 healthy blood donors were found to be  $8.98 \pm 5.23$  ng/ml [143,144]. It is of interest that, taken with the mean pterin oxidation activity (0.21 mU/l) quoted by Yamamoto et al. [132] (see above), this leads to a specific activity of approximately 100 nmol/min/min for circulating human XOR (see "Properties of Purified XOR").

While the reported absolute levels of circulating XOR vary greatly between individual laboratories, there is general agreement concerning the increase in such levels in some disease states, particularly those involving liver damage. Titers are reported to be very high, as much as 1000-fold higher than normal controls in viral hepatitis, especially in the early, acute phase [127,128,130,134, 135,143,144]. Much lower levels are reported in obstructive jaundice, chronic hepatitis, and cirrhosis. That titers in the latter two disease states are only moderately elevated, compared with controls, was confirmed in a recent study [145] which reported the interesting finding of particularly high levels in cholestatic disorders. Levels of circulating XOR have been shown to be elevated in other diseases, including rheumatoid arthritis, mixed connective tissue disease, scleroderma [133], and atherosclerosis [131].

The concept that IR in any tissue might lead to release of XOR into the circulation has been investigated by several groups. Thus, increased plasma levels of XOR have been reported in patients subjected to IR induced by limb tourniquet [136,139], by aortic cross-clamp procedures [138] or liver transplantation [146]. In rats, experimentally-induced IR in excised livers gave rise to major increases in perfusate XOR levels, leading the authors to speculate that widespread endothelial and epithelial damage could result as XOR, with its ROS-generating capacity, spreads through the circulation [112]. This idea was further pursued by perfusion of the effluent from IR liver through isolated rat lung, which suffered allopurinol-inhibitable increases in microvascular and alveolar permeability [147]. Impairment of lung function has similarly been observed following release of XOR from ischemic small intestine [137]. Once in the circulation,

XOR has the capacity to bind to glycosylaminoglycans on the surface of vascular endothelial cells [148,149] and, in concentrated form, to initiate oxidative damage in organs that are not only remote from the original site of damage but may themselves have only a low content of XOR [112,114,150].

The development of damage in organs uninvolved in an initial ischemic insult is known as multiple organ dysfunction syndrome (MODS) and is the major cause of death in critically ill patients, having been cited following IR in gut, liver, skeletal muscle, and heart [124]. While MODS can affect any organ, the lung is most frequently involved and has been best studied in this respect, particularly in the extreme condition of acute respiratory distress syndrome (ARDS) [124,129,151].

Not only XOR but anti-XOR antibodies are present in the circulation of healthy human subjects. Levels of such antibodies are remarkably high, particularly those of IgM class, which represent 3% of total IgM [152]. Titers depend on both age and sex [152] and are significantly elevated in patients with coronary heart disease [153]. It is proposed that these autoantibodies constitute a natural defense against excess levels of XOR, clearing them from the circulation in the form of immune complexes [152]. As an example of beneficial autoantibodies this system is rare, if not unique, and clearly merits further study.

#### **PATHOLOGICAL ROLES OF XOR IN THE VASCULATURE**

Recent research into IR injury has focused on the vasculature, especially on leukocyte–endothelial cell interactions, which are relevant to many vascular diseases [122,125,126].

As noted in “Distribution,” XOR is present both in the cytoplasm and on the outer surface of endothelial cells [37,38]. In the vasculature, such extracellular endothelial XOR could well be supplemented by circulating enzyme, levels of which can vary greatly in certain pathological states (see “Circulating XOR”). XOR is by no means the only source of ROS in the vasculature. Neutrophils contain the complex enzyme, NAD(P)H oxidase, which is a rich source of both superoxide and the potent oxidizing and chlorinating agent, HOCl [104,154]. Interaction of neutrophils with the vascular endothelium involves a complex sequence of rolling, adhesion, and transendothelial migration of neutrophils, mediated by a range of cell adhesion molecules (CAMs) [122,125]. In view of the considerable destructive potential of neutrophils in IR, XOR-derived ROS have been increasingly seen as mediating neutrophil adhesion rather than as primary agents of tissue damage [104,155–158]. Thus, there is evidence for the involvement of XOR-generated ROS

and other pro-inflammatory species in upregulation of CAMs, particularly in the early stages of neutrophil adhesion [104,123,124,159–161]. Thereafter, an increasingly complex chain of events is seen as being set in train (Fig. 5).

A further aspect of ROS involvement in microvascular dysfunction is that of NO inactivation and consequent loss of its endothelium-relaxing properties, following rapid reaction with superoxide [122–124,162–164]. It is likely that ROS derived from neutrophils themselves contribute to this process [161], as indeed do those generated by other vascular NAD(P)H oxidases [165]. NAD(P)H oxidases have been identified in vascular endothelial, smooth muscle, and fibroblast cells. They are similar but not identical to the well-characterized neutrophil enzyme and have become a focus of intense research activity as a source of ROS in cardiovascular disease [166,167].

Despite the overwhelming current interest in NAD(P)H oxidase, several instances of the apparent involvement of XOR in diminished NO activity have been reported. Thus, studies with spontaneously hypertensive rats showed a lowering of blood pressure in response to SOD and to XOR inhibitors [168,169]. Inhibition of XOR also served to reduce the increased production of free radicals in the microcirculation of these animals [170]. In hypercholesterolemic rabbits, XOR inhibitors were found to normalize elevated superoxide production by vascular ring segments [171,172] and to partially restore impaired acetylcholine-dependent vessel relaxation [172]. Specific inhibitors were also used to show that XOR is a significant source of superoxide production in the human vasculature [173], to ameliorate impaired vasodilation in hypercholesterolemic human patients [174] and to improve myocardial efficiency in patients with idiopathic dilated cardiomyopathy [175].

The XOR inhibitors used in the above studies were allopurinol, oxypurinol, or an analogue [169], all of which act at the molybdenum site of the enzyme. It should be kept in mind that XOR can not only accept electrons from xanthine and related substrates, but can also act as an NADH oxidase [70] (see “Properties of Purified XOR”). This latter activity, which also generates superoxide, depends solely on the FAD site of XOR (Fig. 2) and will not be affected by classical molybdenum site inhibitors, exclusive use of which could lead to its being overlooked [73]. There is, unfortunately, no specific inhibitor for this XOR site. While it is blocked by diphenyleiodonium (DPI), so are many other flavoenzymes, including NAD(P)H oxidase and NO synthase. As pointed out by Cai and Harrison [163], these facts, together with the lack of a widely available specific anti-

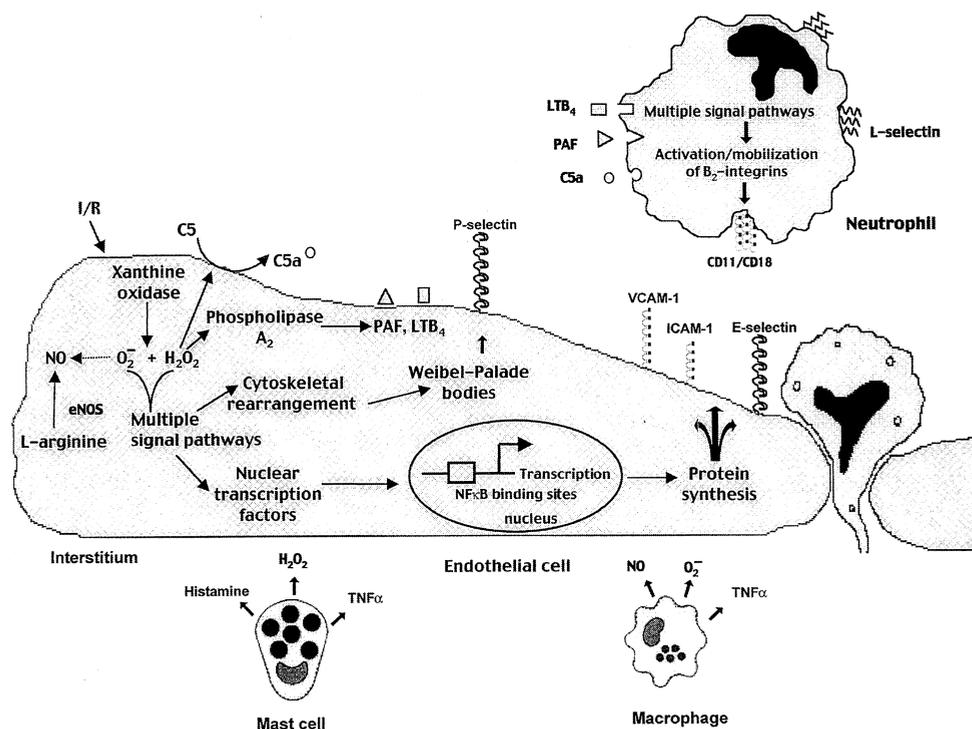


Fig. 5. Proposed involvement of XOR-generated ROS and other pro-inflammatory species in post-capillary venules in response to IR. IR results in increased production of superoxide and hydrogen peroxide by XOR, with a corresponding reduction in the production of NO by endothelial NO synthase (eNOS). The enhanced generation of oxidants results in the activation and deposition of complement, and phospholipase A<sub>2</sub>-mediated production of LTB<sub>4</sub> and PAF. Oxidants also mediate the initial expression and deposition of P-selectin by mobilizing the leukocyte rolling receptor from its preformed pool (Weibel-Palade bodies) in endothelial cells. Firm adhesion of leukocytes, which is mediated by  $\beta_2$ -integrins (CD11/CD18), is introduced by engagement of activated complement, LTB<sub>4</sub> and PAF with their receptors on rolling leukocytes. Sustained rolling and adhesion of leukocytes on endothelial cells are ensured by an oxidant-dependent synthesis of endothelial cell adhesion molecules, such as E-selectin and ICAM-1. Oxidants, derived from either endothelial cells or leukocytes, elicit this biosynthetic response by activating specific nuclear transcription factors (e.g., NFκB). The inflammatory responses to IR are amplified by mediators released from neighboring mast cells and macrophages. From Carden and Granger [124], reproduced with permission.

XOR antibody, have prevented a full understanding of the role of XOR in endothelial dysfunction.

Of relevance to interactions between XOR-derived ROS and NO are recent discussions concerning inactivation of XOR by NO and the consequent possibilities of feedback inhibition of superoxide production. Following reports of NO-induced inactivation of XOR in endothelial cells [176,177], Ichimori et al. [178] demonstrated inactivation of reduced enzyme by NO under anaerobic conditions and discussed the consequences of this for continued generation of superoxide. While the ability of NO to inactivate XOR has been disputed [179,180], indirect support for this idea is provided by the demonstration that XOR is inactivated by NO bound in an enzyme-substrate/product complex [181]; an intermediate in the XOR-catalyzed reduction of inorganic nitrite to NO [182].

The discovery that XOR can itself produce NO [182] (see "XOR-Catalyzed Generation of NO") clearly adds a new dimension to considerations of the enzyme's vaso-

lar role. XOR can be seen as complementary to NO synthase in that, unlike the latter, it is capable of producing NO under anoxic conditions. XOR could, accordingly, promote NO-induced vasodilation in ischemia, when NO synthase activity is low. While  $K_m$  values for nitrite are in the millimolar range, some two orders of magnitude greater than typical tissue levels, this does not, in itself, preclude a physiological role. What is important is the reaction rate, and Zweier and colleagues [183] have argued that XOR is potentially an important source of NO in ischemic biological tissues.

As discussed above, NO and superoxide, of whatever origins, interact rapidly. The resulting product is peroxynitrite, a powerful and destructive oxidant [184,185]. In fact, peroxynitrite can be produced by XOR itself [186] (Fig. 6) or from endothelial NO synthase, when it becomes fully or partially uncoupled from L-arginine or tetrahydrobiopterin [163,187]. This reactive species has recently generated a great deal of clinically-related interest and has been implicated in a range of pathologies,

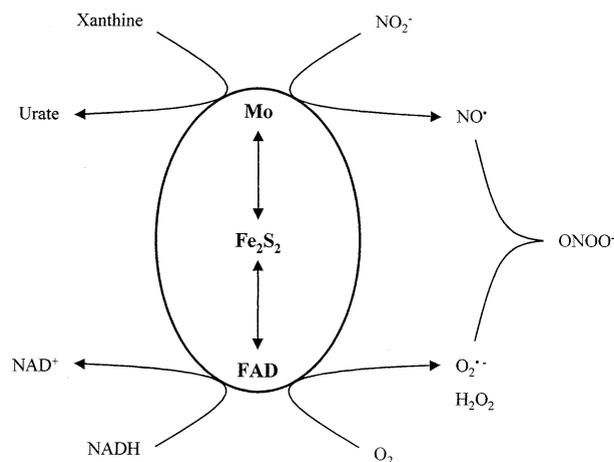


Fig. 6. Schematic diagram showing XOR-catalyzed production of NO and peroxynitrite. Under anaerobic conditions and in the presence of nitrite and a reducing substrate, such as xanthine or NADH, NO is generated at the Mo site. In the presence of molecular oxygen, this is reduced at the FAD to give superoxide, which reacts rapidly with NO to give peroxynitrite.

including arthritis, asthma, sepsis, atherosclerosis, and, particularly, neurological diseases, such as multiple sclerosis, Alzheimer's and Parkinson's diseases, and amyotrophic lateral sclerosis [188,189]. Uric acid, a general antioxidant, is an effective scavenger of peroxynitrite [188] and occurs in relatively high concentrations (50–900  $\mu\text{M}$  in humans) in the plasma of man and the higher primates, which lack urate oxidase activity [117]. Other animals have much lower levels of circulating uric acid and it has been speculated that the loss of urate oxidase activity confers strong evolutionary advantages upon higher primates. Such advantages are seen as primarily accruing from the anti-oxidant and anti-radical properties of uric acid, offering protection against aging and cancer or oxidative stress in the central nervous system [117, 189,190]. In the latter context, it is of interest that uric acid levels have been shown to be significantly reduced in the brains of patients with Alzheimer's [191] and Parkinson's disease [192].

On the other hand, cerebrospinal fluid levels of uric acid have been shown to be elevated in bacterial meningitis and other inflammatory brain diseases, including multiple sclerosis, viral meningitis, and stroke, suggesting that such elevation is at least an initial response to infection [193]. Administration of uric acid to mice before the onset of clinical experimental autoimmune encephalomyelitis (EAE), the animal correlate of multiple sclerosis, was found to prevent development of the disease and to promote recovery in those animals with active EAE [189]. Levels of XOR, the only metabolic source of uric acid, were found to be raised by over 20-fold in brains of bacterial meningitis patients, leading the authors to speculate that the presence and inducibility

of endothelial XOR activity in the brain protects the vascular endothelium from oxidative damage during inflammation [193].

Such a view of XOR as a source of protective uric acid, while addressed by some [29,194] has been generally overlooked by workers in the field. It is, of course, at variance with that generally assumed, throughout this section, whereby XOR is seen primarily as a potentially destructive agent in the vasculature. Clearly the system is complex and deserving of considerable further investigation.

## REGULATION

As noted in "Distribution," XOR activity in human tissues is generally low, compared with other mammalian species. Clearly, enzyme activity is subject to control at several levels, more than one of which appear to be involved in this case. Thus, Xu and colleagues [195] have recently shown that, relative to mice, both transcription rates and core promoter activity of the human gene are repressed. Analysis of human XOR promoter activity in different cell types showed both repressor and activator binding regions regulating core promoter activity and a model, involving interaction of E-box and TATA-like elements, was proposed to explain the repressed expression of human XOR. XOR genes of rat [196] and mouse [78,80] do not possess a TATA-like element. Although the reasons for generalized repression of human XOR expression are not clear, human XOR, like that in other mammals, is known to be activated under a variety of conditions.

Thus, cytokines have been shown to stimulate XOR activity, in a profile consistent with an acute phase response, in cultured rat pulmonary endothelial cells [197], in bovine renal epithelial cells [194], and in the fibroblastic cell line, L929, [198]. In all of these cases, XOR activation was deemed to occur primarily at the transcriptional level. Cytokine-induced activation of human XOR has also been reported. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and particularly interferon- $\gamma$  (IFN- $\gamma$ ) were shown to lead to increased XOR activity in cultured mammary epithelial cells [75]. In this case, there was evidence of both transcriptional and post-translational activation in that IFN- $\gamma$  induced an 8-fold increase in enzyme activity, compared with 2–3-fold increases in either specific mRNA or XOR protein. Support for involvement of human XOR in inflammation and the acute phase response is provided by detection, in the 5'-flanking region of the human XOR gene, of an IL-6 site and of potential TNF, IFN- $\gamma$  and IL-1 responsive elements [77].

Hormones also affect XOR activity. Both enzymic activity and XOR protein levels were monitored in serial

samples of human milk, taken from individual mothers during the first month post-partum [199]. A general pattern emerged whereby enzymic activity peaked during the first 15 d, falling thereafter, by as much as 95%, to subsequently maintained basal levels. XOR protein levels varied little throughout these changes, which were proposed to result from post-translational regulation. Similar time-dependent variation of XOR activity has been reported to occur in mouse mammary glands, in which case, enzyme activity was apparently matched by XOR protein levels [47]. The mouse system has since been examined by using cultured mammary epithelial cells [200]. Combined treatment of HC11 cells with prolactin and cortisol led to progressive 4–5-fold increases in XOR activity, which were attributed to increased de novo synthesis and decreased degradation of XOR protein. Increased de novo synthesis was associated with elevated steady state levels of XOR mRNA. Evidence for the involvement of tyrosine kinase and MAP kinase-dependent pathways was presented.

The effect of reduced oxygen tensions on XOR activity is clearly relevant to the role of the enzyme in IR injury. Indeed, hypoxia has been shown to increase activity in cultured bovine and rat endothelial cells [201–203] and in rat brain slices [204]. Whereas upregulation in rat epididymal fat pad endothelial cells was attributed to changes at the transcriptional level [201], those in bovine aortic endothelial cells [202] and rat brain slices [204] were deemed to be determined solely by post-translational events. In none of these studies was XDH to XO conversion observed. The effects of both hypoxia and hyperoxia have been studied in cultured 3T3 mouse fibroblasts [205]. After 24 h of hypoxia, XOR activity was increased by post-translational mechanisms, as evidenced by unchanged levels of protein synthesis or XOR mRNA. Decreases in XOR activity following 24 h of hyperoxia were similarly attributed to post-translational effects. From 24–48 h, in contrast, the respective increases and decreases of enzyme activity were accompanied by corresponding changes in XOR mRNA levels and protein synthesis, implying pre-translational control. The mechanisms of such control are not known, although the presence of hypoxia inducible-factor-1 (HIF-1)-like sites in the 5'-upstream regions of the mouse and human genes has been proposed [205,206].

Different mechanisms of control of XOR activity clearly occur, even in response to a given effector. The reasons for these variations are unclear, although they may well reflect cell and/or tissue-specific differences [202,206]. In a number of cases, noted above, evidence of post-translational control of XOR activity was cited, and it is of interest to examine mechanisms by which this may occur. One such possibility involves interconversion of the sulfo- and desulfo-forms of XOR. As mentioned in

the section, "Properties of Purified XOR," XOR commonly contains significant proportions of demolybo- and desulfo-forms, which are inactive to Mo-site-directed substrates. Desulfo-XOR can be converted to the active sulfo-form in vitro by incubation of the reduced enzyme with sulfide ion [53]. Moreover, desulfo-sulfo conversion in vivo has been proposed as the basis of activation of liver XOR in response to increased protein diet in chickens [207] and rats [54]. Indeed, it has been suggested that desulfo-sulfo interconversions, possibly enzymically catalyzed, may serve to regulate the activity of several relevant enzymes [208–210]. It has long been known that mutations at the maroon-like locus (*ma-l*) of *Drosophila melanogaster* result in desulfo-XOR [211]. The *ma-l* gene has now been cloned and it has been suggested that the encoded protein sulfurates Mo-co, effectively converting desulfo- to sulfo-enzyme [212]. Homologues have been identified in other species, including man [212–214] and mutations of the gene have been shown to be responsible for classical xanthinuria type II [214]. Type I xanthinuria results from mutations in the XOR gene itself [215,216].

As noted in "Properties of Purified XOR," XOR in human milk, and possibly in most other human tissues, has very low activity toward conventional reducing substrates, such as xanthine, that act at the Mo-site. In the case of, at least, milk XOR, this low activity results primarily from a low content of Mo [50,51,71], and the question arises as to whether the human enzyme is subject to activation via Mo incorporation [73]. As discussed above in this section, evidence has been presented that the true specific activity of breast milk XOR can vary as much as 50-fold in the first few weeks postpartum, presumably in response to hormonal changes [199]. Changes of this magnitude are not attributable solely to desulfo-sulfo conversion in view of the fact that "low activity" XOR, as routinely purified from human milk contains approximately 40% sulfo-form [51]. It is tempting to consider enzymic activation by means of incorporation of Mo, and, indeed XOR of mouse L929 cells has been shown to be activated by addition of Mo, which was proposed to trigger one or more steps of Mo-co biosynthesis [60]. However, L929 cells appear to be unique in this respect [60] and, in our hands, cultured human cells of low XOR activity do not similarly respond [75]. The biosynthesis of molybdopterin and its incorporation, together with Mo itself, into the apoenzyme has been studied in many species, including humans [217,218]. Several enzymes are involved and it is difficult to envisage involvement of the whole system in an activation process. Regulation of XOR activity by means of incorporation of the final, Mo-incorporating step [219] is conceptually more attractive, but presently entirely speculative.

The involvement of protein phosphorylation in post-translational activation of XOR in response to hypoxia has recently been proposed by Kayyali and coworkers [203], working with rat pulmonary artery microvascular endothelial cells. After 4 h of hypoxia, increased enzyme activity was shown to be independent of protein synthesis and to be accompanied by phosphorylation of XOR protein. Evidence was produced for a causal effect of the latter, which was attributed, at least in part, to p38 kinase and casein kinase II. It is of interest that, over 25 years ago, Davis and colleagues [220] reported the presence of a phosphoserine group in purified bovine milk XOR, largely on the basis of nuclear magnetic resonance (nmr) evidence. Two research groups [221,222] subsequently refuted these findings, which were apparently not further explored, apart from a single report, by Schieber and Edmondson [223], of a phosphoserine grouping in chick hepatocyte XOR. The concept of phosphorylation-mediated control of XOR, with its capacity to generate a range of reactive oxygen and nitrogen species, is exciting and deserving of further study.

In the context of post-translational activation of XOR, it is worth discussing XDH to XO conversion, which was central to the original mechanistic hypothesis of IR injury [8–10]. As noted in “Ischemia-Reperfusion Injury,” the extent and timing of this conversion, under ischemic conditions *in vivo*, have increasingly been questioned. Kooij and coworkers [110,115] have maintained that XDH to XO conversion does not occur to any significant extent in rat liver after *in vivo* ischemia, and suggest that contrary findings may be attributable to conversion during homogenization and assay procedures. Indeed, on the basis of cytophotometric studies of rat liver, Frederiks and Bosch [57] question whether the commonly accepted value of 10–20 % for *in vivo* content of XO might not be similarly artifactual. There is no doubt that XOR is at great risk of exposure to proteases during tissue storage and manipulation, even in the presence of inhibitors, and variable results are to be expected.

What has often been overlooked in such debates is the fact that XO is not essential for XOR-catalyzed generation of ROS. Although XDH prefers to donate electrons to  $\text{NAD}^+$ , it will also reduce molecular oxygen, albeit less efficiently than does XO. In the absence of  $\text{NAD}^+$  and the presence of xanthine,  $V_{\max}$  and  $K_m$  values for oxygen are approximately 25 and 600%, respectively, of those for XO [224]. Moreover, both forms of the enzyme show NADH oxidase activity, again with generation of ROS, XDH being rather more effective in this respect [70]. Accordingly, upregulation of overall XOR activity, irrespective of XDH/XO ratios, can serve to increase ROS levels. Indeed, as noted above, just such upregulation has been shown in cultured cells [201–203,205] and tissue slices [204] in response to hypoxia. It is noteworthy

that, in three of these studies [201,202,204], XDH to XO conversion was shown not to occur. Changes in  $\text{NAD}^+/\text{NADH}$  ratios are also a factor to be considered. Thus increased NADH levels in ischemia could serve as a trigger to increase ROS production via NADH oxidase activity of both XDH and XO [70,73]. It has also been argued that increased concentrations of NADH will preferentially inhibit XDH, shifting the flow of purine-generated electrons through XO with consequent increase in ROS production [225].

Cytokine-induced generation of ROS has been seen as dependent on XDH to XO conversion [156]. As discussed above, cytokines have, in many cases, been shown to upregulate overall XOR activity, which, again, might serve as sufficient trigger for increased ROS production. In fact, although  $\text{TNF-}\alpha$  was reported to induce XDH to XO conversion in rat pulmonary artery endothelial cells [226], no such conversion could be detected in human mammary epithelial cells [75].

#### XOR-CATALYZED GENERATION OF NO

##### *Enzymology*

It has recently been established that XOR can act as a source of NO. Initial reports described the detection of NO, by chemiluminescence, following incubation of XOR with NADH and nitrate [227,229] or nitrite [228–230] under hypoxic conditions. The anaerobic XOR-catalyzed reduction of inorganic nitrite to NO was subsequently investigated in detail by Godber et al. [182], who showed xanthine to be a more effective reducing substrate than NADH in this reaction. Steady state kinetic parameters were determined spectrophotometrically for urate production or NADH oxidation catalyzed by both XO and XDH, and Michaelis-Menten kinetics were shown to be followed, apart from when xanthine was treated as the variable substrate, in which case substrate inhibition was evident. Nitrite was shown to be reduced at the molybdenum site of the enzyme, in contrast to  $\text{NAD}^+$  and molecular oxygen, which have long been known to act at the FAD site [1] (Fig. 6). In subsequent support of this, Murray et al. [231] have demonstrated that organically liganded molybdenum can catalyze the reduction of nitrite to NO in the absence of protein. By following the kinetics of NO production and by use of electron paramagnetic resonance spectroscopy, Li and coworkers [183] effectively confirmed all the conclusions of Godber et al. [182]. They went on to argue that XOR-catalyzed reduction of nitrite could generate NO levels comparable with those produced from NO synthase in the ischemic myocardium. It should be stressed that NO generation by this route depends on low oxygen tensions and possibly (because of substrate inhibition) low purine concentrations.

Further independent evidence of a role for circulating nitrite as a vasodilator has recently been reported [232, 233].

Godber and coworkers showed that XOR-catalyzed production of NO fell off as levels of oxygen increased, consistent with the concomitant production of superoxide and interaction of the latter with NO to yield peroxynitrite [182]. They subsequently confirmed the XOR-catalyzed generation of peroxynitrite and showed marked differences between the two forms of the enzyme [186]. With XO, peroxynitrite generation rose to a peak in the presence of about 1% oxygen, decreasing rapidly as oxygen concentrations increased. XDH, on the other hand, catalyzed generally higher rates of peroxynitrite production that rose to maximal values at approximately 10% oxygen and were maintained up to and beyond ambient oxygen tensions. These findings can be qualitatively explained in terms of the fact that XO, with its more facile reduction of molecular oxygen, will have less electrons available for reduction of nitrite; a situation that will be exacerbated as oxygen tensions increase.

The enzyme sites involved in XOR-catalyzed production of NO and peroxynitrite are shown schematically in Fig. 6.

#### *Microbicidal role of XOR*

XOR has long been known to have bactericidal properties in the presence of hypoxanthine [234] and to be activated in response to bacterial infection in vivo [235]. The enzyme is upregulated by proinflammatory cytokines (see "Regulation") and has increasingly been seen as a defensive agent, largely by virtue of its ability to generate ROS. More recently, Umezawa et al. [236] reported induction of both NO and XOR in mice in response to *Salmonella typhimurium* infection and showed that mortality rates were increased by XOR inhibitors. They proposed a bactericidal role, not just for superoxide, but for the product of its reaction with NO, peroxynitrite.

Peroxyntirite is certainly a powerful bactericidal agent [237] and, in the presence of nitrite, can be generated by XOR alone (see above). In fact, this may well explain the presence of the enzyme in milk, a question of many years' standing. Localization of XOR on the MFGM befits a role in sterilizing the neonatal gut. This is particularly so, as pathogenic bacteria, with the capacity to target epithelial membranes of the digestive tract, may well bind to similar antigens on the MFGM, itself of epithelial cell origin [238]. This process will not only divert the bacteria from their primary target but will also bring them into intimate contact with XOR. Contact of this type will be further promoted by the known affinity of XOR for acidic polysaccharides [148], such as occur

in many bacterial capsules [239]. It is noteworthy that the optimal pH for anaerobic XOR-catalyzed generation of NO in the presence of xanthine is pH 6 or less [182]; a value much lower than that (pH 8.8) observed for aerobic xanthine oxidase activity and more appropriate to a role in the digestive tract.

XOR-catalyzed production of NO, a prerequisite for peroxynitrite generation, was shown to occur at maximal rates of approximately  $1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , significant in physiological terms.  $K_m$  values for nitrite were, however, in the millimolar range [182]; levels that would need to be attained in order to achieve maximal rates. While levels of nitrite in the digestive tract are generally two orders of magnitude less than this, they are potentially much higher in the microenvironment of enteric bacteria. At least in anaerobic culture, such bacteria can excrete millimolar levels of nitrite [240], derived from dissimilatory nitrate reductase [241]. It is an intriguing thought that, by way of nitrite excretion, enteric bacteria might initiate their own destruction. As noted above, XDH, in contrast to XO, is capable of generating peroxynitrite across a wide range of oxygen tensions and it is this form of the enzyme that predominates in freshly expressed milk [242].

The above discussion is based on data obtained with the well-characterized bovine milk enzyme, and human milk enzyme, as routinely purified, has very much lower activity in reactions involving the molybdenum center. Nevertheless, activities are exceptionally high in the first few weeks post-partum (see "Regulation"), precisely the period when the antimicrobial activity of XOR is required in the neonatal gut [199,243]. Levels of nitrite are also particularly high at this time [244].

As already noted, the antibacterial properties of XOR/hypoxanthine are well established [234,243], and the resulting hydrogen peroxide has also been implicated in the lactoperoxidase/thiocyanate system in milk [245]. However, despite the circumstantial evidence, outlined above, for a bactericidal role of XOR-derived NO or peroxynitrite in milk, experimental support for this has hitherto been lacking. By making use of *Escherichia coli* transfected with the lux operon, we have recently been able to demonstrate bacteriostatic activity of fresh milk, both bovine and human, which is dependent on both xanthine and nitrite and is inhibited by allopurinol (V. Salisbury, J. Hancock, R. Eisenthal, R. Harrison, unpublished).

Microbicidal activity of milk XOR can be seen as complementary to similar activity of the enzyme in epithelium of the digestive tract (see "Distribution"). Van den Munckhof et al. [22] have identified XOR activity in the cytoplasmic matrix of enterocytes and in the mucous of rat duodenum. Enzyme was also found in the cytoplasm of apical cell layers of epithelia of esophagus and

tongue, with the highest activity in the cornified layer. Of particular interest was their finding that bacteria, present between cell remnants of the cornified layer of the esophagus, were surrounded by XOR. Moreover, many of the bacteria showed signs of destruction and/or cell death. Van den Munckhof and colleagues proposed that epithelial XOR plays an antimicrobial role in the digestive tract by generating superoxide and hydrogen peroxide. Their proposal could well be modified to include XOR-catalyzed production of peroxynitrite, as discussed above for MFGM.

A third line of antimicrobial defense in the digestive tract is suggested by the recent detection of XOR in human bile duct epithelial cells. As noted under "Distribution," enzyme was apparently concentrated in the apical region, suggesting secretion into the bile. Indeed, we have determined the presence, in mouse bile, of XOR at concentrations some two orders of magnitude higher than those found in mouse serum (H. Martin, unpublished).

#### *Role of XOR in the metabolism of nitrovasodilators*

The vasodilatory action of organic nitrates has been recognized for over 100 years and these compounds have been widely used in the treatment of angina, acute congestive heart failure, and hypertensive emergencies. Concerning their mode of action, it is generally agreed that they are metabolized to NO, which stimulates soluble guanylate cyclase and hence upregulation of the vasodilatory second messenger, cyclic guanosine monophosphate. The metabolic route from organic nitrate to NO is, however, still unknown [246–248]. The involvement of sulphhydryl groups in this process has long been discussed and physiological responses to glyceryl trinitrate (GTN) are certainly influenced by thiols [249]. A direct non-enzymic involvement of thiols in the metabolism of organic nitrates now seems unlikely however, and attention has shifted to enzyme-catalyzed mechanisms [246].

Following the initial report [229] that XOR catalyzes the reduction of GTN to NO in the presence of NADH under hypoxic conditions, Doel et al. [250] showed that GTN, isosorbide dinitrate, and isosorbide mononitrates are reduced anaerobically to inorganic nitrite in the presence of XOR. Reduction was much faster with xanthine as reducing substrate, compared with NADH. In the presence of xanthine, urate was produced in nearly 1:1 stoichiometric ratio with inorganic nitrite, further reduction of which was relatively very slow. In fact, although NO could be detected in the presence of NADH this was not the case with xanthine, confirming the earlier report [229]. This was explained in terms of substrate inhibition, by xanthine but not NADH [182], of reduction of

inorganic nitrite to NO under the experimental conditions employed.

In contrast to inorganic nitrate [181] or nitrite [182], organic nitrates are initially reduced at the FAD site of the enzyme [250]. Interestingly, cytochrome P-450 [251] has been shown to catalyze the same reaction, and other flavoproteins, as yet unidentified, may well do the same. Indeed, organic nitrates have been shown to be reduced by flavins alone [252]. Intriguingly, despite the apparent lack of direct involvement of Mo in XOR-catalyzed reduction of organic nitrates, organically liganded Mo alone will effect this reduction [231].

The mechanism of further metabolism of inorganic nitrite to NO is less certain. While XOR is certainly capable of catalyzing this reduction *in vitro* [182], rates of NO production from organic nitrates are slow. This may reflect inhibition by organic nitrates [250] and/or, as noted above, by xanthine, and it is not clear to what extent comparable inhibition may occur with the local concentrations of substrates encountered *in vivo*.

The intermediacy of inorganic nitrite, however, derived, in the metabolism of organic nitrates has been questioned, largely because of the weak vasodilatory properties of inorganic nitrite itself [253]. It is, however, conceivable that the clinical ineffectiveness of nitrite reflects difficulties in crossing the cell membrane, difficulties that could be circumvented if it is generated intracellularly from the relatively lipophilic organic nitrates. Moreover, as noted above, nitrite is increasingly seen as a vasodilator in the absence of drugs [232,233].

Ratz et al. [254] observed that purified XOR catalyzed the anaerobic transformation of GTN to its dinitrate metabolites in the presence of xanthine and that this transformation was blocked by diphenyleneiodonium (DPI), an inhibitor of flavoenzymes. While this is clearly consistent with involvement of XOR in GTN metabolism, the authors went on to provide evidence against such involvement. They showed that a 105,000 g supernatant of rat aorta, supplemented with xanthine or NADH, was unable to biotransform organic nitrates under anaerobic conditions. This experiment was based on the assumption that XOR is primarily cytosolic and its conclusions may be questioned in the light of the likely membrane and/or vesicle association of XOR (see "Distribution"). Less easy to refute, however, are their findings that the specific XOR inhibitor, allopurinol, failed to block the functional relaxation response to organic nitrates.

Indirect evidence in support of involvement of XOR in GTN metabolism is provided by the observation that in the course of anaerobic incubation with GTN and xanthine, XOR is progressively inactivated [250]. This would be consistent with the well-known phenomenon of clinical tolerance to nitrovasodilators, which is believed

by many to reflect inactivation of the metabolizing enzyme(s) [255]. It is likely that the inactivation of XOR in the presence of GTN is primarily associated not with the reduction of GTN to inorganic nitrate, but with the slow further reduction of nitrite to NO [250]. Detailed study of this inactivation has shown that it involves a so-called "suicide" reaction, resulting from interactions within the enzyme-substrate complex [181]. An alternative mechanism, whereby newly-produced free NO inactivates the enzyme [178], was effectively discounted in this case.

Although, as noted above, XOR-catalyzed reduction of GTN to NO *in vitro* is slow, evidence for NO production *in vivo* has been presented by O'Byrne et al. [256], who showed inhibition of platelet aggregation in the presence of GTN and XOR.

It will be clear from the above discussion that, while XOR is capable of catalyzing the biotransformation of organic nitrates to NO, its clinical involvement is as yet unproven. For organic nitrites, the case, from a kinetic viewpoint at least, is simpler. While organic nitrites have received much less research attention in recent years, the clinical use of isoamyl nitrite [257] actually predates that of GTN [258] in the treatment of angina. It is now primarily used as a recreational drug, as is its homologue, isobutyl nitrite [259]. XOR has been shown to catalyze the relatively rapid anaerobic reduction of isoamyl and isobutyl nitrites to NO, in the presence of xanthine [260,261]. From kinetic considerations, it was concluded that the reaction proceeds without the intermediacy of inorganic nitrite. In this case also, the enzyme was progressively inactivated, consistent with the phenomenon of clinical tolerance.

In the context of these discussions, it is worth noting that S-nitrosothiols, possible intermediate sources of NO [232,262], have been shown to be reduced to NO in the presence of XOR [263]. Here, however, the situation was shown to be more complex in that S-nitrosocysteine, but not S-nitrosoglutathione, was directly reduced to NO under anaerobic conditions. Under aerobic conditions, both S-nitroso compounds were reduced non-enzymically by superoxide to NO, which reacted with a second superoxide molecule to yield peroxynitrite. Direct enzymic reduction of S-nitrosoglutathione is presumably inhibited by its greater size and restricted access to the active site [263].

The enzyme sites involved in XOR-catalyzed reduction of organic nitrates and nitrites are shown schematically in Fig. 7.

## CONCLUSION

As noted in the "Introduction," interest in XOR received a major boost in the 1980s, following publication of its hypothetical involvement in IR injury. In fact, and

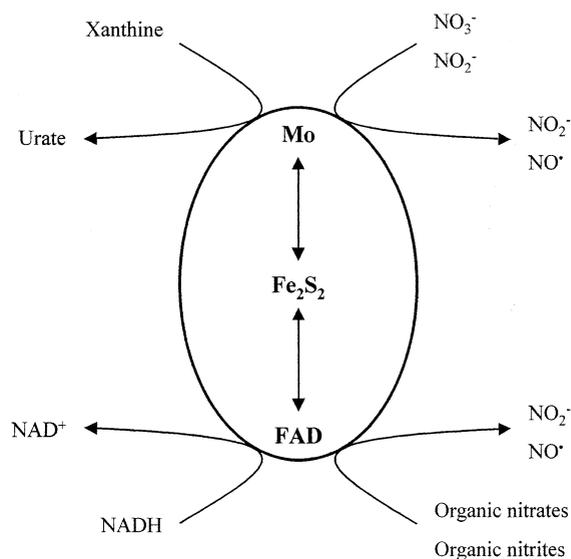


Fig. 7. Schematic diagram showing XOR-catalyzed reduction of nitrates and nitrites. Under anaerobic conditions, organic nitrates are reduced at the FAD site to inorganic nitrite, which can itself be subsequently further reduced to NO at the Mo site. Organic nitrites are similarly reduced at FAD, in this case directly to NO. In contrast to organic nitrites but similarly to inorganic nitrates, inorganic nitrates are reduced at Mo.

not only for this reason, a great deal of new information concerning the enzyme has been gained in the last 20 years. Nevertheless, as will be clear from this review, many uncertainties remain.

Studies of the tissue and cellular distribution of XOR are contradictory and potential reasons for this are discussed in "Distribution." Discrepancies between activity and immunohistochemical data can often be explained in terms of variable specific activities of XOR, especially the human enzyme. Nevertheless, between immunolocalization studies themselves, there are considerable variations that undoubtedly reflect different purities and specificities of the antibodies used. There is certainly a need for widely available monoclonal anti-human XOR antibodies of high avidity. Monoclonals that inhibit either xanthine oxidase or NADH oxidase activity of XOR would be a bonus.

Above all, the roles of XOR *in vivo* are still uncertain. The enzyme is clearly complex and subject to control at many levels, but to what end? It is well established that XOR can act as a source of superoxide and hydrogen peroxide, which could exert protective (e.g., bactericidal) or destructive effects. The results of the latter in the vasculature have been discussed at length, particularly those involving interactions of ROS with NO and the finding that XOR itself can generate NO (or peroxynitrite) adds a whole range of new possibilities. A further complication, that has been seldom considered, is that XOR generates uric acid, a potent scavenger of ROS,

again casting the enzyme as a potentially protective, rather than destructive agent.

While increasing attention is being directed toward circulating XOR, its origins and fate are not well understood. The liver is a likely, but not the only, candidate as a tissue source, and the relative roles of vascular glycosylaminoglycans and circulating anti-XOR antibodies in determining the localization or clearance of XOR are unknown.

The role of ROS in signaling is of increasing interest generally and cytoplasmic XOR could serve as a source of ROS intermediates, mediating gene expression in a host of cellular responses, including growth, survival, apoptosis, etc. [264,265]. Moreover, localization of extracellular XOR on apposing surfaces of cells in culture suggests its involvement in intercellular signaling [37]. There is presently only sparse evidence to implicate XOR in such wider signaling roles, which remain speculative but invite attention.

Ultimately, human XOR is of primary interest and its low conventional activity in most tissues is puzzling. As discussed in "Regulation," transcription of the human gene appears to be repressed compared with that of other mammals, while, in the case of at least the breast milk enzyme, the content of Mo is relatively very low. Like XOR from other species, the human enzyme is subject to control at several levels, in response to a range of factors, including hormones, oxygen tension, and cytokines. In so far as the mechanisms of such control depend on cellular systems, information about XOR purified from different (particularly human) tissues will need to be complemented by studies of the enzyme in its cell environment.

That XOR continues to stimulate research after more than 100 years certainly reflects the complexity of its electron transport arrangements and potential for control at many levels.

In the context of knowledge of the enzyme, realization of its capacity to generate ROS and NO is relatively recent and it may be expected that this capability will be subject to ever increasing attention.

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