Review Articles

ROLE OF MOLYBDENUM HYDROXYLASES IN DISEASES

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تمت مناقشة دور الإنزيمات (الخمائر) المحتوية على الموليبدينوم، وهي ألدهيد أوكسيداز، والزانثين أوكسيداز في إنتاج أنواع الأوكسجين المتفاعل وذلك من وجهة آلية عملها. وعلى نقيض الصبغ الخلوي (سيتوكروم) P450 وأنظمة المونوأوكسيجيناز (المؤكسدات الأحادية) الأخرى، فإنها تقوم بالتفاعل باستخدام الماء عوضاً عن الأكسجين الجزيئي كمصدر لذرة الأوكسجين المدمجة في المركب، وتوليد الإلكترونات بدلاً من استهلاكها. ويختلف الألدهيد أوكسيداز والزانثين أوكسيداز في المواد التي يقومان باستقلابها وفي مثبطاتهما. وبينما الألدهيد أوكسيداز هو الأوكسيداز المهمين، فإن بمقدور الزانثين أوكسيداز التحول المتبادل بين شكلي الأوكسيداز/والديهدروجيناز تحت الظروف المرضية مثل الإسكيمية (فقر الدم الموضعي). ومع ذلك فإن العدد الكبير من الأدوية، والكيماويات الغريبة والداخلية التي تتفاعل مع هذه الإنزيمات، وخصوصاً مع الألدهيد أوكسيداز، توضح أهمية هذه الإنزيمات في أكسدة، وإبطال سمية، وتفعيل الأدوية. وقد ارتبط الألدهيد أوكسيداز والزانثين أوكسيداز ببعض الأمراض مثل اعتلالات الانحلال العصبي والإسكيمية، على التوالي أحيائياً، قد تؤثر أكسدة الأدوية المتفاعلة مع الألدهيد أوكسيداز مثل الأسيتالدهيد المشتق من الإيثانول والريبينال وثنائي نيوكليوتيد النيكوتيناميد والأدنين المختزل على توازن إنتاج أنواع الاكسجين المتفاعل بواسطة هذا الإنزيم مما يؤدي إلى الاعتلالات العصبية، مثل تصلب الضمور العضلي الجانبي، ومرض الرعاش (داء باركنسون)، وانفصام الشخصية. بالإضافة إلى ذلك، فقد تم زج الألدهيد أوكسيداز في الفسيولوجيا المرضية للضرر الذي يصيب الكبد نتيجة للكحول، وفي العمليات البصرية، وفي تخليق حمض الريبينويك، وفي الضرر النسيجي الناتج عن إعادة سريان الدم. وفي الحالات المرضية، فإن مثل هذا الضرر قد يشارك فيه الإنزيمان.

The role of molybdenum-containing enzymes, aldehyde oxidase and xanthine oxidase in the production of reactive oxygen species has been discussed in term of mechanism of action. Unlike cytochrome P450 and other monooxygenase systems, the molybdenum hydroxylases carry out their reactions using water rather than molecular oxygen as the source of the oxygen atom incorporated into the product, and generated rather than consumed electrons. Aldehyde oxidase and xanthine oxidase differ in their substrates and inhibitor specificity. While aldehyde oxidase is a predominant oxidase, xanthine oxidase can undergo inter-conversion between oxidase/ dehydrogenase forms under pathological conditions such as ischaemia. Nevertheless, the wide range of drugs, xenobiotics and endogenous chemicals that interact with these enzymes, particularly aldehyde oxidase, highlight the importance of these enzymes in drug oxidation, detoxification and activation. Aldehyde oxidase and xanthine oxidase have been linked to some diseases such as neurodegenerative and ischaemia disorders, respectively. *In vivo,* oxidation of aldehyde oxidase-substrates such as ethanol-derived acetaldehyde, retinal and NADH may alter the balance of ROS production by this enzyme leading to neurological disorders, such as amyotrophic lateral sclerosis, Parkinson's disease and schizophrenia. In addition, aldehyde oxidase has been implicated in pathophysiology of alcohol liver injury, visual processes, synthesis of retinoic acid and reperfusion tissue injury. Under pathological conditions, such as ischaemia-reperfusion injury, both enzymes may participate.

Key words: Molybdenum hydroxylases, aldehyde oxidase, xanthine oxidase, neurodegenerative disorders, amyotrophic lateral sclerosis (ALS), molybdenum center.

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Introduction

Molybdenum hydroxylases are a group of enzymes that share the same transition metal molybdenum (Mo^{VI}). The principal mammalian molybdenum-containing enzymes are aldehyde oxidase (EC 1.2.3.1), xanthine oxidase (EC 1.1.3.22), xanthine dehydrogenase (EC 1.1.1.204) and sulphite oxidase (EC 1.8.3.1). Xanthine oxidase/dehydrogenase is the key enzyme in the sequential metabolism of hypoxanthine to xanthine and uric acid (1). Calzi *et al.* suggested that aldehyde oxidase is an important enzyme in detoxification of foreign xenobiotics (2). This hypothesis has been supported by studies on the distribution of this enzyme in liver and lung (2). However, molybdenum hydroxylases have been implicated as key oxidative enzymes in some diseases e.g. ischaemia and reperfusion injury (3). Limited results are available on the concentration and distribution of molybdenum hydroxylases. However, there is some interspecies variation in the expression of aldehyde oxidase more than that of xanthine oxidase. In human, the highest xanthine oxidase expression and activities are found in proximal intestine, lactating mammary gland and liver, whereas high aldehyde oxidase levels are consistently found in the liver, lung, kidney and brain (2).

Molybdenum hydroxylases generally catalyze hydroxylation reactions. The reaction stoichiometry is unusual among other hydroxylation reactions in that reducing equivalents are generated, rather than consumed in the reaction, and water, rather than molecular oxygen, is the ultimate source of oxygen atom incorporated in the substrate (4).

1.1. Molybdenum cofactor of molybdenum hydroxylases:

The transition element molybdenum is essential for almost all organisms and occurs in more than thirty enzymes catalysing diverse redox reactions (5). However, only three molybdenum containing enzymes have been found in mammals (6); xanthine oxidase which is essential for purine catabolism, aldehyde oxidase which is involved in the oxidation/reduction of very diverse substrates, and sulphite oxidase which catalyzes the terminal step in oxidative degradation of sulphur-containing amino acids (7). The molybdenum atom seems to be biologically inactive unless it is complexed as a cofactor, to form molybdopterin (Figure 1). The cofactor is a reduced form of 2-amino-4-hydroxypteridine, with a four carbon side-chain in position six. This chain bears a phosphorylated primary alcohol in the terminal position and *cis*-dithiolene moiety to which the molybdenum atom is co-ordinated (8). A significant feature of the molybdenum center is that co-ordination to the *cis*-dithiolene sulphur atoms is the only mechanism for anchoring the metal to the protein (8).

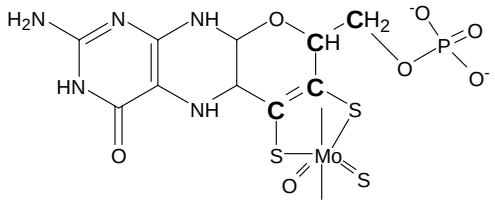

Figure 1. Structure of the molybdopterin cofactor from xanthine oxidase (9).

Molybdopterin can be embedded within different apoproteins and is responsible for the correct positioning and anchoring of the molybdenum center within the holo-enzyme. The structure of molybdopterin has been established crystal-ographically in the molybdenum-containing aldehyde oxidoreductase from *Desulfovibrio gigas* (9). Although, the pterin cofactor is frequently referred to as "molybdopterin", it has also been found in a variety of tungsten-containing enzymes (9). The core structure of molybdopterin is highly conserved in all organisms (10).

The molybdopterin cofactor modulates the reduction potential of the molybdenum center. Particularly significant is the observation that the sulphur-sulphur distance of the dithiolene moiety increases from 3.0 to 3.5 Å (Figure 1), with the S-Mo-S bond angle also increasing considerably, upon reduction of the enzyme (11). This configuration facilitates interaction between the molybdenum center and substrate.

1.2. Structure of molybdenum hydroxylases:

Aldehyde oxidase and xanthine oxidase are metalloflavoproteins. These enzymes are homodimers of around 300 kDa, depending on species. Each subunit contains an active site, but it is thought that the monomers are not independently active (7). Molybdenum hydroxylases have in common a folding pattern that gives, from the N-

terminus, two discrete iron-sulphur centers, [2Fe-2S] domains, followed by a flavin domain and finally the molybdenum-binding portion of the protein (12).

There are three different types of redox center in each subunit, consisting of one molybdopterin cofactor, one FAD, and two [2Fe-2S] centers (8,9). The first three-dimensional structure for molybdenum hydroxylases using X-ray crystal-ography is that described by Romão *et al.* for aldehyde oxidoreductase from *Desulfovibrio gigas* (9).

Until recently (13), there was some discrepancy about the number of ligands attached to molybdenum atom (4,14). However, the molybdenum atom is believed to be co-ordinated to six-ligands; a sulphido and oxo ligand, two ligands with the dithiolate pterin cofactor, and two unspecified ligands, one of which is thought to be the catalytically labile site which may be an oxo ligand, a water ligand or a metal co-ordinated hydroxide (4,14,15). Each iron-sulphur center is believed to function as a one-electron acceptor, whereas the other two centers, FAD and molybdenum, function as two-electron acceptors. Olson *et al.* have concluded that the iron-sulphur centers act as electron sinks serving to keep molybdenum oxidised and FAD reduced during turnover (16). These redox centers mediate electron transport around the enzyme, as shown in Figure 2 (7).

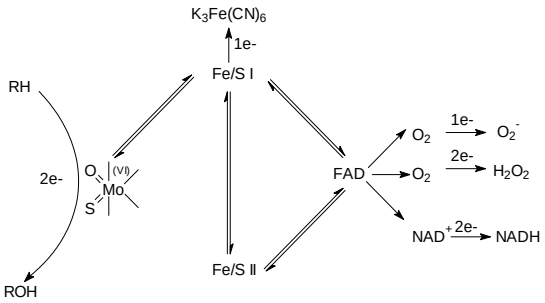

Figure 2. Site of substrate interaction with the molybdenum hydroxylases and the intra-molecular transfer of electrons between the various redox groups (7).

The sites of interaction of reducing and oxidising substrates have been investigated by spectrophotometric or electron paramagnetic resonance (EPR) spectroscopy (16,17). These studies have shown that substrate oxidation occurs predominantly at the molybdenum site, with the exception of NADH, which reacts directly with FAD in xanthine oxidase. *In vivo*, electrons are passed from the enzyme to the physiological electron acceptors, oxygen or NAD^+, *via* the FAD center (Figure 2).

There are three main inactive forms of xanthine oxidase or aldehyde oxidase: desulpho, demolybdo and deflavo enzyme (18-20). Desulpho and demolybdo forms are thought to occur *in vivo*, whereas, the deflavo form is an artefact of enzyme purification (18-20). In the desulpho form, the Mo=S ligand in the molybdenum active site is replaced by Mo=O. It is possible that this form may serve to regulate aldehyde oxidase, being reactivated in the presence of sulphur incorporating enzymes such as rhodanese (20). The demolybdo form has no molybdenum atom but still contains the molybdopterin cofactor (9).

Desulpho and demolybdo forms are inactive with respect to the oxidation of substrates, such as N^1-methylnicotinamide (21). On other hand, deflavo enzyme is devoid of oxygen reductase activity, which indicates the necessity for FAD in oxygen reduction (19).

Electron egress from the enzyme can be followed using electron acceptors, that react at different redox centers. Dichlorophenolindophenol (DCIP) and potassium ferricyanide ($K_3Fe(CN)_6$) accept electrons from molybdenum, and iron-sulphur centers, respectively, whereas cytochrome c is reduced *via* superoxide anion generated at the FAD site (19,22). The reduction of DCIP, potassium ferricyanide and cytochrome c can be followed spectrophotometrically at 600 nm, 420 nm and 550 nm, respectively.

They are very useful probes for determining enzyme activity and electron flow in multicofactor-containing enzymes in both their native and modified forms using spectrophotometric techniques. Deflavo aldehyde oxidase only reduces DCIP and potassium ferricyanide but shows no reaction with cytochrome c (22). Cytochrome c reductase activity is dependent on oxygen reduction that is absent in deflavo enzyme (19).

1.3. Specificity of aldehyde oxidase:

1.3.1. Substrate specificity:

Aldehyde oxidase catalyzes nucleophilic attack at an electron-deficient carbon atom adjacent to a ring nitrogen atom in N-heterocyclic compounds which are oxidised to cyclic lactams (7). In addition,

aldehydes are converted to carboxylic acids (7). Generally, under physiological conditions, the majority of aldehydes are metabolised to carboxylic acids rather than to alcohols, because the reduction of aldehydes to alcohols is readily reversible while the oxidation of aldehydes to carboxylic acids are not (23).

Aldehyde oxidase has a broad substrate specificity that includes endogenous as well as exogenous compounds (7,24). It catalyzes the hydroxylation of a wide range of substrates, including aliphatic and aromatic aldehydes, in addition to charged and uncharged nitrogenous heterocyclic compounds, including drugs such as methotrexate, quinine, quinidine, acyclovir and famciclovir (24). It is also involved in oxidation of reactive iminium ions to more stable lactams.

Aldehyde oxidase also reacts with metabolites of other drug metabolising enzymes, such as oxidation of tamoxifen, a triarylethylene anti-tumour agent, to tamoxifen acid (25) and citalopram, tertiary amine antidepressant, to citalopram proprionic acid (26), after their conversion to aldehydes by cytochrome P450 and monoamine oxidase, respectively.

Endogenous substrates of aldehyde oxidase include retinal, homovanilly aldehyde, vanillin, dihydromandelaldehyde and pyridoxal (Figure 3) (27,28). Thus, it is possible that aldehyde oxidase plays a role in physiologically significant processes such as vision and synaptic transmission (29).

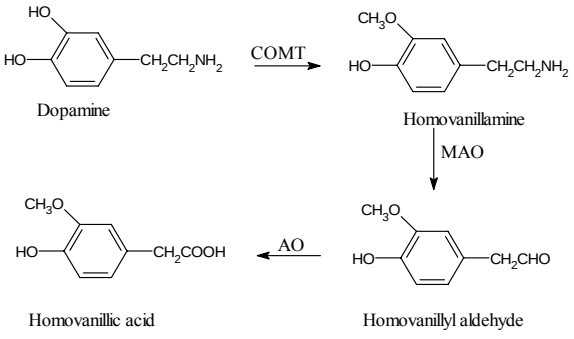

Figure 3. Pathway of dopamine catabolism in mammalian systems by catechol-O-methyltransferase (COMT), monoamine oxidase (MAO) and aldehyde oxidase (AO) (32).

The main classes of reducing substrates for aldehyde oxidase include oxime, azo dyes, aromatic and heterocyclic hydroxamic acid and benzisoxazoles such as zonisamide, a novel anticonvulsant. K_m values for these compounds range from around 1 mM with acetaldehyde to less than 1 µM with phenanthridine. Wright *et al.* Mira *et al.* showed that NADH is also oxidised by aldehyde oxidase (30,31).

In addition, it is interesting that aldehyde oxidase is able to catalyze the reduction of variety of compounds such as sulphoxides, *N*-oxides, nitrosamines, hydroxamic acids, azo dyes, oximes, epoxides, aromatic nitro compounds and 1,2-benzisoxazole derivatives (reference 32 and references therein) However, oxidation reactions are prevalent to reductive reactions *in vivo*.

1.3.2. Inhibitor specificity:

Aldehyde oxidase inhibitors may be categorized according to their mode of interactions with aldehyde oxidase into three main classes.

The first class are reagents that interact with the substrate-binding site to yield inactive molybdopterin cofactor; these include cyanide, arsenite, 4-(chloromercuri)benzoate and methanol. Consequently, these inhibitors are common for both aldehyde oxidase and xanthine oxidase. The enzymes are rendered non-functional upon reaction with cyanide, which reacts with the Mo=S group to yield one equivalent of thiocyanate. However, it has been found that aldehyde oxidase reacts several orders of magnitude more rapidly with cyanide to give the desulpho form of the enzyme than does xanthine oxidase, in an irreversible manner (21). The site of interaction is thought to be at the substrate-binding site due to the protection effect of quinacrine, a competitive aldehyde oxidase inhibitor (21,33). The inhibition of aldehyde oxidase by either arsenite or the thiol reagent, 4-(chloromercuri)benzoate, is reversible and competitive with respect to reducing substrate, while both compounds are irreversible inhibitors of xanthine oxidase exhibiting uncompetitive inhibition with respect to xanthine as substrate (43). Both aldehyde oxidase and xanthine oxidase undergo progressive inhibition by methanol, which is dependent on enzyme turnover.

The second class includes inhibitors that are structurally similar to aldehyde oxidase substrates and are also thought to act at the molybdenum

center. Consequently, chlorpromazine (Figure 4, [1]) (33), amsacrine [2] (35,36), hydralazine [3] (37) and isovanillin [4] (38) are potent aldehyde oxidase inhibitors that resemble *N*-methylphenothiazine, phthalazine, vanillin and *N*-[(2'-dimethylamino)-ethyl]acridine-4-carboxamide (DACA), respectively, (Figures 4). Inhibitor constants (K_i or IC_{50}) for the above inhibitors is in the order of 1 µM with amsacrine, chlorpromazine, hydralazine and isovanillin using hepatic aldehyde oxidase from different species.

The third class includes inhibitors of the internal electron transport system and FAD site. This class includes menadione (Figure 4, [5]) (21), and β-estradiol [6] (33). Menadione is one of the most potent and selective inhibitors for aldehyde oxidase (38). It has been suggested that menadione exerts its effect through blocking of electron transfer from the FAD center (39). This is in agreement with the ability of menadione to serve as an electron acceptor for xanthine oxidase and other flavoproteins and even to enhance their reaction (39). It is interesting to note that aldehyde oxidase, purified from maize seedlings, although very similar to that found in mammalian systems is not inhibited by menadione (40). There are differences in the NAD-binding site between the mammalian and plant aldehyde oxidase genes that may be responsible for the inability of menadione to inhibit maize aldehyde oxidase (41). β-Estradiol, an estrogen, has been shown to be a potent progressive inhibitor of liver aldehyde oxidase *in vitro* (33). By using different artificial electron acceptors, it has been shown that DCIP, but not potassium ferricyanide nor cytochrome c, could be reduced in the present of estrogens (38). It has been proposed that β-estradiol exerts its inhibitory effect through interaction with electron flow within the enzyme beyond molybdenum center (38).

It should be noted that the extent of aldehyde oxidase inhibition by some of the aforementioned inhibitors depends on the species under test. For example, methadone (Figure 4, [7]) was found to be very potent inhibitor of rat liver aldehyde oxidase, but a poor inhibitor of guinea pig liver aldehyde oxidase (42).

Allopurinol, a selective inhibitor of xanthine oxidase, is a moderate substrate for aldehyde oxidase (43). Allopurinol is traditionally used as a specific xanthine oxidase inhibitor both *in vivo* and *in vitro*, whereas menadione is often employed *in vitro* as a specific aldehyde oxidase inhibitor (39).

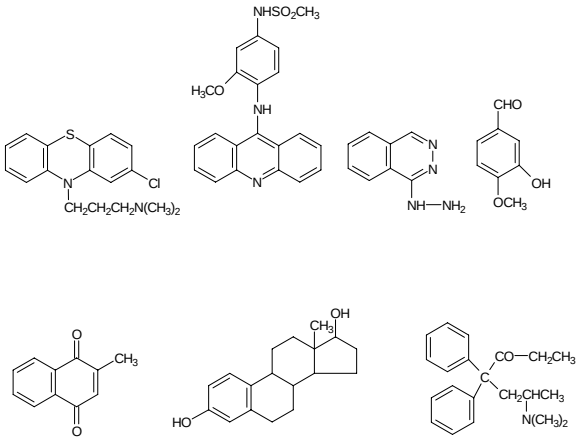

Figure 4. Structures of some potent aldehyde oxidase inhibitors.

1.4. The mechanism of action of molybdenum hydroxylases

Since xanthine oxidase is readily available from the bovine milk, most of mechanistic studies on the oxidative reaction of molybdenum hydroxylases have been carried out using a xanthine oxidase/xanthine system (4,14). However, it has been assumed that aldehyde oxidase has a very similar mechanism of action to xanthine oxidase (44). The overall mechanism is ping-pong, with initial reduction of the enzyme by reducing substrate and product formation occurring at the molybdenum site, prior to reduction of the oxidising substrate (by oxygen) at the FAD site (16).

1.4.1. Role of molybdenum center in substrate binding:

In most mechanistic studies it has been proposed that the initial step involves the binding of substrate to the molybdopterin site of the enzyme. Although the oxygen atom incorporated into product is ultimately derived from water, rather than molecular oxygen, studies indicate that the primary source of the oxygen atom could be either an oxo ligand (Mo=O) (14), "buried" water or, more recently, metal bound hydroxide (Mo-OH) (4). Hille and Sprecher investigated the source of oxygen incorporated in to product, using either $H_2{}^{18}O$-labelled water or ^{18}O-labelled enzyme at substoichiometric xanthine concentrations; they found that oxygen is transferred originally from a

catalytically labile site in the enzyme (45). However, the precise nature of the oxygen function within the enzyme is still equivocal.

After binding to the molybdenum center, xanthine is rapidly oxidized to uric acid with a concomitant two-electron reduction of the molybdenum from Mo^{VI} to Mo^{IV}. This is followed by a series of one-electron transfers from the molybdenum center to other sites and molybdenum is re-oxidized to Mo^{VI}. When an EPR signal was generated using [8-^{13}C]-xanthine, strong isotropic splitting of a Mo^{V} signal was observed, demonstrating that the purine nucleus was an integral component of the signal-giving species (46).

Furthermore, when a signal was generated using enzyme labelled with ^{33}S strong anisotropic coupling was observed, indicating that the catalytically essential sulphur was a Mo=S ligand (47). Similarly, when the signal was generated in $H_2{}^{17}O$, strong but approximately isotropic splitting was observed.

As Mo=O has been identified in the molybdopterin cofactor of xanthine oxidase, it was initially thought that the oxo ligand is the catalytically labile site in the enzyme. However, this mechanism requires immediate regeneration of Mo=O, *via* solvent, prior to further substrate turnover (14).

An alternative mechanism has been proposed for both aldehyde oxidase and xanthine oxidase which involves a "buried" water molecule as the initial oxygen source (11). This is supported by crystallographic data for the *Desulfovibrio gigas* aldehyde oxidoreductase which indicate the presence of three "buried" water molecules located in a side pocket within the protein structure.

In the mechanism proposed by Howes *et al.* for xanthine oxidase the molybdenum oxo-ligand plays no active part in the reaction (15). This mechanism was supported by an earlier study of xanthine oxidase reduction by ^{13}C-labelled aldehydes (48). In this study, Mo-^{13}C bond formation was detected using electron-nuclear double resonance (ENDOR) spectroscopy technique (48).

Catalysis is thought to proceed *via* deprotonation at the C-8 position of xanthine and reduction of the Mo=S ligand to Mo-SH. Reduction of water with the enzyme-substrate complex is thought to generate product and initiate electron transfer to other redox groups.

More recently, it has been suggested that the catalytically labile site of the enzyme is a metal-coordinated hydroxide ligand rather than "buried" water or an oxo-ligand (4). Alternate mechanisms in which a Mo-OH group, rather than Mo=O, represents the catalytically labile oxygen have been considered. Subsequently, Xia *et al.* proposed a base-assisted hydroxylation at an electron deficient carbon *via* Mo-OH that precedes hydride transfer to the sulphido ligand of molybdenum (4).

In fact, Greenwood *et al.* (49) have previously suggested that a metal-bound hydroxide might represent the catalytically labile oxygen on the basis of ^{17}O-EPR studies of model compounds relevant to the enzyme active site.

The most recent crystallographic information suggest that Mo=O group occupies the apical position and favors the reaction mechanism shown in Figure 5. In this mechanism an active site base abstracts the proton from the Mo-OH group, which then undertakes a nucleophilic attach on the C-8 position of substrate (4).

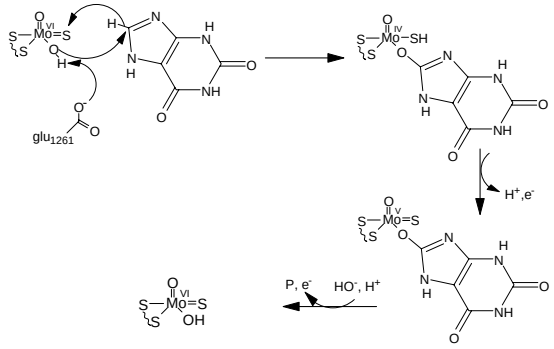

Figure 5 Proposed mechanism of xanthine oxidase action based on metal Mo-OH group (4).

Concomitant hydride transfer to the Mo=S group yields an $Mo^{IV}O(SH)(OR)$ species as shown, with OR representing product coordinated to the metal vial the newly introduced hydroxyl group. This initial intermediate breaks down by electron transfer to other redox-active centers in the enzyme, with the transient formation of a paramagnetic Mo^{V} species detectable by EPR spectroscopy, followed by displacement of product by hydroxide from solvent to return to starting $Mo^{VI}OS(OH)$ state (4).

1.4.2. Intra-molecular electron transfer:r

As shown in Figure 3, reducing equivalents react with molybdenum hydroxylases *via* the molybdenum

center, which subsequently transfers electrons to other redox groups. Up to six electrons per subunit can be accepted *via* reducing substrates under normal conditions, although eight electrons may be accepted during reduction by dithionite (16).

These electrons can be transferred to the other electron centers, with the iron-sulphur centers probably acting as an electron sink to maintain the molybdenum and flavin as Mo^{VI} and as $FADH_2$, respectively. These two states are required for effective reacting enzyme (16).

A detailed mechanism for electron distribution within xanthine oxidase has been proposed by Olson *et al.* called "rapid equilibrium hypothesis" (50). They suggest that internal electron transfer between the redox centers in xanthine oxidase is always faster than the rates of reduction and re-oxidation of the enzyme by xanthine and oxygen, respectively (50). The oxidation states of the redox groups during the catalytic cycle should be determined solely by their relative reduction potentials.

Barber and Siegel have compared relative reduction potentials for aldehyde oxidase and xanthine oxidase (see Table 1) (51). They indicate that the Fe/S I potential, when measured in the same buffer, is considerably more positive in aldehyde oxidase (-207 mV) than xanthine oxidase (-280 mV). In contrast, the Fe/S II potential in aldehyde oxidase (-310 mV) is more negative than in xanthine oxidase (-245 mV). Thus, whereas Fe/S II is reduced prior to Fe/S I in xanthine oxidase, it is reduced after Fe/S I in aldehyde oxidase. Moreover, the reduction potential of FAD is much more positive in aldehyde oxidase at pH 7.8 than in xanthine oxidase (see Table 1).

Table 1 Midpoint reduction potentials for prosthetic groups of rabbit liver aldehyde oxidase and bovine milk xanthine oxidase (pH 7.8, Mean ± 15 mV) (15).

Prosthetic group	*Potential (mV)*	
	Aldehyde oxidase	**Xanthine oxidase**
$FAD/FADH^{\cdot}$	-258	-310
$FADH^{\cdot}/FADH_2$	-212	-220
Fe/S I (ox/red)	-207	-280
Fe/S II (ox/red)	-310	-245
Mo^{VI}/Mo^{V} rapid	-359	-355
Mo^{V} rapid/Mo^{IV}	-351	-335
Mo^{VI}/Mo^{V} slow	-439	-354
Mo^{V} slow/Mo^{IV}	-401	-386

Recent studies have reported, the midpoint reduction potentials for redox centers in prokaryotic xanthine oxidase which were found to be very similar to those of the corresponding centers in eukaryotic enzyme (52).

The relative reduction potentials or electron affinity constants should control intramolecular electron transfer. As shown in Table 1, the electronegativity of the redox groups in the molybdenum hydroxylases can be approximately ordered from high- to low-potential giving: FAD > Fe/S > Mo.

Turnover in modified enzyme can be severely attenuated when the internal equilibrium distribution of reducing equivalents becomes unfavourable (53). By using series of enzyme forms possessing chemically-modified flavins, it was found that Vmax values for substrate oxidation depended exponentially on the flavin midpoint potential, with enzyme possessing low-potential flavin derivatives exhibiting less than 10% of the catalytic power of enzyme possessing high-potential derivatives (53). This has been shown to be due to the accumulation of reduced molybdenum (Mo^{IV}) and oxidised flavin (FAD) at steady state as a result of unfavourable distribution of reducing equivalents in the partially reduced enzyme species (53).

In aldehyde oxidase and xanthine oxidase, the midpoint reduction potentials of the native flavin center are sufficiently negative (-212 and -220 mV) to be a good electron donor for oxygen, while the potentials of the molybdenum center are sufficiently more negative (-359 and -355 mV) to assure that the enzymes do not become trapped in the unfavourable condition of stabilising reduced molybdenum (Mo^{IV}) and oxidised flavin (FAD).

Rate constants for electron transfer between specific pairs of redox-active centers within xanthine oxidase have been examined by flash photolysis, a pH-perturbation method and pulse radiolysis (54,55). Using radiolytically-generated deazariboflavin radical as a reductant, it was reported that the rate constants between Fe/S and FAD centers are 120 s^{-1} and 218 s^{-1} at pH 6.0 and 8.5, respectively (55). By using iodoacetamide to alkylate the flavin center, electron transfer from Fe/S to FAD centers was prevented and electron transfer between the two Fe/S centers could be determined (90 s^{-1}) (55).

When radiolytically generated N^1-methyl-nicotinamide radical was used as selective reductant

for the molybdenum center in xanthine oxidase, rate constants of 8,500 s^{-1} and 125 s^{-1} were measured for electron transfer from the initially reduced molybdenum center to one of Fe/S centers and from Fe/S I to FAD, respectively, at pH 6.0 (55).

Studies by Mondal and Mitra have shown that the rate constant for electron transfer from the xanthine to xanthine oxidase is the rate-limiting step (56). Thus, the value of the rate constant for electron transfer from the substrate to enzyme (7.7 s^{-1}) is slower than the rate constant for intramolecular electron transfer (≥90 s^{-1}) at 20 °C and pH 6.0 (55,56).

2. Pathophysiology of molybdenum hydroxylases:

It can be seen from Section 1.3.1 that aldehyde oxidase is important in the detoxification and clearance of many drugs and endobiotics. However, reduction of oxygen, during substrate turnover, leads to the formation of superoxide anion and hydrogen peroxide (3). This capacity has attracted attention to the possible role of aldehyde oxidase as a source of reactive oxygen species (ROS). *In vivo,* it seems that aldehyde oxidase and xanthine oxidoreductase are, quantitatively, the most important cellular sources for ROS (3).

Consequently, aldehyde oxidase has been implicated in pathophysiology of alcohol liver injury (31), visual processes, synthesis of retinoic acid (28) and reperfusion tissue injury (57). Although xanthine oxidase generates ROS, it should be noted that *in vivo,* the enzyme exists predominantly as a dehydrogenase, reacting with NAD^+, whereas aldehyde oxidase reacts exclusively with oxygen. Consequently, aldehyde oxidase, rather than xanthine oxidoreductase, appears to be responsible for ROS formation while under pathological conditions, such as ischaemia-reperfusion injury, both enzymes may participate (57).

It has been suggested that ethanol may cause tissue injury *via* the production of ROS and subsequent lipid peroxidation (58). Furthermore, it has been proposed that ROS production *via* aldehyde oxidase may cause liver and brain injury during alcohol metabolism (59). Oxidation of ethanol to acetaldehyde is catalyzed by alcohol dehydrogenase and/or cytochrome P450 (CYP2E1) (60). The production of ROS by aldehyde oxidase could occur *via* the oxidation of acetaldehyde to acetic acid or NADH to NAD^+ (30,31,59). It has also been proposed that ROS arising from the action of aldehyde oxidase on acetaldehyde and/or NADH (produced *via* alcohol dehydrogenase/ethanol reaction) may be a contributing factor to breast cancer (4). The mechanism of the ROS tissue-injury during alcohol toxicity has been shown to be mediated through an iron-catalyzed process that suggests a role for hydroxyl radicals in this injury (31).

One physiological role of aldehyde oxidase may be the oxidation of retinal, vitamin A, to retinoic acid (61). Retinoic acid is an important mediator in the differentiation and development of neurons and glia (see below) as well as cell-cell signalling in the central nervous system (29). Recently, it has been shown that altered retinoic acid synthesis could be implicated in the aetiology of Parkinson's disease and schizophrenia (62,63). Alternatively, aldehyde oxidase is a source of oxygen radicals, which may contribute to these diseases (31).

Glial cells form 90% of the cells within the nervous system (64). They are thought to support the environment around nerve cells; for example, by regulating the concentrations of free ions in the extracellular spaces of spinal cord, transporting materials to and from blood vessels, and providing metabolic support for the neuronal membrane. Berger found that aldehyde oxidase was highly expressed in glial cells of the lateral motor column; the region of the spinal cord which degenerates in amyotrophic lateral sclerosis (ALS) (65). This, together with the fact that aldehyde oxidase is selectively localised in the motor neurons of mouse brain and spinal cord, suggest that the enzyme could play a role in the homeostasis of motor neurons (66).

It has been suggested that ALS, a neurodegenerative disorder resulting in motor neuron death, is associated with oxidative damage induced by ROS (3,67).

Consequently, it has been proposed that aldehyde oxidase is linked to ROS generation in ALS (31). In fact, recent genetic evidences has implicated aldehyde oxidase in the genesis of the familial recessive form of ALS (65). In this hereditary disease, the candidate gene maps on chromosome 2q33-2q35, the same locus for human aldehyde oxidase (67,68). In support of this hypothesis it has been recently demonstrated, by *in situ* hybridisation experiments, that mouse aldehyde oxidase is selectively localised in the cranio-facial and schleletric motor neurons (66).

However, ALS is potentially a useful model for more common neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and Huntington's chorea due to several reasons. First, a common feature of these disorders is selective neuronal death. Therefore, Doble has proposed that the proximate cause of neuronal death may differ in these diseases, but the final common pathway is likely to be similar (69). Secondly, the involvement of motor system in ALS permits simpler and more direct diagnosis than do extrapyramidal changes or dementia in Parkinson's disease and Alzheimer's disease, respectively (70). Thirdly, the relatively rapid onset (less than five years) and stereotyped nature history facilitate clinical monitoring of the disease (71).

The causes of ALS are still unknown, but there is increasing evidence that two pathogenic mechanisms, namely excitotoxicity and oxidative stress, participate in this disorder (72,73). However, physiological activation of motoneurons by glutamate (one of the main mediators of the nervous system) has been coupled to abnormal activity of cytoplasmic superoxide dismutase (SOD) (74). It has also been reported that excitotoxicity can be increased by oxidative stress (72). Consequently, Kalra *et al.* have proposed that these two mechanisms may participate in a "positive feedback" manner in which one potentiates the other (75). If this is the case, then it is possible that antioxidants and glutamate antagonists could have either additive or synergistic effects in the treatment of ALS. Furthermore, ROS producing systems, such as aldehyde oxidase and xanthine oxidase could participate in the aetiology of this disorders.

A link between ROS and ALS is supported by the selective vulnerability of motor neurons to free radical damage (76). Recent reports have indicated oxidative changes in proteins, lipids and DNA in the CNS of patients with ALS (73,77). For this reason, antioxidants like procysteine and TR500 (glutathione repleting agents) are in phase I and recombinant SOD is in phase II trials for ALS (78,79).

Most patients (85-90%) present with "sporadic ALS" while the remaining 10% of ALS cases are of familial origin (80). Both sporadic and familial ALS manifest the same clinical and pathological symptoms. Approximately 15-20% patients with "familial ALS" pattern show mutations in the gene expressing SOD which supports a role for ROS in ALS (81). The genetic evidence suggests that sporadic cases may also result from dysfunction of free radical homeostatic pathways (82). Mutations of the SOD gene are found in approximately 2% of cases of sporadic ALS (80). Such mutations are dominant and the gene is located on chromosome 21q22.1 (80). Recently, it has been found that both recombinant SOD and catalase prolong the survival of ALS mice models after disease onset (83). Jean-Pyolee (74) has suggested that mutant SOD is not deficient in ALS but is hyperactive which leads to a greater accumulation of hydrogen peroxide than normal. The use of human antioxidant enzymes, obtained by gene-technology, may permit the treatment of a variety of clinical conditions associated with oxidative stress.

A second mutation locus responsible for familial ALS has been mapped to chromosome 2q33, which is inherited in an autosomal recessive pattern (84). The aldehyde oxidase gene, which is also mapped to 2q33, has been coupled to familial ALS (85).

At present, the aetiopathologies of Parkinson's disease, Alzheimer disease and Huntington's chorea are unknown. However, the most accepted hypothesis is selective oxidative stress in the CNS (76).

Studies indicate that dopaminergic neurons in Parkinson's disease may be more susceptible to oxidative stress due to reduced glutathione levels and excessive free iron content (76). Dopamine generates free radicals and hydrogen peroxide by auto-oxidation or through normal enzymatic processing by monoamine oxidase (Figure 3). Consequently, high levels of hydrogen peroxide are present in the substantial nigra.

It has also been suggested that the neuropathology of Huntington's chorea involves oxidative stress, although most of the evidence is indirect (76). Post-mortem brains from patients with Huntington's chorea show an increase in oxidised DNA indicative of oxidative stress damage coupled with reduced levels of SOD and oxidised glutathione (86).

Evidence for the role of oxidative stress in Alzheimer's disease aetiology is accumulating (87,88). Various products of oxidation reactions, e.g. oxidised glutathione molecules, and mediators of oxidative stress, e.g. accumulation of free fatty acids, are found in brain of patients with Alzheimer's disease (88). Basically, most of cellular macromolecules (DNA, protein, and lipids) can be found in an oxidised form in Alzheimer's disease

brain tissue. Other studies indicate that SOD activity is decreased in Alzheimer's disease brain although these results are not substantiated in other studies (89).

Riluzole [2-amino-6- (trifluoromethoxy) benzo-thiazole], an inhibitor of glutamatergic transmission in the CNS, is currently given to patients with ALS to improve their prognosis (90,91). The mechanism by which riluzole acts as a neuroprotective is presently unknown. It has been found that the drug specifically blocks inactivated sodium channels in myelinated nerve fibre, with IC_{50} of 65 μM (91). Alternatively, Oturan *et al.* have shown that riluzole traps hydroxyl radicals, produced from the decomposition of hydrogen peroxide, to give two hydroxylated products (91). Consequently, antioxidants and antiglutamate agents may have additive or synergistic effects in the treatment of ALS.

The interaction of riluzole with aldehyde oxidase in the CNS may contribute to its neuroprotective effect by inhibiting ROS production or altering the balance between hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). Our studies showed that riluzole inhibits initial rates of superoxide anion production during phthalazine oxidation by aldehyde oxidase but the overall formation of superoxide anion appeared to be enhanced (non-competitive inhibition, K_i = 53 μM) (93,94). A more pronounced inhibitory effect was observed with hydrogen peroxide formation (competitive inhibition, K_i = 9.3 μM). Accordingly, it appears that, *in vitro,* riluzole may decrease hydrogen peroxide production but increase superoxide anion production. This may contribute to the variability in response to riluzole in ALS patients, who may also have varying SOD activity (95). It would be of interest to measure the aldehyde oxidase status of patients with ALS. N^1-Methylnicotinamide has been used as a probe aldehyde oxidase substrate in patients with classical xanthinuria type II (96). This is a rare autosomal recessive disorder in which both aldehyde oxidase and xanthine oxidase are absent. However, the distribution of aldehyde oxidase within a normal population has, to date, not been investigated.

In addition to its therapeutic effects in ALS, riluzole is neuroprotective in models of brain ischaemia, Parkinson's disease, Alzheimer disease and Huntington's chorea (76,97). Riluzole is in phase III trials for Parkinson's disease, phase II trials for Alzheimer disease and phase I trial for Huntington's chorea (98-100). It is possible that riluzole, or other aldehyde oxidase inhibitors that pass the blood brain-barrier, may also reduce oxidative damage in the CNS generated by the oxidation of neurotransmitters catalysed by aldehyde oxidase.

Conclusion

Aldehyde oxidase has a broad substrate specificity and thus catalyzes the oxidation of a wide range of endogenous compounds and xenobiotics; in addition to cytochrome P450, it is a major defense mechanism for the removal of drugs/xenobiotics from the body (22). Consequently, as an obligate aerobe, aldehyde oxidase is one of the most important sources of ROS (3,30,31). In contrast to xanthine oxidoreductase, which can be converted from a dehydrogenase to oxidase form, aldehyde oxidase seems to be a permanent oxidase, with no activity towards NAD^+. Furthermore, aldehyde oxidase generates hydrogen peroxide and superoxide anion under normal physiological conditions whereas most other sources produce ROS only in certain pathological conditions such as ischaemia. In addition, aldehyde oxidase generates both hydrogen peroxide and superoxide anion simultaneously, whereas the majority of other systems produce either hydrogen peroxide or superoxide anion.

There is a lot of evidence to indicate that many physiological and pathological conditions such as ageing, inflammation, viral infections and neurodegenerative diseases may develop through the action of ROS (This was reviewed in reference 3). Neurodegeneration of motor neurons in the CNS is symptomatic of the progressive disease ALS. Some forms of the ALS have been linked, *via* genetic mapping and ROS generation, to aldehyde oxidase (30,31). This is supported by the fact that the aldehyde oxidase gene is highly expressed in motor neurons and glial cells of the spinal cord.

This manuscript indicates that molybdenum hydroxylases, particularly aldehyde oxidase and xanthine oxidase, contribute directly to the human disorder in several levels. This has been supported by the fact that these enzymes produce reducing equivalents rather than consume as seen from their mechanism of action.

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