Physicochemical and Kinetic Properties of Purified Sheep's Milk Xanthine Oxidoreductase

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ABSTRACT

Xanthine oxidoreductase (XOR) was purified for the first time from sheep's milk. The ultraviolet-visible absorption spectrum was essentially identical to those of the corresponding bovine, human, and goats' milk enzymes and showed an A_{280}/A_{450} ratio of 5.35 ± 0.24 , indicating a high degree of purity. Like milk XOR from other species, sheep's milk enzyme showed a single band on SDS-PAGE corresponding to a subunit with approximate M_r 150,000. Xanthine oxidase activity of purified sheep's milk XOR ($0.69 \pm 0.04 \mu\text{mole urate min}^{-1} \text{mg}^{-1}$) was low relative to that of the bovine milk enzyme ($1.83 \pm 0.02 \mu\text{mole urate min}^{-1} \text{mg}^{-1}$), but higher than those of human or goats' milk XOR. As in the latter 2 cases, the low activity of sheep's milk XOR can be attributed to its relatively low molybdenum content (0.18 atoms per subunit), compared with that of the bovine milk enzyme (0.56 atoms Mo per subunit). Consistent with this, NADH oxidase activity of sheep's milk XOR was similar to that of enzymes purified from bovine, human, or goats' milk. The presence of desulpho-enzyme in sheep's milk XOR was demonstrated by re-sulfuration experiments, whereby xanthine oxidase activity was increased by approximately 75%.

(Key words: molybdenum, NADH oxidase, sheep's milk, xanthine oxidoreductase)

Abbreviation key: MFGM = milk fat globule membrane, $\text{NAD}^+(\text{H})$ = nicotinamide adenine dinucleotide (reduced), PFR = protein/flavine ratio, ROS = reactive oxygen species, XDH = xanthine dehydrogenase, XO = xanthine oxidase, XOR = xanthine oxidoreductase.

INTRODUCTION

Xanthine oxidoreductase (XOR), a member of the molybdenum hydroxylase flavoprotein family, is a complex

enzyme long known to be present in the bovine milk fat globule membrane (MFGM) (Patton and Keenan, 1975). Because of this ready availability, XOR has been studied for over 100 yr (Massey and Harris, 1997), and its enzymology is well characterized (Bray, 1975; Hille, 1996). It comprises 2 subunits (M_r 150,000), each of which contains one flavin adenine dinucleotide, one Mo, and 2 Fe_2S_2 redox centers. The enzyme occurs in 2 interconvertible forms, dehydrogenase (XDH, EC 1.1.1.204) and oxidase (XO, EC 1.1.3.22), both of which reduce molecular oxygen to the reactive oxygen species (ROS), superoxide anion and hydrogen peroxide. XDH, but not XO, reduces NAD^+ .

The generally accepted physiological role of XOR is in purine catabolism, where it catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid, with concomitant reduction of NAD^+ or molecular oxygen (Bray, 1975). Nevertheless, the enzyme has a somewhat specialized distribution, being especially rich in endothelial and epithelial cells, and other functions have been sought, particularly those involving its generation of ROS (Harrison, 2002).

Together with the less-studied rat liver enzyme (Amaya et al., 1990), which has very similar properties, bovine milk XOR has provided a basis for discussions of XOR in humans. However, human milk XOR has surprisingly low XO activity (Abadeh et al., 1992; Sanders et al., 1997), resulting from its low content of molybdenum, which is less than 5% theoretical (Godber et al., 1997; Bray et al., 1999). This low enzymatic activity raises questions about the physiological role of human XOR (Harrison, 1997) and the potential for posttranslational activation in vivo. Goats' milk XOR has recently been shown to have similarly low XO activity and Mo content (Atmani et al., 2004), albeit a little higher than that of the human enzyme, and it was of interest to examine XOR from other mammals. We now describe, for the first time, purification and characterization of XOR from sheep's milk.

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MATERIALS AND METHODS

Purification of Sheep's Milk XOR

Xanthine oxidoreductase was purified from fresh sheep's milk by ammonium sulphate fractionation, followed by affinity chromatography on heparin and ion-exchange fast protein liquid chromatography, essentially as described for bovine (Godber et al., 2000), human (Sanders et al., 1997) and goats' milk (Atmani et al., 2004). In a minor modification, 10 mM, rather than 5 mM dithiothreitol was added to the cream.

Concentration of enzyme was determined from the UV-visible spectrum by using an absorption coefficient of 36 mM subunit⁻¹ cm⁻¹ at 450 nm (Bray, 1975). Protein concentrations were determined as described by Bradford (1976).

The oxidase content of XOR was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295 nm, using an absorption coefficient of 9.6 mM cm⁻¹ (Avis et al., 1956). Assays were performed at 25 ± 0.2°C in air-saturated 50 mM Na/bicine buffer, pH 8.3, containing 100 μM xanthine. The sum of oxidase and dehydrogenase contents was determined as above but in the presence of 0.5 mM NAD⁺.

Molybdenum content of purified XOR was determined by a modification (Atmani et al., 2004; Baghiani et al., 2003) of the colorimetric procedure of Hart et al., (1970).

Steady-State Kinetic Studies

With xanthine as reducing substrate, urate was determined as described above for determination of oxidase plus dehydrogenase content of XOR. 1U is defined as 1 μmole of urate produced per minute. With NADH as reducing substrate, NADH utilization was followed at 340 nm, in air-saturated 50 mM sodium phosphate buffer, pH 7.2, at 25 ± 0.2°C, by using an absorption coefficient of 6.22 mM⁻¹ cm⁻¹ (Horecker and Kornberg, 1948). 1U is defined as 1 μmole of NADH consumed per minute.

Desulfuration and Sulfuration of XOR

Purified sheep's milk XOR was converted to its desulfo-form by incubation with KCN (Massey and Edmondson, 1970; Godber et al., 2000). Desulfo-XOR or native enzyme were (re)sulfurated by incubation with methyl viologen and sodium sulfide in a modification (Godber et al., 2000; Baghiani et al., 2003) of the procedure described by Wahl and Rajagopalan (1982).

SDS-PAGE

SDS-PAGE was performed on a Bio-Rad Protean II mini-gel apparatus according to the method of Laemmli (1970), using a 5% concentrating gel and 10% separating gels. Samples and standards of known molecular weights (MW-SDS-200 Kit, Sigma) were run at 100 (stacking gel) and 200 V (running gel). Gels were stained for protein with Coomassie brilliant blue.

RESULTS

Five separate purifications yielded 22.6 ± 3.3 mg (mean ± SD) of XOR per liter of sheep's milk. Purified enzyme showed a characteristic UV-visible spectrum (Figure 1), with A_{280}/A_{450} (protein to flavin, **PFR**) ratio of 5.34 ± 0.24 (mean ± SD, n = 5), indicating a high degree of purity (Bray, 1975). The PFR values for bovine, human, and sheep's milk enzymes prepared in parallel were very similar (Table 1).

Enzyme purity was confirmed by SDS-PAGE, which showed a single major protein band with M_r approximately 150,000 (Figure 1, inset). Around 25% of the purified enzyme was in the dehydrogenase form.

Xanthine oxidase activity of purified sheep's milk XOR was 0.69 ± 0.04 U/mg of protein, compared with respective V_{max} values of 1.83 ± 0.02, 0.06 ± 0.01, and 0.27 ± 0.01 U/mg of protein for bovine, human, and goats' milk, similarly prepared in the present study (Table 2). The K_m values for XOR from sheep's, human, and goats' milk were similar, being some threefold higher than that of the bovine milk enzyme (Table 2). The V_{max} and K_m values for NADH oxidation were similar for all 4 species (Table 2).

The molybdenum content of purified sheep's milk XOR, 0.18 ± 0.04 atoms per subunit, is compared with those previously reported for enzymes of other species in Table 1.

As shown in Table 3, incubation of the sheep's milk enzyme with KCN led to total loss of XO activity, consistent with its conversion to desulfo-enzyme. Either desulfo- or native XOR could be resulfurated, whereby the native enzyme regained 173% of its original xanthine oxidase activity. Purified bovine and human milk XOR behaved similarly, demonstrating the presence of significant contents of desulfo-form in preparations of native enzyme from all 3 species.

DISCUSSION

Xanthine oxidoreductase has been purified, for the first time, from sheep's milk. The purification procedure involved chromatography on heparin, as described previously for the bovine (Godber et al., 2000), human (Sanders et al., 1997), and goats' milk (Atmani et al.,

Figure 1. Ultraviolet-visible spectrum of purified sheep milk xanthine oxidoreductase (XOR) showing the three classic characteristic maxima at 280, 350, and 450 nm and SDS-PAGE (10%) of sheep milk XOR. Lines: 2 = pure sheep milk XOR; 3 = sheep XOR sample before heparin affinity chromatography; and 1 = molecular weight markers (myosin, 290,000; β -galactosidase, 135,000; albumin, 82,000; ovalbumin, 53,000; carbonic anhydrase, 29,500; trypsin inhibitor, 23,500; β -lactalbumin, 13,000; and aprotinin, 7500).

2004) enzymes. The UV-visible spectrum (Figure 1) was characteristic of XOR and showed a PFR ratio close to 5.0, generally accepted as a criterion of purity (Bray, 1975; see also Atmani et al., 2004). Like other XOR, freshly purified sheep's milk enzyme showed a single band on SDS-PAGE corresponding to a subunit of approximately 150 kDa. Traces of degradation bands appeared on storage (cf. Abadeh et al., 1992; Atmani et al., 2004).

The specific XO activity of purified sheep's milk XOR was found to be $0.69 \mu\text{mol of urate min}^{-1} \text{mg}^{-1}$. This can be compared with corresponding values for the purified human, goats', and bovine milk enzymes (0.06, 0.27,

and $1.83 \mu\text{mol of urate min}^{-1} \text{mg}^{-1}$, respectively), which have been explained (Atmani et al., 2004) in terms of their molybdenum contents (0.03, 0.09, and 0.55 atoms per subunit, respectively). The molybdenum content of sheep's milk XOR was shown here to be 0.18 atoms per subunit, which is roughly consistent with its specific activity. It is worth noting that, although xanthine is reduced directly at the Mo site (Bray, 1975), molybdenum content is not alone in determining XO activity. Xanthine oxidoreductase contains variable amounts of 'desulfo' enzyme, which contains molybdenum but is inactive because of 'replacement' of an Mo=S grouping by Mo=O (Gutteridge et al., 1978; Wahl and Rajagopa-

Table 1. Kinetic parameters for xanthine oxidation and NADH oxidation of xanthine oxidoreductase (XOR) purified from sheep's, human, goats', and cows' milks.¹

	Sheep's milk XOR	Human milk XOR	Goats' milk XOR	Cows' milk XOR
Xanthine oxidation				
$K_m (\mu M)$	7.14 ± 0.9	7.74 ± 0.06	6.33 ± 0.08	2.15 ± 0.05
V_{max}	0.69 ± 0.04	0.06 ± 0.01	0.27 ± 0.01	1.83 ± 0.02
NADH oxidation				
$K_m (\mu M)$	2.19 ± 0.10	2.52 ± 0.08	4.12 ± 0.06	2.74 ± 0.12
V_{max}	0.21 ± 0.04	0.29 ± 0.03	0.27 ± 0.04	0.25 ± 0.05

¹Values are expressed as mean $\pm$ SD ($n = 5$). V_{max} values are in units (1 U = $\mu\text{mole of urate production or NADH utilization}$) per mg of enzyme (see Materials and Methods Section).

Table 2. Contents of Mo in xanthine oxidoreductase (XOR) purified from sheep, goat, cow, and human milk.

	Sheep	Goat	Cow	Human
PFR ¹	5.35 ± 0.24	5.20 ± 0.12	5.28 ± 0.17	5.39 ± 0.23
Mo content, atoms/subunit	0.183 ± 0.041	0.091 ± 0.050	0.548 ± 0.050	0.029 ± 0.006
Demolybdo form, %	81.7	90.9	45.2	97.1

¹PFR = Protein to flavin ratio. Molybdenum contents are expressed as means ± SD (n = 4).

lan, 1982). Consequently, ratios of Mo contents are not directly comparable to ratios of xanthine oxidase activities. In fact, sulfuration of purified sheeps' milk XOR resulted in an increase in specific activity of 73% (Table 3). Assuming that resulfuration is only 50% efficient (Nishino et al., 1983), this translates into a desulfo-content of approximately 60%. While this value is far from precise, it represents a significant component.

Corresponding values for bovine and human milk XOR, calculated from the data in Table 3 are approximately 40 and 70% desulfo-content, respectively. As shown in Table 3, treatment of XOR with KCN led to complete loss of xanthine oxidase activity, which could be restored by sulfuration under the same conditions as for the native enzymes. That the restored activity was, in all 3 cases, less than that generated by sulfuration of native enzyme can be explained in terms of inactivation during incubation with KCN.

In contrast to XO activity, NADH oxidase activity of sheep's milk XOR (0.21 μ mole of NADH consumed $\text{min}^{-1} \text{mg}^{-1}$) was similar to those previously determined (Atmani et al., 2004) for human, goats', and cows' milk enzymes (0.29, 0.27, and 0.25 μ mole of NADH consumed $\text{min}^{-1} \text{mg}^{-1}$, respectively). This is entirely consistent with the fact that NADH oxidase activity of XOR involves donation of electrons to the flavin adenine dinucleotide site and is not directly influenced by Mo content (Bray, 1975).

An obvious question concerns the physiological relevance of XOR with low XO activity. In the case of the milk fat globule membrane, XOR has long been recognized as a major protein component (Patton and Keenan, 1975) and has been implicated in the process of milk lipid secretion (Mather and Keenan, 1998; Keenan, 2001). Very recently, McManaman and colleagues

(2002, 2003) provided evidence that XOR mediates interaction between butyrophilin (in the apical membrane of the secretory cell) and adipophilin (on the lipid droplet) during the envelopment of cytoplasmic lipid droplets by the apical membrane. The involvement of XOR in this process was further supported by Vorbach et al. (2002), who observed defective lactation in XOR mice and demonstrated, by electron microscopy, that XOR is necessary for envelopment of lipid droplets by the secretory cell membrane.

Interestingly, the latter authors provided evidence that XOR participates in milk secretion by virtue of its protein structure rather than its enzymatic activity, possibly explaining the predominance of 'inactive' XOR in human, goat, and sheep milks. What, then, is the role of XO activity in this context? A possible answer lies in microbicidal activity of MFGM in the neonatal gut, involving XOR-catalyzed reduction of nitrite yielding nitric oxide and peroxynitrite (Hancock et al., 2002; Harrison, 2002; Atmani et al., 2004). Such activity clearly requires active enzyme, and particularly an active molybdenum site, which is essential for nitrite reduction (Godber et al., 2000). It is noteworthy that, in the first few weeks postpartum, both XO (Brown et al., 1995) and nitrite reductase (Stevens et al., 2000) activities of human milk are much higher than in subsequent fractions, from which enzyme is commonly purified. In some cases, XO activity was seen to rise to a peak immediately after birth (Brown et al., 1995). Throughout these variations in enzymatic activity, XOR protein levels remained relatively constant, suggesting posttranslational activation-deactivation. It is an attractive idea that the process of milk lipid secretion depends solely on XOR protein, which is later activated in order to fulfill a microbicidal function in the neonate.

Table 3. Total specific activity (μ mol of urate/min per mg) of native and potassium cyanide (KCN)-inactivated sheeps', bovine, and human milk xanthine oxidoreductase (XOR) before and after resulfuration.

Enzyme sample	Before resulfuration		After resulfuration	
	Native	KCN-treated	Native	KCN-treated
Sheep's milk XOR	0.6347 ± 0.0451	ND ¹	1.0892 ± 0.0387	0.8654 ± 0.1145
Bovine milk XOR	2.1107 ± 0.0245	ND	2.8935 ± 0.0874	2.1875 ± 0.0637
Human milk XOR	0.0644 ± 0.0127	ND	0.1282 ± 0.0295	0.0896 ± 0.0764

¹ND = Not detectable.

This could be seen as fulfilling an evolutionary advantage by sparing metabolically expensive processes (e.g., incorporation of Mo?) until required. While highly speculative, such ideas suggest experimental investigation.

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