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NADH oxidation and superoxide production by caprine milk xanthine oxidoreductase

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Abstract

Xanthine oxidoreductase (XOR) was purified from caprine milk where more than 90% exists in demolybdo 'inactive' form. The dehydrogenase form of the enzyme, oxidises nicotinamide adenine dinucleotide (reduced) (NADH), in the presence of oxygen, and generates superoxide anion radical ($O_2^{\bullet -}$) significantly faster than does the oxidase form. The corresponding forms of human and bovine milk enzymes behaved similarly. The steady-state kinetics of NADH oxidation and $O_2^{\bullet -}$ production, in the absence and presence of NAD^+, by the dehydrogenase form of XOR from the three species, are analysed. Allopurinol, oxypurinol and amflutizole blocked the reducing substrates that act at the molybdenum site, and all of which were ineffective in the case of NADH oxidase activity. However, and as expected, diphenyleneiodonium was a powerful inhibitor of NADH oxidation. The possible physiological and pathological significance of reactive oxygen species, especially $O_2^{\bullet -}$, arising from NADH oxidation by caprine XOR is discussed.

Keywords: Caprine milk; NADH oxidase; Reactive oxygen species (ROS); Superoxide anion; Xanthine dehydrogenase (XDH); Xanthine oxidoreductase (XOR)

1. Introduction

Xanthine oxidoreductase (XOR) is a homodimer enzyme of approximately 300 KDa. Each subunit contains four redox centres: one molybdenum cofactor (Mo–co), one flavin adenine dinucleotide (FAD) and two iron sulphur (Fe-2S) sites (Bray, 1975; Hille, 1996). In milk, the major source of the enzyme, XOR, is associated with the milk fat globule membrane (MFGM), derived from the mammary epithelial cell (Patton & Keenan, 1975). The enzyme has broad specificities for both reducing and oxidising substrates, including purines, pyrimidines and pterines (Bray, 1975). The sites of interaction of the various substrates have been well investigated by spectrophotometric and electron paramagnetic resonance spectroscopy studies (e.p.r) using various inactive forms of the enzyme (Komai, Massey, & Palmer, 1969; Olson, Ballou, Palmer, & Massey, 1974; Bray, 1975). These studies have shown that most reducing substrates interact at the molybdenum site, with the exception of NADH, which reacts directly with the FAD (Hunt & Massey, 1994). In vivo, electrons are passed to the physiological electron acceptors (molecular oxygen or NAD^+) via the FAD centre. In vivo, the D form (dehydrogenase; xanthine

Abbreviations: EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol; MES, 2-(*N*-morpholino)-ethane-sulphonic acid; MFGM, milk fat globule membrane; NAD(H), nicotinamide adenine dinucleotide (reduced); PFR, protein/flavine ratio; ROS, reactive oxygen species; PMSF, phenylmethylsulphonyl fluoride; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase

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dehydrogenase (XDH), EC 1.1.1.204) predominates and can be converted to the O form (oxidase; XO, EC 1.1.3.22) either irreversibly by proteolysis (Dela Corte & Stripe, 1972; Nishino & Tamura, 1991) or reversibly by sulphydryl oxidation (Amaya et al., 1990).

The conventionally accepted role of XOR is in purine catabolism, where it catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid while concomitantly reducing NAD^+ (type D) or molecular oxygen (type O). NAD^+ is always reduced divalently to produce NADH. However, molecular oxygen (O_2) may be reduced either divalently to produce hydrogen peroxide (H_2O_2) or univalently to generate superoxide anion radical ($O_2^{\bullet -}$) (McCord & Fridovich, 1968; Fridovich, 1970). The potential to generate these reactive oxygen species (ROS) has led to a particular research interest in the enzyme as a destructive agent in many instances of the vasculature, especially on leucocyte–endothelial cell interactions, which are relevant to many vascular diseases including ischaemia reperfusion (Granger et al., 2001; Krieglstein & Granger, 2001).

We have recently described the purification and partial characterisation of XOR from caprine (Atmani, Benboubetra, & Harrison, 2004) and sheep (Benboubetra, Baghiani, Atmani, & Harrison, 2004) milks. As for the purified human enzyme (Abadeh, Killacky, Benboubetra, & Harrison, 1992; Sanders, Eisenthal, & Harrison, 1997), the caprine milk enzyme has very low activity towards most reducing substrates, including hypoxanthine and xanthine. As much as 91%, 82% and 97% of purified caprine, sheep and human milk enzyme is inactive toward conventional reducing substrates (Atmani et al., 2004) compared to about 60% on the purified bovine milk enzyme (Bray, 1975; Godber, Sanders, Harrison, Eisenthal, & Bray, 1997; Bray, Lowe, Godber, Harrison, & Eisenthal, 1999). This inactive enzyme is made up of two forms; demolybdo-XOR which lacks Mo or possibly molybdopterin and the desulpho-XOR in which the Mo=S grouping, essential for the catalytic activity, is replaced by Mo=O (Gutteridge, Tanner, & Bray, 1978; Wahl & Rajagopalan, 1982). However, in the presence of O_2, the caprine milk enzyme catalyses the oxidation of NADH and generates $O_2^{\bullet -}$. Such NADH oxidase activity is exhibited by milk XOR from other species including human (Sanders et al., 1997; Zhang et al., 1998). In the relative very low activity towards conventional reducing substrate, the presence of a NADH oxidase activity in caprine XOR suggests a possible functional role. We investigated the NADH oxidase activity of both the dehydrogenase and oxidase forms of caprine enzyme with a particular emphasis on $O_2^{\bullet -}$ production. The results are compared with those obtained with the corresponding forms of bovine and human milk enzyme.

2. Materials and methods

2.1. Materials

Fresh bovine and caprine milks were obtained from farms local to Bath (UK). Unwanted human breast milk was kindly donated by mothers in the Special Care Baby Unit of the Southmead Hospital, Bristol and The Royal United Hospitals (UK). For convenience, human and caprine milks were kept frozen and thawed the day of the purification whereas bovine milk was used fresh. All chemicals, unless otherwise stated, were from Sigma (Poole, Dorset, UK).

2.2. Methods

2.2.1. Purification of XOR from caprine milk

XOR was purified from caprine milk according to the method recently reported by Atmani et al. (2004). Frozen milk (3 L) was allowed to thaw at room temperature. 1 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM phenylmethylsulphonyl fluoride ($PhMeSO_2F$) and 1.25 mM sodium salicylate were added and the mixture was stirred for 30 min before centrifugation at 4000*g* for 20 min. The cream was collected, resuspended in 0.2 M potassium phosphate buffer, containing 1 mM EDTA and 5 mM dithiothreitol, and stirred for 120 min at 4 °C. All subsequent steps were carried out at 4 °C, unless otherwise specified. The mixture was centrifuged at 4000*g* for 20 min and the aqueous layer was carefully filtered through a small plug of glass wool. Butanol, kept at −4 °C, was added to the filtrate at a level of 15% (v/v), followed by slow and progressive addition of ammonium sulphate powder to 15% (w/v) and the whole was stirred for 90 min, before centrifugation at 12,000*g* for 20 min. The upper butanol layer was discarded and the aqueous phase was filtered again through a plug of glass wool. Ammonium sulphate powder was added at a level of 25% (w/v) to the filtrate while stirring continuously over a period of 60 min before centrifugation at 14,000*g* for 30 min. The protein precipitate was collected, dissolved in a small volume of 50 mM 2-(*N*-morpholino)-ethane-sulphonic acid (MES) buffer, pH 6.3, and dialysed overnight against the same buffer (3 L). After centrifugation at 48,000*g* for 60 min, the supernatant was applied to a column (1 × 6 cm) of heparin immobilised on cross-linked 4% (w/w) beaded agarose (Sigma, type 1) previously equilibrated in the same buffer. The column was washed with MES buffer, containing 0.1 M-NaCl, and XOR was eluted with the same buffer containing 0.3 M-NaCl. The column eluate was dialysed overnight against 50 mM-Bicine buffer, pH 8.3, and applied to an equilibrated Hi-Trap mono Q ion exchange column (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), at room temperature, for fast protein liquid