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Studies of the ferroxidase activity of native and chemically modified xanthine oxidoreductase

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The O_2-utilizing (type O, oxidase) form of xanthine oxidoreductase is primarily responsible for its ferroxidase activity. This form of xanthine oxidoreductase has 1000 times the ferroxidase activity of the serum ferroxidase caeruloplasmin. It has the ability to catalyse the oxidative incorporation of iron into transferrin at very low Fe^{2+} and O_2 concentrations. Furthermore, the pH optimum of the ferroxidase activity of the enzyme is compatible with the conditions of pH that normally exist in the intestinal mucosa, where it has been proposed that xanthine oxidoreductase may facilitate the absorption of ionic iron. Modification of the molybdenum (Mb) centres of the enzyme *in vitro* by treatment with cyanide, methanol or allopurinol completely abolishes its ferroxidase activity. The feeding of dietary tungsten to rats, which prevents the incorporation of molybdenum into newly synthesized intestinal xanthine oxidoreductase, results in the progressive loss of the ferroxidase activity of intestinal-mucosa homogenates. Removal of the flavin centres from the enzyme also results in the complete loss of ferroxidase activity; however, the ferroxidase activity of the flavin-free form of the enzyme can be restored with artificial electron acceptors that interact with the molybdenum or non-haem iron centres. The presence of superoxide dismutase or catalase in the assay system results in little inhibition of the ferroxidase activity of xanthine oxidoreductase.

Studies *in vivo* and *in vitro* from a number of laboratories have demonstrated that caeruloplasmin (serum ferroxidase-I) facilitates the mobilization of iron from liver stores (Osaki & Johnson, 1969; Osaki *et al.*, 1970, 1971; Roeser *et al.*, 1970; Evans & Abraham, 1973; Williams *et al.*, 1974; Topham *et al.*, 1980). Considerable evidence suggests that caeruloplasmin facilitates iron mobilization by promoting the oxidative incorporation of iron into plasma transferrin (Osaki *et al.*, 1966, 1970, 1971; Osaki & Johnson, 1969; Roeser *et al.*, 1970; Topham *et al.*, 1980). Although caeruloplasmin facilitates the release of iron from liver stores, it appears to play no role in the uptake of iron by the intestinal cell, the processing of iron within the mucosal cell or the regulation of intestinal iron absorption (Lee *et al.*, 1968; Ragan *et al.*, 1969; Brittin & Chee, 1969; Roeser *et al.*, 1970). An iron-binding protein that has properties very similar to, but not identical with, those of plasma transferrin has been isolated and purified from intestinal-mucosal homogenates (Huebers *et al.*, 1971; 1976; Worwood & Jacobs, 1971; Halliday *et al.*, 1976; Valberg *et al.*, 1977). Numerous studies suggest that this protein, designated mucosal transferrin, mediates the transcellular transport of iron in the mucosal cell (Huebers *et al.*, 1971; Pollack *et al.*, 1972; Halliday *et al.*, 1976; El-Shobaki & Rummel, 1977; Savin & Cook, 1980).

A non-caeruloplasmin enzyme that promotes the oxidative incorporation of iron into transferrin has been isolated from intestinal-mucosal homogenates (Topham, 1978). This intestinal ferroxidase has been purified and identified as xanthine oxidoreductase (Topham *et al.*, 1981). Recent studies *in vivo* suggest that intestinal xanthine oxidoreductase may facilitate the transcellular transport of ionic iron in the mucosal cell by promoting its oxidative incorporation into mucosal transferrin (Topham *et al.*, 1982). The studies presented here were undertaken to learn more about the nature of the ferroxidase activity of xanthine oxidoreductase through comparison of its kinetic properties with those of caeruloplasmin, comparison of the relative ferroxidase activities of the two forms (oxidase and dehydrogenase) of xanthine oxidoreductase, and by chemical modification of its prosthetic groups *in vitro* and *in vivo*.

EXERIMENTAL PROCEDURES

Materials

Xanthine oxidoreductase. Highly purified milk xanthine oxidoreductase was generously given by Dr. Michael J. Barber, Department of Biochemistry, Duke University Medical Center and Veterans Administration Hospital, Durham, NC, U.S.A. This enzyme was obtained as 25 μl pellets that had been prepared in liquid N_2, and the pellets were stored at −30 °C until used. In these studies, unless otherwise specified, pellets were diluted 500-fold with 0.05 M-Hepes buffer, pH 7.4, to yield a final concentration of approx. 1 μM-xanthine oxidoreductase. This enzyme preparation exhibited a single protein band upon polyacrylamide-gel electrophoresis. The specific ferroxidase activity of milk xanthine oxidoreductase has been shown to be equivalent to that of highly purified intestinal xanthine oxidoreductase (Topham *et al.*, 1981).

Apo-transferrin. For the assay of ferroxidase activity, a 2.0% (w/v) solution of iron-free transferrin (apotransferrin; Calbiochem–Behring, La Jolla, CA, U.S.A.) was prepared in deionized glass-distilled water and extensively dialysed as previously recommended (Johnson

Abbreviations used: NBT, nitroblue tetrazolium; MB, Methylene Blue; TNBS, trinitrobenzenesulphonic acid; PMS, phenazine methosulphate.

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et al., 1970). The apo-transferrin was 98–99% pure as judged by polyacrylamide-gel electrophoresis.

Reducing substrates and electron acceptors. Hypoxanthine, xanthine, NAD, NBT, MB, TNBS and PMS were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Chromatographic materials. Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) was swollen in distilled water and equilibrated with either 0.05 M-Hepes buffer, pH 7.4, or 0.05 M-Hepes buffer, pH 7.4, containing 0.5 M-KCl before use.

Methods

Assay of ferroxidase activity. Ferroxidase activity was assayed spectrophotometrically by monitoring Fe^{3+}-transferrin formation as the absorbance change at 460 nm, the wavelength at which Fe^{3+}-transferrin has its maximal absorption. This spectrophotometric assay has been described in detail and validated in numerous previous reports (Osaki *et al.*, 1966, 1971; Johnson *et al.*, 1967; Osaki & Johnson, 1969; Topham & Johnson, 1974). The application of this assay specifically for the analysis of the ferroxidase activity of purified xanthine oxidoreductase and xanthine oxidoreductase in tissue homogenates has been recently reported (Topham, 1978; Topham *et al.*, 1981, 1982). In assays containing NBT, MB, TNBS or PMS as artificial electron acceptors, the final concentration of each of these acceptors was 50 μM. Control assays were run without Fe^{2+} and apo-transferrin, but with each artificial acceptor, to ascertain if the reduction of the acceptors resulted in absorbance changes at 460 nm. If so, absorbance changes resulting from reduction of the electron acceptor were subtracted to obtain the true enzymic ferroxidase activity. All ferroxidase assays were performed in triplicate and all values of ferroxidase activity reported in the Tables and Figures represent averages for at least three assays.

Assay of xanthine oxidoreductase activity. Xanthine oxidoreductase activity was assayed spectrophotometrically by monitoring uric acid formation from hypoxanthine or xanthine at 295 nm as previously described (Stirpe & Della Corte, 1969; Waud & Rajagopalan, 1976). The amount of each form (dehydrogenase or oxidase) of the enzyme was determined by performing the assay in the presence and absence of 0.5 mM-NAD, as recommended by Della Corte & Stirpe (1972). All assays were performed in triplicate, and the values reported in the Tables and Figures represent averages for at least three assays.

Steady-state kinetic analyses. For determination of the K_m for Fe^{2+}, the initial rate of Fe^{3+}-transferrin formation was measured at initial Fe^{2+} concentrations from 10 to 120 μM. For determination of the K_m for O_2, the ferroxidase activities were measured polarographically as previously described (Topham & Johnson, 1974) with assay mixtures containing initial O_2 concentrations from 2.1 to 236 μM with a constant Fe^{2+} concentration of 120 μM. Steady-state kinetic parameters were computed by linear-regression analyses of double-reciprocal kinetic plots.

Preparation of flavin-free xanthine oxidoreductase. The flavin centres of xanthine oxidoreductase can be selectively removed under conditions of high ionic strength (Komai *et al.*, 1969; Kanda *et al.*, 1972). The flavin-free enzyme used in the present study was prepared by a modification of the procedure of Kanda *et al.* (1972). Samples of xanthine oxidoreductase (2 mg/ml) were incubated at 25 °C with various concentrations of KI for different periods of time. After treatment of the xanthine oxidoreductase with KI, flavin and KI were removed from the samples by gel filtration on a column of Sephadex G-25 rather than by extensive dialysis. The eluent for the Sephadex G-25 column was 0.05 M-Hepes buffer, pH 7.4, containing 0.5 M-KCl.

Preparation of fresh homogenates of intestinal mucosa. Female Fischer rats (CDF strain; 150 g; Charles River Laboratories, Wilmington, MA, U.S.A.) were used in the present studies. Food was removed 18 h before the animals were used. As previously suggested by Battelli *et. al.* (1972), each animal received an intragastric dose of 15 mg of trypsin inhibitor (Type I-S; Sigma Chemical Co., St. Louis, MO, U.S.A.) in 3 ml of saline (0.9% NaCl) 15 min before they were killed. This minimizes the irreversible proteolytic conversion of the dehydrogenase form of xanthine oxidoreductase to the oxidase form during homogenate preparation. Immediately after the animals had been killed, the small intestine from the gastric pylorus to the ileocaecal value was excised and separated from adhering pancreatic tissue. The gut was flushed with cold saline containing the trypsin inhibitor (8 mg/ml), blotted to remove excess fluid, placed on a stainless-steel tray, slit open, mucosa was removed by gently scraping with a glass microscope slide. A 20% (w/v) homogenate of the mucosa was prepared in 0.05 M-Hepes buffer, pH 7.4, containing 250 mM-sucrose and trypsin inhibitor (8 mg/ml). The homogenate was centrifuged for 15 min at 25000 *g* and the resulting supernatant filtered through glass wool. The filtered supernatant was then centrifuged at 105000 *g* for 1 h.

Partially purified intestinal xanthine oxidoreductase was prepared from the 105000 *g* supernatant by $(NH_4)_2SO_4$ fractionation. As previously reported (Kaminski & Jezewska, 1979), the pellet from the 1.6 M–2.4 M-$(NH_4)_2SO_4$ fraction contained the intestinal xanthine oxidoreductase. This pellet was dissolved in a volume of buffer [0.05 M-Hepes buffer (pH 7.4)/250 mM-sucrose] equal to one-half the original tissue weight.

Inhibition of the oxidase activities of intestinal xanthine oxidoreductase in rats by dietary tungsten. Adult female Fischer rats (150 g, CDF strain; Charles River) were used in the present study. These animals were initially placed on a normal protein diet for 2 weeks. At this time, control animals were killed and cytosol (105000 *g* supernatant) was prepared from homogenates of the mucosal tissue as described in the previous subsection. This cytosol was analysed for hypoxanthine oxidase and ferroxidase activity. The remaining animals were placed on a tungsten-supplemented diet (normal protein diet plus 0.7 g of sodium tungstate/kg of diet). Animals were periodically killed and the mucosal cytosol preparations from these animals were analysed for hypoxanthine oxidase and ferroxidase activity. At least six animals were killed at each time interval. The diets

used in these studies were prepared by the Nutritional Biochemical Division, ICN Pharmaceuticals, Cleveland, OH, U.S.A., and were identical in composition except for the tungsten supplementation.

RESULTS

Relative ferroxidase activities of the two forms of xanthine oxidoreductase

Xanthine oxidoreductase may be isolated from various mammalian tissues as one of two interconvertible forms, namely a dehydrogenase (NAD^+-dependent, form D) or an oxidase (O_2-utilizing, form O) (Stirpe & Della Corte, 1969). The interconversion of the two forms of enzyme has been extensively studied (Stirpe & Della Corte, 1969; Della Corte & Stirpe, 1972; Waud & Rajagopalan, 1976). Conversion of the dehydrogenase (type D) into the oxidase (type O) can be brought about by proteolysis, heating, storage at −20 °C, aerobiosis, organic solvents, incubation with subcellular fractions and thiol-group-modifying reagents. Except in the case of type-O forms obtained by proteolysis or *N*-ethylmaleimide treatment, reconversion into the type-D form can be effected by treatment with dithiothreitol or dithioerythritol.

As described in detail under 'Methods', partially purified intestinal xanthine oxidoreductase was prepared from rats that had received an intragastric dose of trypsin inhibitor to minimize the irreversible conversion of type-D into type-O enzyme. Initially these preparations contained 60% type-D and 40% type-O enzyme and possessed ferroxidase activity (Fig. 1). Heat treatment of these preparations resulted in the conversion of type-D into type-O enzyme and this conversion was accompanied by a corresponding increase in the ferroxidase activity. Treatment of the heated sample with 1 mM-dithioerythritol for 30 min at 37 °C resulted in substantial reconversion of type-O into type-D enzyme with a corresponding loss of ferroxidase activity.

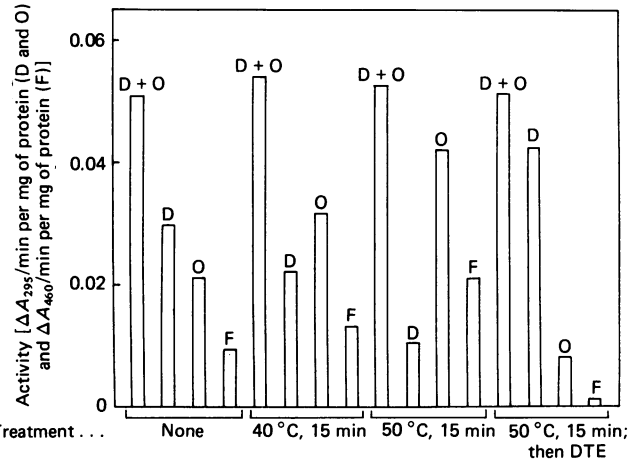

Fig. 1. Changes in ferroxidase activity upon interconversion of the two forms of xanthinine oxidoreductase

Partially purified xanthine oxidoreductase was obtained from freshly prepared intestinal-mucosa cytosol of control rats as described in detail under 'Methods'. To minimize proteolytic interconversion of the two forms of the enzyme during this procedure, these rats received intragastric doses of trypsin inhibitor before they were killed and trypsin inhibitor was included in the homogenizing medium. The relative amounts of the two forms of the enzyme and the ferroxidase activity associated with each sample after treatment were determined as described under 'Methods'. After treatment of the sample at 50 °C for 5 min, reconversion of oxidase to dehydrogenase was accomplished by treatment of the sample with 1 mM-dithioerythritol (DTE) for 30 min at 37 °C. Dithioerythritol was removed from the sample by gel filtration on Sephadex G-25 before analyses of enzyme activities. D+O, total xanthine oxidoreductase activity (both O_2 and NAD^+ present); O, xanthine oxidase activity (only O_2 present); D, xanthine dehydrogenase activity (difference between total and oxidase activity); F, ferroxidase activity.

Steady-state kinetic comparison of the ferroxidase activities of xanthine oxidoreductase and caeruloplasmin

The pH optimum for the ferroxidase reaction catalysed by xanthine oxidoreductase was 7.4. Saturation kinetics were observed in respect to Fe^{2+} and O_2. A summary of the kinetic properties determined for the ferroxidase reaction catalysed by xanthine oxidoreductase and a comparison of these properties with those previously reported (Osaki, 1966) for the serum ferroxidase, ceruloplasmin, can be found in Table 1.

Effect of chemical modification of the molybdenum centres on the ferroxidase activity of xanthine oxidoreductase

Reaction of xanthine oxidoreductase with a number of agents results in chemical modification of specific redox centres. Cyanide, methanol and allopurinol modify the molybdenum centres and destroy the ability of the enzyme to oxidize hypoxanthine or xanthine. Cyanide inactivation results from the abstraction of sulphur, as thiocyanate, from the enzyme (Massey & Edmondson, 1970). These 'cyanolysable' sulphur groups are located at or near the molybdenum centres and are essential for fully functional enzyme. Inactivation by methanol results from its conversion at the molybdenum centres to formaldehyde, with subsequent formylation of the cyanolysable sulphur atoms (Pick *et al.*, 1971). Inactivation by allopurinol results from enzymic oxidation of allopurinol to oxypurinol, which forms a complex with enzyme-bound molybdenum in the quadrivalent state (Massey *et al.*, 1970).

Treatment with cyanide, methanol or allopurinol completely abolished the ferroxidase activity of xanthine oxidoreductase (Table 2).

Table 1. Comparison of the kinetic properties of the ferroxidase reaction catalysed by caeruloplasmin and xanthine oxidoreductase

The values presented for caeruloplasmin were those previously reported by Osaki (1966). The kinetic properties for the ferroxidase reaction catalysed by xanthine oxidoreductase were determined as described under 'Methods'.

Property	Caeruloplasmin	Xanthine oxidoreductase
pH optimum	6.5	7.4
K_m (s) for Fe^{2+} (μM)	0.6, 50	46
K_m for O_2 (μM)	9.0	50
Molar ferroxidase activity (μM-Fe^{3+}-transferrin/min per μM-enzyme)	550	500000

Table 2. Effect of modification of the molybdenum centres on the ferroxidase and hypoxanthine oxidase activity of xanthine oxidoreductase

The treatments of xanthine oxidoreductase with cyanide and methanol were performed as described by Coughlan & Ni Fhaolain (1979). The treatment with allopurinol was conducted as described by Massey *et al.* (1970).

Modifying agent	Specific activity: Ferroxidase (ΔA_{460}/min per mg of protein)	Specific activity: Hypoxanthine oxidase (ΔA_{295}/min per mg of protein)
None	4.64	2.90
Cyanide	0.00	0.00
Methanol	0.00	0.00
Allopurinol	0.00	0.00

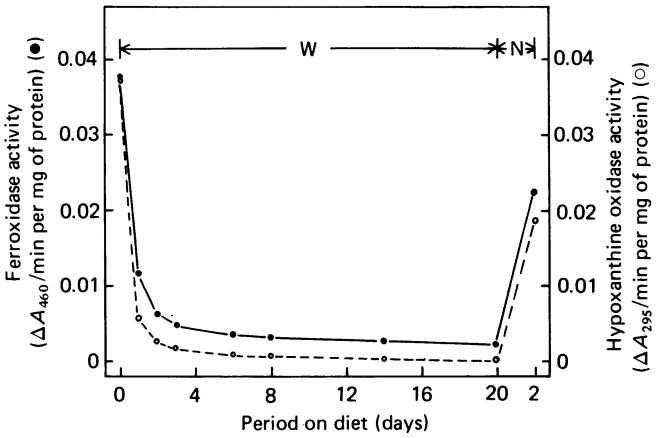

Fig. 2. Comparison of the effect of dietary tungsten on the hypoxanthine oxidase and ferroxidase activities of xanthine oxidoreductase in rats

The dietary regimen utilized in the animals, the preparation of mucosal cytosol from each group of animals, and the assay of the hypoxanthine oxidase and ferroxidase activities were carried out as described under 'Methods'. At least six animals were killed at each time interval. W, period when animals maintained on the tungsten-supplemented diet; N, period when animals returned to the control diet.

Comparison of the effect of dietary tungsten on the hypoxanthine oxidase and ferroxidase activities of intestinal xanthine oxidoreductase

The dietary administration of tungsten to rats has previously been shown to result in marked inactivation of the hypoxanthine and xanthine oxidase activity of xanthine oxidoreductase in the liver, kidney and intestine (Johnson *et al.*, 1974*a*). This inactivation has been demonstrated to result specifically from the inhibition of the incorporation of molybdenum into newly synthesized enzyme (Johnson *et al.*, 1974*b*). The effect of tungsten was extremely specific, and tungsten-treated rats lacked any signs or symptoms of toxicity from the metal itself. A comparison of the rate of loss of ferroxidase activity and hypoxanthine oxidase activity was made in the cytosol of intestinal mucosa from rats given tungsten-supplemented diets for various periods of time. Both activities were rapidly lost in an approximately parallel fashion after the introduction of tungsten into the diet (Fig. 2).

Table 3. Effect of flavin removal on the ferroxidase activity of xanthine oxidoreductase

The treatments with KI to remove flavin from xanthine oxidoreductase and the assays of ferroxidase and hypoxanthine oxidase activities of the treated samples were performed as described under 'Methods'.

[KI] (M)	Period of treatment (min)	A_{280}/A_{450}	Specific activity: Ferroxidase (ΔA_{460}/min per mg of protein)	Specific activity: Hypoxanthine oxidase (ΔA_{295}/min per mg of protein)
0	5	7.92	4.92	2.67
2	5	11.0	2.96	1.15
3	5	25.7	1.44	0.392
3	60	106	0.00	0.00

Studies of the ferroxidase activity of flavin-free xanthine oxidoreductase

The progressive removal of flavin from xanthine oxidoreductases was paralleled by a loss of ferroxidase activity (Table 3). Most, if not all, of the ferroxidase activity could be restored to the flavin-free enzyme with artificial electron acceptors that have previously been proposed (Coughlan & Ni Fhaolain, 1979) to interact with the molybdenum or non-haem iron centres (Table 4). In the presence of NBT, the flavin-free enzyme actually had greater ferroxidase activity than the native enzyme, and the presence of all artificial electron acceptors enhanced the ferroxidase activity of native enzyme. These artificial electron acceptors have been shown to have similar effects on the hypoxanthine/xanthine oxidase and dehydrogenase activities of native and flavin-free xanthine oxidoreductase (Kanda *et al.*, 1972).

Possible involvement of reactive forms of oxygen in the ferroxidase activity of xanthine oxidoreductase

Superoxide and hydrogen peroxide are generated upon the reoxidation of xanthine oxidoreductase by molecular oxygen (Fridovich, 1970). These reactive forms of oxygen could oxidize iron and contribute to the ferroxidase activity of xanthine oxidoreductase. If so, superoxide dismustase and catalase should inhibit the ferroxidase activity of xanthine oxidoreductase. Inclusion of large quantities of superoxide dismutase (1.0 mg, 3000 units) or catalase (1.0 mg, 32000 units) in ferroxidase mixtures of a final volume of 1.1 ml resulted in little inhibition of the ferroxidase reaction catalysed by xanthine oxidoreductase (Table 5).

DISCUSSION

On the basis of the investigations of Stirpe & Della Corte (1969) and of Della Corte & Stirpe (1972), it has been speculated that xanthine oxidoreductase exists *in vivo* in many mammalian tissues primarily as the NAD^+-linked dehydrogenase. However, even though

Table 4. Ferroxidase specific activities of native and flavin-free xanthine oxidoreductase with various electron acceptors

The assays of ferroxidase activity in the presence of O_2 and the artificial electron acceptors were conducted as described under 'Methods'. The final concentration of each of the electron acceptors other than O_2 in the assay mixture was 50 μM. Control assays were run without Fe^{2+} and apo-transferrin, but with each artificial electron acceptor to ascertain if the reduction of the acceptor resulted in absorbance changes at 460 nm. If so, the absorbance change resulting from the reduction of the electron acceptor was subtracted to obtain the true enzymic ferroxidase activity.

	Ferroxidase specific activity (ΔA_{460}/min per mg of protein)	
	Native	Flavin-free
O_2	4.92	0.00
NBT	5.20	8.49
MB	4.63	3.21
TNBS	5.86	5.26
PMS	6.19	4.36

Table 5. Effect of catalase and superoxide dismutase on the ferroxidase reaction catalysed by xanthine oxidoreductase

Ferroxidase activity was assayed as described under 'Methods'. The final concentration of xanthine oxidoreductase in all assay mixtures was 7.5 μg/ml. The final concentration of catalase and superoxide dismutase in the assay mixtures was 1 mg/ml.

Sample	Ferroxidase specific activity (ΔA_{460}/min per mg of protein)
Xanthine oxidoreductase	5.01
+ Catalase	4.91
+ Superoxide dismutase	4.85

rats were given intragastric doses of proteolytic inhibitor before they were killed and proteolytic-enzyme inhibitor was included in the homogenizing medium to minimize proteolytic conversion of the dehydrogenase to the oxidase, fresh homogenates of intestinal mucosa contained oxidase-type activity. These results were consistent with the previous studies of Battelli *et al.* (1972). Krenitsky & Tuttle (1978) presented evidence for the existence of two catalytically and immunologically different types of xanthine oxidoreductase in mouse small intestine. One of these is a dehydrogenase that is convertible to an oxidase, whereas the other is a stable oxidase not derived from the dehydrogenase.

Conversion of the dehydrogenase form of the enzyme into the oxidase form in mucosal homogenates was accompanied by a large increase in the ferroxidase activity, whereas reconversion of the oxidase into the dehydrogenase in these homogenates resulted in a loss of ferroxidase activity. Thus the oxidase form of the xanthine oxidoreductase would appear to be primarily responsible for the ferroxidase activity. If the oxidase form of xanthine oxidoreductase plays a role in the processing of dietary iron by the intestinal-mucosal cell, as has previously been suggested (Topham *et al.*, 1982), then the interconversion of the two forms of the enzyme could alter the ferroxidase activity present in this cell in response to changes in the iron status of the animal. This could result in changes in the amount of iron incorporated into mucosal transferrin, which would, in turn, alter the amount of iron transported to the bloodstream.

On the basis of molar activity, xanthine oxidoreductase is a significantly (1000-fold) more effective ferroxidase than the serum ferroxidase caeruloplasmin. Like caeruloplasmin, it possesses low K_m values for Fe^{2+} and O_2 and has a pH optimum that would permit it to catalyse effectively the ferroxidase reaction under the conditions that exist in the cytosol of the intestinal-mucosa cell. It is also noteworthy that both of these enzymes that catalyse the ferroxidase reaction are also able to catalyse the oxidation of a number of organic substrates.

Superoxide and hydrogen peroxide are generated upon the reoxidation of xanthine oxidoreductase by molecular oxygen. These reactive forms of oxygen could contribute to the ferroxidase activity of this enzyme. In fact, Fried *et al.* (1973) have suggested that an important function of xanthine oxidoreductase may be the generation of these reactive forms of oxygen that would be available for coupled oxidation reactions. The observation that the presence of neither superoxide dismutase or catalase significantly diminished the ferroxidase activity of xanthine oxidoreductase would make the participation of these reactive forms of oxygen unlikely.

The flavin chromophores of xanthine oxidoreductase are the sites of interaction with O_2 and thus are the sites where the superoxide and hydrogen peroxide are generated upon reoxidation of the enzyme. Removal of flavin from the enzyme did result in the loss of ferroxidase activity; however, the ferroxidase activity could be restored to the flavin-free enzyme with the artificial electron acceptors that have been proposed previously (Coughlan & Ni Fhaolain, 1979) to interact with either the molybdenum or non-haem iron centres. This also would argue against reactive forms of oxygen contributing to the ferroxidase activity. Furthermore, the presence of these artificial electron acceptors in assay mixtures containing native xanthine oxidoreductase enhanced the ferroxidase activity of the native enzyme. These artificial electron acceptors may permit the removal of electrons directly from the molybdenum and non-haem iron centres of the native enzyme during the ferroxidase reaction, thus by-passing the movement of electrons through the flavin centres to oxygen.

Since the ferroxidase activity can be restored to the flavin-free enzyme with artificial electron acceptors that interact with the molybdenum or non-haem iron centres, then these centres are the most probable site of entry of electrons into the enzyme during the ferroxidase reaction. Modification of the molybdenum centres of the enzyme *in vitro* and *in vivo* resulted in the complete loss of ferroxidase activity. This would suggest that the molybdenum centres are involved in the ferroxidase reaction; however, it does not definitely establish that the molybdenum centres are the site of entry of electrons

into the enzyme during the ferroxidase reaction. It has been demonstrated that the redox centres of xanthine oxidoreductase are arranged in a non-linear manner and that reversible electron transfer between the centres can take place (Edmondson *et al.*, 1973). At present there are no known procedures for specific modification or removal of the non-haem iron centres that do not result in irreversible denaturation of the enzyme.

In summary, the results presented here demonstrate that xanthine oxidoreductase kinetically compares very favorably as a ferroxidase with the serum ferroxidase caeruloplasmin and that it should be capable of catalysing the ferroxidase reaction under physiological conditions of pH and oxygen concentration. The results also suggests that the ferroxidase activity exhibited by xanthine oxidoreductase is most probably mediated through the prosthetic groups of the enzyme and not by the superoxide and hydrogen peroxide that are generated upon reoxidation of the enzyme. The mechanism by which xanthine oxidoreductase promotes the oxidative incorporation of iron into transferrin and the possible physiological significance of this activity require further investigation.

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