A Flavin-Dependent Sulfhydryl Oxidase in Bovine Milk[†]

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ABSTRACT: Both metal and flavin-dependent sulfhydryl oxidases catalyze the net generation of disulfide bonds with the reduction of oxygen to hydrogen peroxide. The first mammalian sulfhydryl oxidase to be described was an iron-dependent enzyme isolated from bovine milk whey (Janolino, V.G., and Swaisgood, H.E. (1975) *J. Biol. Chem. 250*, 2532−2537). This protein was reported to contain 0.5 atoms of iron per 89 kDa subunit and to be completely inhibited by ethylenediaminetetraacetate (EDTA). However the present work shows that a soluble 62 kDa FAD-linked and EDTA-insensitive sulfhydryl oxidase apparently constitutes the dominant disulfide bond-generating activity in skim milk. Unlike the metalloenzyme, the flavoprotein is not associated tightly with skim milk membranes. Sequencing of the purified bovine enzyme (>70% coverage) showed it to be a member of the Quiescin-sulfhydryl oxidase (QSOX) family. Consistent with its solubility, this bovine QSOX1 paralogue lacks the C-terminal transmembrane span of the long form of these proteins. Bovine milk QSOX1 is highly active toward reduced RNase and with the model substrate dithiothreitol. The significance of these new findings is discussed in relation to the earlier reports of metal-dependent sulfhydryl oxidases.

Both metal- and flavin-dependent sulfhydryl oxidases catalyze disulfide bond formation at the expense of molecular oxygen:

$$2\ \text{R-SH} + \text{O}_2 \rightarrow \text{R-S-S-R} + \text{H}_2\text{O}_2$$

In the former category, copper-dependent oxidases from skin (*1*, *2*), kidney (*3*), and antibody-producing tumor tissue (*4*) have been described, although the metal dependence of the skin enzyme is now in doubt (*5*). Swaisgood and colleagues, following earlier work by Kiermeier and co-workers (*6*−*9*), have conducted a range of studies on a sulfhydryl oxidase that they isolated from bovine milk. These investigations have included enzyme specificity (*10*−*14*) and steady-state kinetics (*15*), together with analysis of molecular weight (*10*, *16*, *17*), amino acid composition, and metal content (*10*, *17*). Their 89 kDa milk enzyme contained 0.5 equiv of iron per subunit (*10*) and was completely inhibited by 1 mM EDTA (*10*, *17*). Activity was regained upon the addition of iron and, to a lesser extent with divalent copper and manganese (44 and 33% of the recovery with iron, respectively) (*10*, *17*). These data suggest a metal binding site of considerable accommodation. Alternative purification methods of the enzyme have been presented by the same laboratory (*16*, *18*−*20*). The enzyme has yet to be sequenced or cloned. An important contribution of this pioneering work was the suggestion that the sulfhydryl oxidases may play significant roles in oxidative protein folding in mammals (*10*, *12*, *17*, *21*).

The second mammalian sulfhydryl oxidase to be purified was isolated from rat seminal vesicles by Kistler and colleagues (*22*, *23*) and was later found to be a member of a newly recognized family of flavoenzymes termed Quiescin-sulfhydryl oxidases (QSOX[1]) (*24*, *25*). Vertebrates have two QSOX paralogues (*26*−*29*) of which QSOX1 is the only one that has received detailed enzymological scrutiny. Two alternate splice forms of QSOX1 exist: a long form (∼ 80 kDa) which retains a single transmembrane span at its C-terminus, and a shorter, soluble, form (∼63 kDa) truncated before this feature (*26*, *28*, *30*). QSOX enzymes are particularly associated with cells bearing a heavy secretory load (*26*−*28*, *31*). QSOX has been found extracellularly (*23*, *25*, *31*−*34*) and in a variety of intracellular locales (*26*, *28*−*31*, *35*).

Several recent findings raised the possibility that milk might contain QSOX1 in addition to the iron-dependent oxidase described above. First, immunohistochemistry of human mammary tissue using an anti-peptide antibody directed against residues 494−507 of human QSOX1 (ref Seq NP_002817) showed strong staining of the apical caps of breast secretory epithelial cells (*28*). Second, an expression

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; GSH, reduced glutathione; KP_i, potassium phosphate; QSOX, flavin-dependent sulfhydryl oxidases homologous to Quiescin Q6; SAGE, serial analysis of gene expression.

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profiling database queried for QSOX1 suggested marked expression levels in mouse mammary tissue (*36*). Finally, a SAGE expression database of mouse tissues showed the highest level of QSOX1 was found in lactating mammary gland (*37*).

We therefore considered the possibility that QSOX might be released from mammary epithelial cells during formation of the skim or cream phases of milk. To estimate the relative contribution of metal- and flavin-dependent sulfhydryl oxidases in milk, we planned to exploit their differential sensitivities to metal chelators. As mentioned earlier, the iron-dependent enzyme was reported to be completely inhibited by EDTA (*10*) while the flavin-dependent QSOX enzymes from egg white and mouse seminal vesicles are unaffected by this chelating agent (*23*, *33*). However, in multiple experiments performed with batches of fresh milk obtained over a 2-year period, we found that EDTA had essentially no effect on the sulfhydryl oxidase activity of whole or skim milk. This suggested that the metal-dependent enzyme reported by Swaisgood and colleagues contributed little to the overall sulfhydryl oxidase activity of milk under our assay conditions.

Herein, we report the isolation and characterization of a QSOX1 from bovine milk and develop a purification procedure that is both robust and reproducible. In addition to providing a potentially useful new source of mammalian QSOX, we hope that this work will encourage renewed investigation of sulfhydryl oxidases whose activity is believed to be metal-dependent.

EXPERIMENTAL PROCEDURES

Materials. Most reagents were obtained as described previously (*5*, *33*, *38*−*41*): 4-Aminoantipyrine and ferrozine were from Sigma, phenol was from Fisher, and trishydroxypropylphosphine (THP) was obtained from Calbiochem.

General. Absorbance and fluorescence instrumentation was as described earlier (*40*). Concentrations of purified bovine QSOX1 were determined at 458 nm using an extinction coefficient determined for the corresponding avian enzyme (12.5 mM^{-1} cm^{-1}; (*33*). Unless otherwise stated, potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, was used. Experiments were performed at 25 °C unless otherwise noted.

Enzyme Assays. Two procedures were used to assay for sulfhydryl oxidase activity: mesurement of oxygen uptake using an oxygen electrode to characterize the kinetic parameters of the purified enzyme (see below), and a simple discontinuous screening assay to handle the large numbers of chromatographic fractions generated during purification of the enzyme. In the latter, aliquots of suitable volume (for example 180 μL for the CM52 column in Figure 2) were added to 20 μL of 3 mM DTT in the wells of a 96-well plate. The top of the plate was securely sealed with Parafilm to prevent evaporation. The plate was incubated at room temperature and quenched after 1 h with 20 μL of 10 mM DTNB in 50 mM phosphate buffer containing 1 mM EDTA. Absorbance values were recorded with a Perkin-Elmer Fusion plate-reader using a 405 nm filter. However, sulfhydryl oxidase-containing wells were readily apparent by eye because they showed a decrease, or absence, of the strong yellow background absorbance of the thionitrobenzoate anion. As the purification of sulfhydryl oxidase progressed, smaller aliquots were added to the wells, with the balance made up to 180 μL with phosphate buffer. Peroxidase-containing fractions were also identified in 96-well plates. In this case, 200 μL of a working solution (containing 2 mM 4-aminoantipyrene (*42*) and 20 mM phenol in 20 mM KP_i with 1 mM EDTA at pH 7.5) were added to wells containing 5 μL of each fraction. The reaction was started by the addition of 10 μL of freshly prepared 0.3% hydrogen peroxide. Peroxidase-containing wells showed a deepening wine-red color as the quinoneimine derivative (510 nm (*42*)) was generated.

Oxygen electrode assays were performed at 25 °C as described previously (*33*) in either a standard glass chamber with a 2 mL volume or one modified to accept a 1 mL volume by the inclusion of a circular depression impressed in the base of the well (sized to accommodate an 8 × 2 mm stir bar). Buffers contained 50 mM potassium phosphate, with or without 1 mM EDTA, pH 7.5. For milk samples, assays were initiated by the addition of 5 mM DTT or GSH (concentrated stock solutions of GSH in water were carefully adjusted to pH 6.0 with KOH). All thiol substrates were standardized with DTNB before use. Reduced RNase was prepared, characterized, and stored, as described earlier (*33*). Assays were routinely performed in duplicate and typically contained 10−50 nM sulfhydry oxidase.

Sulfhydryl Oxidase Purification. Four liters of whole milk (combined from eight Holstein cows) were obtained from the University of Delaware farm at the 7 AM milking. The warm milk was transferred to 500 mL bottles and centrifuged at 1060*g* for 20 min in a Sorvall RC-5B centrifuge using a fixed-angle GSA rotor. The skim milk was siphoned from the bottles without disturbing the cream layer on the top and sides of the bottle. The volume of skim milk recovered was approximated 3400 mL. Casein was precipitated by adding about 190 mL of 1 M HCl to the stirred skim milk over about 5 min at room temperature to a final pH of 4.6. After centrifugation at 4200*g* for 15 min at 4 °C, the stirred clear yellow acid-whey was returned to pH 7.4 by the addition of 96 mL of 1 M KOH (correspondingly less base is required for this step because of the prior removal of caseins). The cloudy solution was supplemented with a protease inhibitor cocktail tablet (Roche) and EDTA to a concentration of 1 mM. The stirred solution was brought to 65% saturation in ammonium sulfate at 4 °C and then left to settle overnight. The majority of the pale yellow supernatant was removed by siphon, and the precipitated material was resuspended in the remaining liquid and recovered by centrifugation (20 min at 4200*g*). The supernatants were discarded, and the precipitates kept in four centrifuge bottles maintained in the dark at 4 °C until needed.

Table 1 refers to a purification during which one-half of the precipitated protein was used, and so the volumes of whole and skim milk have been adjusted accordingly. The precipitate from two bottles was gently resuspended in a minimal volume of 20 mM phosphate buffer, pH 6.0, containing 1 mM EDTA. This suspension (65 mL) was dialyzed against two 4 h changes of 1 L of the same buffer and then overnight against a third 1 L of buffer supplemented with a protease inhibitor tablet. Stirring was then stopped, allowing a white granular precipitate to settle to the bottom of the dialysis tubing. Incomplete dialysis leads to problems clearing the solution of this precipitate. The supernatants were

Table 1: Purification of a Sulfhydryl Oxidase from Bovine Milk

step	volume, mL	total protein, mg/mL	total SOX activity, units	yield, %	specific activity, units/mg	fold purification
Skim	1682	33.0	18600	100	0.33	1.0
Whey	1582	9.0	14300	77	1.00	3.1
65% $(NH_4)_2SO_4$ pellet	159.0	37.0	19700	106	3.4	10.0
CM52	121.2	0.76	10600	57	115	340
butyl-Sepharose	0.800	1.35	3530	19	3270	9770
Source S30	0.655	0.72	2430	13	5160	15400

centrifuged at 2600*g* for 20 min to remove any additional precipitate, and the straw-colored, opalescent, supernatant (about 105 mL) was collected and applied to a 5 × 24 cm CM52 cation exchange column (bed volume 350 mL) equilibrated in 20 mM phosphate buffer, pH 6.0, containing 1 mM EDTA. The column was developed with a gradient of KCl as follows: the low-salt reservoir (800 mL of stirred 20 mM buffer) supplied the head of the column at 156 mL/h and was replenished at the same flow rate with a solution of 1 M KCl dissolved in the same buffer. Six-minute fractions were collected and analyzed by UV/vis spectrum, and small aliquots from each tube were screened for sulfhydryl oxidase and peroxidase activity in a 96-well format. Once zones of sulfhydryl oxidase activity had been identified, they were more accurately quantitated with the oxygen electrode using both GSH and DTT substrates.

Pooled fractions were brought to 40% saturation with ammonium sulfate and then the solution applied at 1 mL/min to a butyl-Sepharose column (2.5 × 7.5 cm) equilibrated at room temperature with 40% saturated ammonium sulfate in 20 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The top of the column was supplied at 1 mL/min from a 50 mL volume of stirred 40% ammonium sulfate in 20 mM phosphate that was progressively diluted with ammonium sulfate-free buffer at the same flow rate. Fractions (6 mL) were collected and screened with the microplate assays as described above and by UV/vis absorbance. Suitable fractions were pooled, concentrated, and washed three times with 20 mM phosphate buffer, pH 7.0, containing 1 mM EDTA using a centrifuge ultrafiltration device (Centriprep YM-30).

The concentrated protein (0.3 mL) was applied to a 0.5 × 5 cm Source 30S cation exchange column equilibrated with 20 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. The column was developed at 0.5 mL/min in a linearly increasing gradient to 1 M KCl in 20 mM phosphate buffer formed by an AKTA-FPLC instrument. Fractions (0.5 mL) were collected and assessed for purity by UV/vis spectrum and enzymatic activity. Suitable fractions were combined, concentrated, washed with 50 mM phosphate buffer, pH 7.5 containing 1 mM EDTA, and stored at −80 °C.

Flavin Identification. The purified enzyme (10 μM in 0.1 mL of 50 mM phosphate buffer, pH 7.5, 1 mM EDTA in a microcentrifuge tube wrapped with aluminum foil) was placed in a boiling water bath for 5 min. The tube was cooled in ice and then centrifuged. Aliquots of the supernatant (50 μL) were applied to a C18-reverse phase column developed with a linear acetonitrile gradient (from 100% of 0.1% trifluoroacetic acid (TFA) in water to 100% 0.1% TFA in 90% acetonitrile) over 50 min. Elution profiles were compared with those for riboflavin, FMN, and FAD.

Iron Analyses. A series of iron standards (0−20 μM) were prepared by diluting 10 mM ferrous ammonium sulfate in degassed distilled water into 50 mM Tris buffer, pH 7.5. Sulfhydryl oxidase was used in the same buffer at a concentration of 10 μM. The following procedure is based on that described by Rebouche et al. (*43*). Aliquots (30 μL) of standards, or protein, were thoroughly mixed with an equal volume of 2 M HCl in 5 × 60 mm glass tubes, placed in a boiling water bath for 1 min, cooled, and mixed again. A 50 μL volume of these solutions were then diluted with 50 μL of a ferrozine working solution, which was made by dissolving 0.5 mM ferrozine in 1.5 M sodium acetate and then adding 0.1% v/v β-mercaptoethanol immediately before use. After 30 min at room temperature, protein samples were briefly centrifuged to minimize light scattering material, and the absorbance at 562 nm was recorded in self-masking microcells. Samples of Tris buffer without added iron were carried through this procedure and served as blanks.

Iron Supplementation Experiments. Purified bovine sulfhydryl oxidase was washed by ultrafiltration into 50 mM Tris buffer at pH 7.5 without EDTA, and then 5 μM enzyme was treated at room temperature for a total of 30 min with 10 μM ferrous iron added from a degassed solution of ferrous ammonium sulfate in water. Oxygen electrode assays of the oxidase, using 5 mM DTT, were followed 1 and 30 min after iron treatment and were compared with traces obtained on the addition of either enzyme or ferrous iron alone.

Gel Filtration of Skim Milk Fractions. A 3 × 68 cm column of Superdex 200 size exclusion resin was equilibrated at 90 mL/h using 50 mM phosphate buffer, pH 7.5, with or without EDTA. Samples (2 mL) of skim milk were injected and 3 mL fractions collected for analysis. Eluates were monitored continuously at 700, 450, and 280 nm. The column was standardized using 2 mL of thyroglobulin (670 000), catalase (250 000), aldolase (158 000), bovine serum albumin (67 000), ovalbumin (43 000), and cytochrome c (12 400 Da) at concentrations of approximately 0.5 mg/mL. A linear standard curve ($R^2 = 0.993$) was generated by plotting the logarithm of the molecular weight against the elution time.

Trypsin Digestion and LC-MS/MS Analysis of Digests. Protein samples were run on a 10% SDS-PAGE as detailed above. Coomassie-stained protein bands were excised, chopped into approximately 1 × 1 mm cubes, and placed in a 1.5 mL polypropylene centrifuge tube. The pieces were covered with methanol and vortexed intermittently for 5 min. Subsequently, the following washing steps were performed: with 30% methanol/70% water for 5 min; with two washes of water for 10 min; three washes with 100 mM ammonium bicarbonate/30% acetonitrile for 10 min. The entire procedure was repeated until the gel pieces were entirely cleared of the dye. A blank piece of gel containing no protein bands served as a control. The gel pieces were then covered with

acetonitrile and vortexed for 30 s and then excess liquid was removed. Samples were dried in a Vacufuge (Eppendorf) and incubated for 60 min at 56 °C in a solution of 10 mM tris(hydroxypropyl)phosphine in 100 mM NH_4HCO_3 solution, followed by centrifugation to aid the removal of excess liquid. The gel pieces were then incubated for 45 min at room temperature in the dark with 10 mg of iodoacetamide in 1 mL of 100 mM ammonium bicarbonate. The gel pieces were washed with 100 mM ammonium bicarbonate for 15 min and dehydrated with acetonitrile followed by complete drying in a Speed-Vac. The material was rehydrated on ice for 45 min by adding a sufficient volume of 50 mM ammonium bicarbonate containing 13 μg trypsin/mL to just cover the gel. Excess trypsin solution was removed, replaced with 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. The tube was centrifuged briefly and the solution transferred to a clean centrifuge tube. The gel pieces were further incubated for 15 min at 37 °C with intermittent vortexing and the supernatant combined with the trypsin digest. This procedure was repeated with ammonium bicarbonate and then with two further washes using 5% formic acid in 50% acetonitrile. The combined trypic peptides were vacuum-dried and stored at −80 °C before analysis.

The trypsin digests of purified proteins were each analyzed twice by liquid chromatography−tandem mass spectrometry as follows. The digests were diluted to an approximate concentration of 100 fmol/μL in 0.1% formic acid and 2% acetonitrile (solvent A). The peptide samples were sampled by a Spark Holland (Emmen, NL) Endurance autosampler using a 10 μL microliter-pickup injection method and separated on a 15-cm C18 capillary column (75 μm internal diameter, 5 μm particle size, 300 Å pore size, Micro-Tech Scientific, Vista, CA) over a gradient from 5 to 40% solvent B (0.1% formic acid, 95% acetonitrile) at a flow rate of 400 nL/min on a Micro-Tech Scientific XtremeSimple ultrahigh-pressure splitless nanoflow liquid chromatography system. The eluting peptides were analyzed by a Thermo Scientific (San Jose, CA) LTQ linear ion trap mass spectrometer equipped with a dynamic nanospray probe using an uncoated 10 μm ID SilicaTip PicoTip nanospray emitter (New Objective, Woburn, MA), a source voltage of 2.0 kV, and a heated capillary temperature of 200 °C. MS and MS/MS spectra were acquired using Xcalibur 1.4 software. Tandem mass spectrometry was performed by selecting the top five ions of each full scan (400−1800 m/z) for MS/MS sequencing, with dynamic exclusion of ions that had been selected three times in 15 s for 15 s. An MS/MS/MS scan was triggered if, among the three most abundant ions in the MS/MS scan, a neutral loss of 98, 49, or 32.7 Da was detected (corresponding to loss of phosphoric acid on singly, doubly, and triply charged precursor ions). Other parameters used for generating MS/MS data were an isolation width of 3.0 m/z, a collision energy of 24% (MS/MS) and 35% (MS/MS/MS), a minimum MS signal count of 500, a minimum MS/MS signal count of 100, and an activation time of 120 ms (MS/MS) and 30 ms (MS/MS/MS).

Mass Spectrometry Data Analysis. The ipi.BOVIN.v3.04 database was searched against the raw spectral data files using Sorcerer SEQUEST Version 3.3 (SageN, San Jose, CA) with a peptide mass tolerance of 1.5 amu and differential modifications of M +15.9949 and C +57.021464. Prior to the search, NCBI sequence XP_601276 (similar to quiescin [*Bos taurus*]) was appended to the database, since there are two areas in which this version of the QSOX1 sequence differs from the IPI.bovine.v3.03 version of QSOX1 (IPI00704610). Spectra of matched peptides were examined to ensure that the b- and y-ions and the major ions in the spectra corresponded.

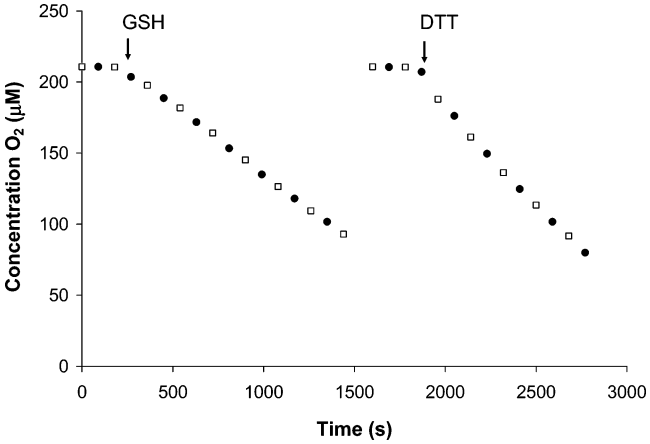

FIGURE 1: Oxygen electrode assays of skim milk for sulfhydryl oxidase activity using glutathione and DTT as substrates. Assays contained 1 mL of skim milk and 1 mL of 50 mM phosphate buffer with (circles) or without (squares) 1 mM EDTA. At the arrows, assays were started by the addition of 5 mM GSH (left panel; see Experimental Procedures) or DTT (right panel).

RESULTS AND DISCUSSION

Sulfhydryl Oxidase Activity in Skim Milk. Our preliminary data using glutathione as a substrate (see Experimental Procedures) confirmed that the bulk of sulfhydryl oxidase activity in whole milk was present in the skim milk fraction (not shown). However, we could not reproduce two key findings of the early studies. The first is the substrate specificity pattern shown by unfractionated skim milk. The earlier work reported that DTT was not a substrate of the milk sulfhydryl oxidase either in skim milk or in a purified form (*13*, *15*, *20*, *44*). However, under the conditions of Figure 1, DTT is a better substrate than GSH (~1.5-fold) in skim milk. This observation was repeatedly confirmed using several batches of fresh milk collected over a 2-year period. In our hands, sulfhydryl oxidase activity is present at rather low levels in milk, such that one-half of the assay volume in Figure 1 was milk, with the balance made up with phosphate buffer. Control experiments, omitting milk, showed the expected small background oxygen consumption due to nonenzymatic thiol oxidation (not shown).

A more important issue is the effect of the metal chelator, EDTA, on sulfhydryl oxidase activity. We found an insignificant effect on the rate of oxygen consumption with either GSH or DTT (Figure 1). In this experiment, skim milk was exposed to EDTA for approximately 20 min. To evaluate whether longer incubation times would reveal noticeable inhibition, we treated skim milk overnight with 1 mM EDTA before assay. This pretreatment had no significant effect over an untreated control (not shown). Similar data were obtained with partially purified enzyme prepared in the absence of EDTA (not shown). In summary, we could find no evidence for an effect of EDTA on the activity of skim milk sulfhydryl oxidase.

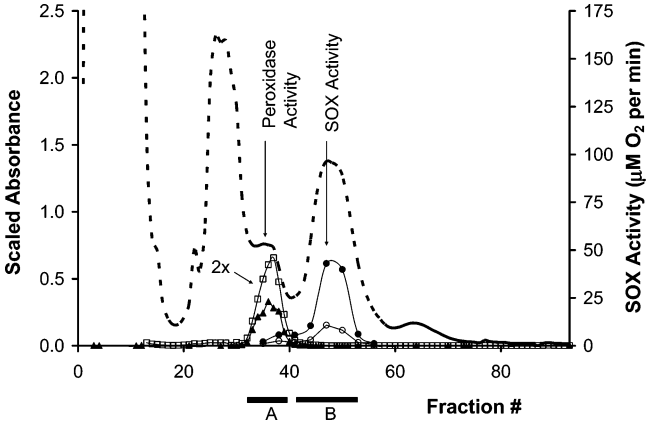

FIGURE 2: Cation-exchange chromatography (CM52) of skim milk membranes at pH 6.0. The dashed line indicates absorbance at 280 nm with contribution from turbidity (fraction 1−15). Solid and open circles represent DTT and GSH oxidase activity, respectively, squares Soret absorbance at 415 nm, and triangles, peroxidase activity (see Experimental Procedures). Absorbance values at 415 nm for fractions 1−12 are not included because these initial fractions were turbid. The column was developed with a gradient of 0−1.0 M KCl in 20 mM phosphate buffer, pH 6.0.

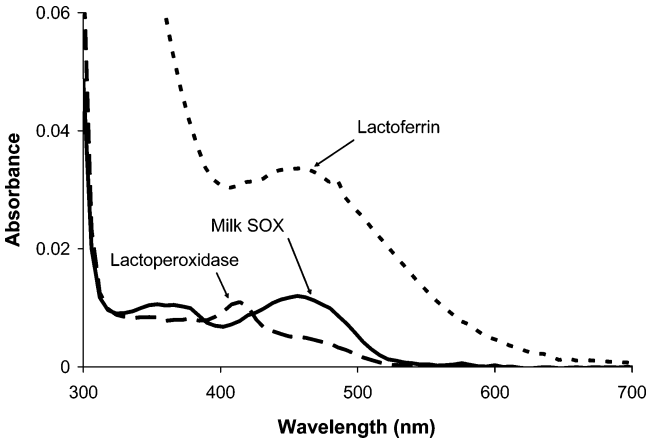

FIGURE 3: Visible spectra of three species eluting from butyl-Sepharose hydrophobic interaction chromatography. Spectra of fractions 16 (lactoferrin), 19 (lactoperoxidase), and 22 (sulfhydryl oxidase) were recorded without dilution and were adjusted to zero at 800 nm to correct for small levels of light scattering between samples.

Purification of Milk Sulfhydryl Oxidase. For the reasons mentioned above, we decided to develop a new purification scheme for bovine milk sulfhydryl oxidase (Table 1), rather than follow the original method or its subsequent modifications (*10*, *16*, *18*−*20*, *45*). Because our results are so different from the earlier work, key chromatographic separations are documented in Figures 2 and 3. In our procedure, whey was prepared by precipitating caseins from stirred skim milk at room temperature by adding 1 M HCl until the pH reached 4.6 (see Experimental Procedures). The whey was recovered from the casein precipitate by centrifugation at 4 °C, and then the supernatant was brought to pH 7.4 by the addition of 1 M KOH. It should be noted that EDTA (at 1 mM) interferes with the acid precipitation of casein and so was only added after the voluminous granular precipitate had been removed. Further, in our hands, the alternative procedure to precipitate casein using chymosin (*10*, *16*, *45*) resulted in lower recovery of activity (not shown).

The neutralized whey, now containing 1 mM EDTA and a protease inhibitor cocktail, was brought to 65% saturation with ammonium sulfate, and the precipitate collected by centrifugation. Resuspension of this material in phosphate buffer followed by extensive dialysis at pH 6.0 gave an opalescent fraction containing the bulk of sulfhydryl oxidase activity (Table 1). Instead of the initial gel filtration step adopted previously (*10*), we wanted to exploit a robust high-capacity procedure early in the purification procedure. To this end, we found that cation-exchange chromatography at pH 6.0 provided significant purification of the sulfhydryl oxidase activity (Figure 2; Table 1).

Strongly opalescent fractions emerged first from the CM52 cation-exchange column. These lipid-rich aggregates could be sedimented at 20 000*g* (2 h, 4 °C) and were shown by transmission electron microscopy to be a heterogeneous mixture of membrane fragments and vesicles (supplementary Figure S1). Importantly, this light-scattering material contained no detectable sulfhydryl oxidase activity (Figure 2). The column was developed with a gradient of KCl leading to the emergence of two overlapping bands of sulfhydryl oxidase activity, with 10% of the activity in fraction "A" and 90% in fraction "B" (Figure 2). To provide an independent benchmark for this key chromatographic step, we also show the elution profile for lactoperoxidase, a well-known peroxidase found in milk. As expected, the Soret absorbance band (maximal at 415 nm) and the peroxidase activity (Figure 2) superimposed.

Part of the minor sulfhydryl oxidase activity in fraction A may reflect the ability of heme-dependent peroxidases, including bovine lactoperoxidase, to catalyze the oxidation of thiols, including GSH and DTT (*46*):

$$2\ \text{R-SH} + \text{O}_2 \rightarrow \text{R-S-S-R} + \text{H}_2\text{O}_2$$

However the apparent sulfhydryl oxidase activity (with either DTT or GSH) of fraction A does not exactly superimpose with the lactoperoxidase peak (Figure 2). We leave this issue, and the previous suggestion that milk sulfhydryl oxidase and lactoperoxidase interact (*47*), for further work. The bulk of the sulfhydryl oxidase activity was found in fraction B. Fractions 47−51 were pooled, adjusted to pH 7.5, brought to 40% saturation (at 25 °C) in ammonium sulfate, and applied to a butyl-Sepharose hydrophobic interaction column. This separation was performed at 22 °C: the chromatographic resolution was noticeably inferior at 4 °C. The sulfhydryl oxidase-containing fractions absorbed to the top of the butyl-Sepharose gel as a pale yellow-orange band and were eluted with a decreasing gradient of ammonium sulfate. The first colored band to emerge was a pink species whose visible spectrum (Figure 3, fraction 16) and apparent molecular weight on SDS-PAGE (ca. 81 kDa) matched an authentic sample of lactoferrin. Next, came an orange-brown band showing a distinct Soret peak (fraction 19; Figure 3), which was identified as lactoperoxidase. Finally, the sulfhydryl oxidase activity was associated with a yellow band, with the spectrum shown in Figure 3 (fraction 22).

Sulfhydryl oxidase-containing fractions were pooled and subjected to a second cation-exchange separation using Source 30S resin at pH 7.0. Following this step, the combined fractions containing sulfhydryl oxidase activity had a UV/

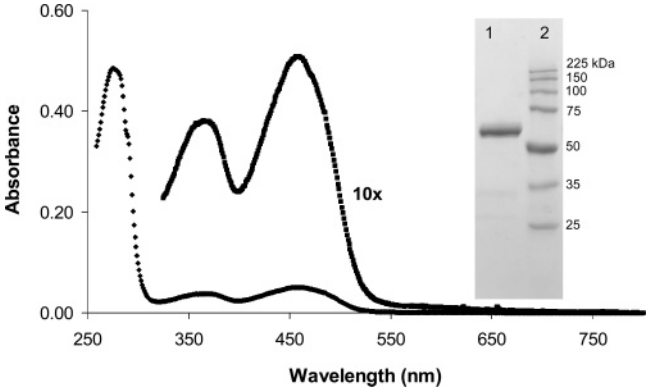

FIGURE 4: UV/vis spectrum of purified milk sulfhydryl oxidase. Spectra were recorded in 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The 280/458 nm ratio was 9.4. A 10-fold amplification of the absorbance in the visible region highlights the spectrum of bound flavin. Lanes 1 and 2 in the inset show separation of the purified enzyme by reducing SDS-PAGE and a mixture of protein molecular weight markers, respectively.

vis spectrum showing an unresolved flavin envelope with maxima at 458 and 365 nm (Figure 4). At this stage the enzyme separated as essentially a single band of protein on SDS-PAGE, with a molecular weight corresponding to ~62 kDa (see inset Figure 4). A sample of the protein was boiled and the released flavin shown to be FAD by comparison with the elution times of riboflavin, FMN, and FAD on reverse phase HPLC (see Experimental Procedures).

Table 1 summarizes the purification developed here: a greater than 15 000-fold purification is required to obtain substantially pure protein. Previously a 3000-fold to 4830-fold purification (*10*, *16*, *45*) from skim milk was reported to yield a single band on SDS-PAGE with an apparent molecular weight of about 89 kDa (*10*, *16*, *19*). A major contaminant we observed after the CM52 step showed an apparent molecular weight of about 81 kDa (a comparison of the purities after each column step is presented in supplemental Figure S2). Sequencing of tryptic peptides showed that this impurity was bovine lactoferrin: an abundant milk protein with the ability to bind up to two iron atoms per monomer (see later (*48*−*50*).

Iron Content of Milk Sulfhydryl Oxidase. We next considered experiments in which ferrous iron was added to the purified sulfhydryl oxidase solutions. In the previous study (*10*), sulfhydryl oxidase preparations, in phosphate buffer, were treated with EDTA and then dialyzed against 1 μM ferrous sulfate before assessing enzyme activity in 50 mM phosphate buffer pH 7.0. This protocol raises several issues. First, the solubility product of ferrous phosphate is very low ($\sim 10^{-32}$ (*51*)) suggesting that an almost stoichiometric depletion of iron from solution would occur. Second, ferrous salts rapidly oxidize in neutral aerobic solutions yielding insoluble ferric hydroxide aggregates (*52*). Finally, the use of a vibrating platinum electrode for oxygen concentration measurements (*10*) is of some concern. Platinum surfaces are prone to thiol-mediated passivation (from glutathione and other substrates) unless coated with cellulose acetate or collodion (*53*). Further, added transition metals can promote an electrochemical response at a bare platinum electrode that may interfere with oxygen quantitation.

Iron analysis of our purified flavoenzyme using ferrozine (see Experimental Procedures) showed minor levels of iron

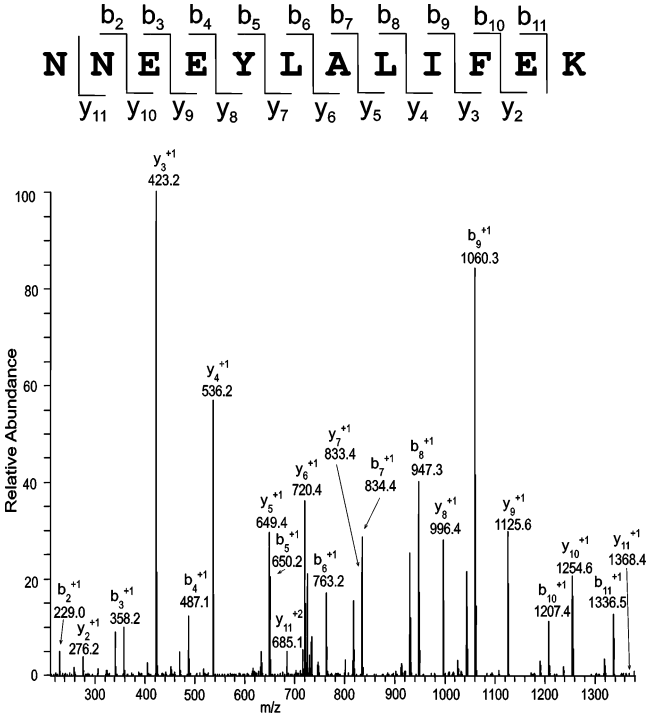

FIGURE 5: Example of spectrum obtained for the peptide sequence ^{184}NNEEYLALIFEK^{195} from the identification of bovine QSOX 1 by LC-MS/MS. Peptides were produced by in-gel tryptic digestion and analyzed by LC-MS/MS as described in Experimental Procedures and in Supporting Information. Sequences were searched against a bovine database (see the text). In addition to the complete series of +1 b- and y-ions, a complete set of +1 b-NH_3 ions may also be observed 17 amu to the left of the +1 b_3−b_{11} ions.

(0.04 ± 0.02) iron atoms/per monomer). To evaluate the effect of added iron, we incubated 5 μM enzyme for 30 min with 10 μM of ferrous ammonium sulfate in Tris buffer (50 mM, pH 7.5; in the absence of EDTA). Assays were then conducted in the same Tris buffer. No significant change in activity, over controls without added metal, was obtained in duplicate experiments using DTT as a substrate (data not shown). In aggregate, the UV−vis spectrum of the purified protein (Figure 4), the presence of only small levels of iron in the enzyme (consistent with adventitious metal binding), the absence of stimulation when ferrous iron is added, and the lack of EDTA inhibition indicate that the activity we have followed from skim milk to essential purity is an iron-independent flavin-containing oxidase. A final piece of evidence is provided in the next section: the milk enzyme isolated here is a member of the QSOX family of flavin-dependent sulfhydryl oxidases. These enzymes have been already demonstrated to bind metal ions adventitiously but are not themselves dependent on transition metals for their biological activity (*5*).

Sequence of the Flavin-Dependent Sulfhydryl Oxidase. The 62 kDa band was excised from SDS-PAGE gels (e.g., inset Figure 4) and destained and the protein digested with trypsin (See Experimental Procedures). A search of the spectral data obtained from LC-MS/MS analysis of the flavoprotein band against the ipi_BOVIN_v_3_04 database determined that the protein was QSOX1 ("similar to quiescin Q6 isoform a": IPI00704610**)**. One contaminating protein, heparanase, was also identified at very low abundance (about 1%): an average of 9218 QSOX peptide spectra were identified in each run, in comparison to only 122 heparanase spectra. Details of

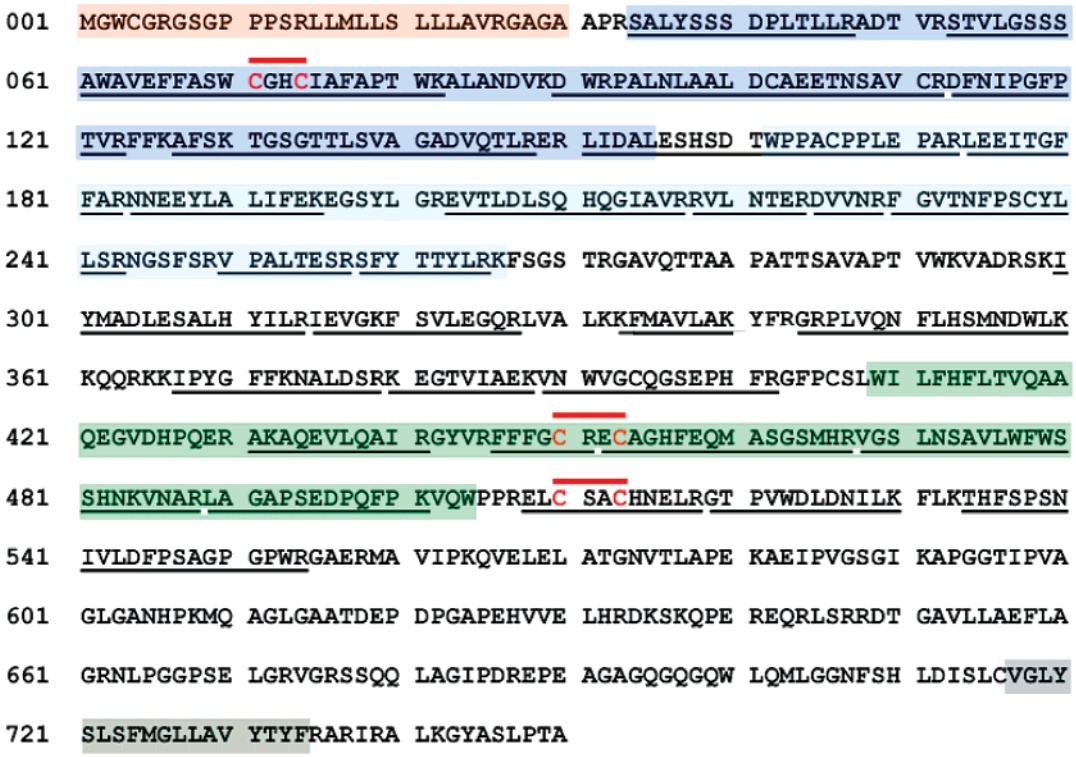

FIGURE 6: Full length sequence of bovine QSOX1 and placement of peptides therein. The N-terminal region (residues 1−90) corresponds to IPI00704610 version 3.04. The remaining sequence corresponds to NCBI XP_601276 (see Supporting Information). Peptide sequence coverage is shown underlined. Each peptide sequenced is detailed in supplementary Table 1. Assuming a molecular weight for the mature protein of 62 kDa (with a start at residue 31 (prediction using Phobius (*67*)) implies a C-terminus close to residue 590 (corresponding to a sequence coverage of approximately 72%). The three conserved CxxC motifs are highlighted in red and three domains of known structure are shown in blue (Trx1), light blue (Trx2), and green (ERV/ALR). The putative signal sequence and the C-terminal transmembrane span of the long form of QSOX1 are shown in orange and gray, respectively (prediction using Phobius (*67*)). A plain text version of this complete sequence is presented as supplementary Figure S3.

Table 2: Pairwise Sequence Alignments of Selected QSOXs to Bovine Milk QSOX1[a]

organism	QSOX 1	QSOX 2
Bos taurus	100	-
Homo sapiens	76	36
Mus musculus	74	37
Gallus gallus	46	37

[a] Alignments were performed using the ClustalW program (*68*). The bovine QSOX2 sequence is currently unavailable.

the mass spectral analysis are presented in Supporting Information (supplementary Tables S1 and S2). An indication of the quality of the MS sequencing data is presented for the tryptic peptide NNEEYLALIFEK in Figure 5.

Since the available database sequences for bovine QSOX1 contained discrepancies, we compared these to other mammalian QSOX1 sequences (not shown) and to the substantial sequence coverage that our MS/MS analysis affords. In this way we can report a reliable consensus sequence for bovine QSOX1 in Figure 6. Additional information for this sequence is presented in Supporting Information. Clearly the bovine QSOX secreted in milk is the lower molecular weight short form lacking the weakly conserved variable C-terminal stretch including the single transmembrane spanning region (Figure 6). The bovine sulfhydryl oxidase is clearly QSOX1 and not QSOX2: a comparison of pairwise sequence identities between the short-form paralogues of human, bovine, mouse, and avian QSOX is presented in Table 2. Milk QSOX has all of the sequence elements previously believed to be catalytically important in the avian QSOX1 enzyme (*26*−*28*, *54*). In particular, it has the three conserved CxxC motifs found in all QSOXs (highlighted in red), together with two thioredoxin domains (in blue) and an Erv/ALR flavin binding domain (in green).

We wished to also identity the 81 kDa contaminant that was prominent at earlier stages of the purification. MS/MS sequencing showed this protein to be lactoferrin (gi:2781197; 84% sequence coverage from 150 unique peptides; supplemental Table S3). Wilcox et al. (*55*) have reported the association of a sulfhydryl oxidase activity with CD36, an 85 kDa membrane glycoprotein secreted in association with bovine fat globule and skim milk membranes (*56*). While we did not observe this protein in our purification procedure, perhaps QSOX1 can associate noncovalently with multiple milk components under certain conditions. Indeed, evidence that the flavin-linked sulfhydryl oxidase is found in multimeric assemblies in untreated skim milk is presented below.

Bovine Sulfhydryl Oxidase Does Not Tightly Associate with Skim Milk Membranes in Milk. It has previously been stated that "Bovine milk sulfhydryl oxidase (SOX) is a metalloglycoprotein that resides as an integral enzyme in the plasma membrane of mammary secretory cells" (*16*). In earlier studies, the source of sulfhydryl oxidase was "skim milk membranes", which are often concentrated from milk whey by precipitation using 50% saturated ammonium sulfate (*20*, *57*). This precipitate contains membrane vesicles, but it will also logically include proteins precipitated by salting-out that are not necessarily originally membrane-associated. Two observations show that the sulfhydryl oxidase isolated in this

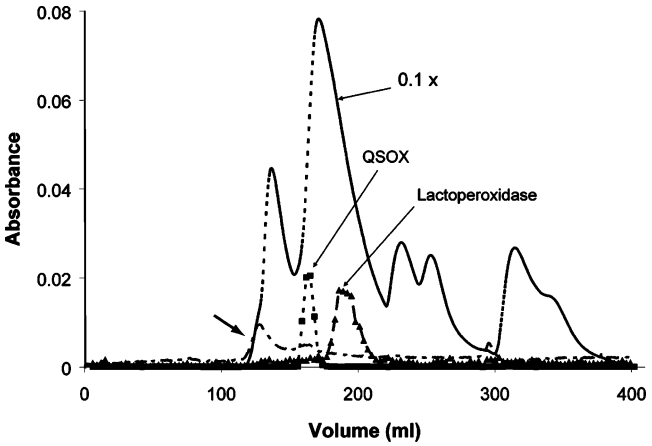

FIGURE 7: Gel filtration exclusion elution profiles of skim milk. A 3.0 × 70 cm Superdex 200 column was loaded with 2 mL of freshly prepared skim milk and developed at 22 °C in 50 mM phosphate buffer with 1 mM EDTA. Light scattering material (followed at 700 nm, dash-dot line), including membrane fragments, elute in the void volume of the column at the bold arrow. Sulfhydryl oxidase and lactoperoxidase levels in each fraction were assessed as in Experimental Procedures. Absorbance values were scaled by the factors shown for each curve (e.g., the peak 280 nm absorbance value at 170 mL was 0.78). Assays values for QSOX and lactoperoxidase were scaled by 0.0067 and 0.125 times, respectively.

work is not tightly associated with skim milk membranes. First, membrane vesicles are not retained on cation-exchange chromatography (Figure 2 and supplemental Figure S1). These fractions contained no detectable sulfhydryl oxidase activity using either glutathione or DTT (Figure 2). It is important to note that the amounts of ammonium sulfate used to precipitate whey proteins differ between the present and earlier purification procedures. We brought acid whey to 65% saturation in ammonium sulfate, whereas the earlier investigators used whey prepared by coagulating the caseins with chymosin and precipitating protein from the cleared whey with ammonium sulfate at 50% saturation (*20*). Major differences in the outcome of these procedures are therefore plausible. However, both methods concentrate whey protein with membrane fragments using ammonium sulfate precipitation.

To address the fundamental question of whether QSOX1 associates with skim milk membrane fragments in *untreated* skim milk, we used a large size-exclusion column of Superdex 200 (see Experimental Procedures). Elution from this calibrated column was monitored by absorbance at 280 nm, by light scattering (assessed at 700 nm) and by activity measurements for sulfhydryl oxidase and lactoperoxidase (to serve as a landmark). Opalescent fractions containing membranes eluted in the void volume (at ~130 mL), considerably in advance of the single peak of QSOX activity centered at 165 mL. Prior calibration of the column with proteins from 670 to 12 kDa showed that bovine QSOX activity in skim milk eluted with an apparent MW of 354 kDa. This is in marked contrast to the purified protein, which eluted as a monomer with an apparent molecular weight of about 77 kDa (not shown). The reason for these size differences is not clear and beyond the scope of this present investigation. The gel-filtration elution profile of skim milk in Figure 7 was essentially identical when samples were separated in the absence of EDTA (not shown).

Besides the gel-filtration data, there is another reason to believe that QSOX1 is not tightly associated with skim milk membranes. These membranes are often prepared on a small scale by sedimentation of skim milk at 100 000 g for 1−2 h (*58*, *59*). When we centrifuged fresh skim milk at 100 000 g for 2 h, we obtained the expected compact white casein precipitate, overlaid with a fluffy layer of skim milk membranes. Assays of the resuspended fluffy layer, and the supernatant fractions, showed that essentially all (>90%) of the SOX activity was in the supernatant (not shown). Similarly, Isaac observed no loss of SOX activity in supernatants obtained after human skim milk was centrifuged for 90 min at 100 000*g* (*60*). In summary, we conclude that the QSOX activity in untreated skim milk is not tightly associated with the structures traditionally regarded as skim milk membranes.

Catalytic Activity of Bovine Milk QSOX. The k_{cat}/K_m values for the oxidation of DTT, glutathione, and reduced RNase by bovine QSOX1 are marginally higher than those of their egg white counterpart (Table 3). Like the egg white enzyme, bovine QSOX1 oxidizes reduced RNase efficiently with a K_m per thiol of 60 μM. Glutathione is a relatively poor substrate of the milk enzyme, with a K_m of 4.9 mM. This value is some 4-fold lower than the avian enzyme (*33*). The general catalytic similarities between the two enzymes (Table 3) suggest that insights gained from the avian enzyme will be applicable to the bovine oxidase.

Conclusions. This is the first report of the purification of a flavin-linked sulfhydryl oxidase from milk. The finding of QSOX1 in milk is consistent with expression and SAGE databases in human and mouse mammary tissue and the immunohistochemical studies of human breast tissue using an anti-QSOX1 peptide antibody (*28*, *36*, *37*).

QSOX enzymes have been localized to the endoplasmic reticulum, Golgi complex, secretory granules, and the plasma membrane (*26*, *28*, *29*, *31*, *61*, *62*) and are also secreted from cells. For example, QSOX1 appears in rat seminal vesicle secretions (*23*, *25*, *30*, *63*), human tears (*54*), bovine blood (*34*), avian egg white (*24*, *33*), and, as shown here, in bovine milk. The reasons for the secretion of QSOX enzymes into such diverse biological fluids are unclear. One possibility is that QSOX is secreted alongside some of the disulfide-

Table 3: Comparison of Steady-State Catalytic Parameters for QSOX1 Enzymes Isolated from Bovine Skim Milk and Avian Egg White[a]

	milk QSOX1			egg QSOX1[b]		
substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
DTT	1880	0.086	3.66×10^5	2060	0.15	2.30×10^5
GSH	1760	4.9	5.94×10^3	2780	20	2.32×10^3
rRNase	1340	0.060	3.74×10^5	1220	0.115	1.76×10^5

[a] All assays were performed at 25 °C in 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. k_{cat} values are expressed, not as disulfide bonds formed per min (as previously), but as thiols oxidized per min (to allow an appropriate comparison of k_{cat}/K_m values). [b] Data from Hoober et al. (*39*) now listed as thiols oxidized per min.

bridged proteins it has helped to generate because it has additional extracellular roles, possibly disulfide generation, in the formation of hydrogen peroxide for antimicrobial effects (*23*, *25*, *26*) or for signaling (*27*, *64*). Human milk sulfhydryl oxidase is rather stable at pH 2.5 and is resistant to degradation by pepsin, trypsin, and chymotrypsin. Consequently, it has been suggested that it may function in protecting the gastrointestinal tracts of newborns as a component of the innate immune system (*65*).

We were unable to verify the existence of significant additional sulfhydryl oxidase activities in bovine milk, including the membrane-bound iron-binding oxidase described by Swaisgood and co-workers. We can explain neither the EDTA inhibition of this activity nor its partial reconstitution by the addition of a variety of redox-active transition metals. Nor can we reconcile why the QSOX1 activity, which we have purified, is freely soluble, whereas the putative iron-binding enzyme is apparently associated with membranes in skim milk. As documented above, the two preparations also have markedly different substrate specificities. In addition, a 3000- to 4830-fold purification of the iron-binding enzyme from skim milk yielded an apparently homogeneous preparation, whereas the QSOX1 enzyme, required an over 15 000-fold purification from the same starting material. Until the final stages of purification, lactoferrin was a persistent contaminant. Thus, it is possible that some of the properties previously associated with the iron-dependent oxidase might have reflected the presence of lactoferrin.

Parallel uncertainties extend to a copper-dependent sulfhydryl oxidase from mammalian skin (*1*). The skin oxidase was also reported to be completely inhibited by EDTA and reactivated by added copper ions. Yet when a skin sulfhydryl oxidase was cloned, it was found to be the flavoenzyme, QSOX1 (*66*). While we cannot exclude the possibility that there are both flavin and metal-dependent oxidases in skin, we have found that both holo- and FAD-free QSOX proteins, with their clusters of CxxC motifs, are able to efficiently bind transition metals including zinc and copper (*5*). In our hands, copper-binding inhibits, rather than stimulates, sulfhydryl oxidase activity (*5*). Finally, there are at least three additional reports of copper-dependent sulfhydryl oxidases that are inactivated by EDTA and reactivated by the addition of Cu^{2+} ions: enzymes present at the basal lateral surfaces of the small intestine (*3*), kidney (*3*), and an activity partially purified from antibody-producing tumors (*4*). Given the burgeoning interest in oxidative protein folding, a reinvestigation of these poorly understood metalloenzyme sulfhydryl oxidases is clearly warranted.

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SUPPORTING INFORMATION AVAILABLE

Reconstruction of the bovine QSOX1 sequence; LC-MS/MS data for bovine QSOX, heparanase, and lactoferrin tryptic peptides; transmission electron micrograph of membrane fraction; SDS-PAGE evaluation of purification steps; amino acid sequence of full-length bovine QSOX1. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Yamada, H. (1989) Localization in skin, activation and reaction mechanisms of skin sulfhydryl oxidase, *Nippon Hifuka Gakkai Zasshi 99*, 861−869.
2. Goldsmith, L. (1987) Sulfhydryl Oxidase from Rat Skin, *Methods Enzymol. 143*, 510−515.
3. Lash, L. H., and Jones, D. P. (1983) Characterization of the membrane-associated thiol oxidase activity of rat small-intestinal epithelium, *Arch Biochem. Biophys 225*, 344−352.
4. Roth, R. A., and Koshland, M. E. (1981) Identification of a lymphocyte enzyme that catalyzes pentamer immunoglobulin M assembly, *J. Biol. Chem. 256*, 4633−4639.
5. Brohawn, S. G., Rudik, I., and Thorpe, C. (2003) Avian sulfhydryl oxidase is not a metalloenzyme: adventitious binding of divalent metal ions to the enzyme, *Biochemistry 42*, 11074−11082.
6. Kiermeier, F., and Ranfft, K. (1970) About Some Characteristics of Sulfhydryloxidase in Milk, *Zeitschrift Lebensm. Unters. Forsch. 143*, 11−15.
7. Kiermeier, F., and Petz, E. (1967) A sulfhydryl group-oxidizing enzyme in milk. I. isolation and characterization of the enzyme, *Z. Lebensm. Unters. Forsch. 132*, 342−352.
8. Kiermeier, F., and Petz, E. (1967) A Sulfhydryl group-oxidizing Enzyme in milk: II Influence of heating on milk and whey, *Z. Lebensm. Unters. Forsch. 134*, 97−102.
9. Kiermeier, F., and Petz, E. (1967) A Sulfhydryl group-oxidizing Enzyme in milk: III. Effect of heating temperature and heating time, *Z. Lebensm. Unters. Forsch. 134*, 149−156.
10. Janolino, V. G., and Swaisgood, H. E. (1975) Isolation and characterization of sulfhydryl oxidase from bovine milk, *J. Biol. Chem. 250*, 2532−2538.
11. Janolino, V. G., Swaisgood, H. E., and Horton, H. R. (1985) Renaturation of soluble and immobilized ribonuclease: are the polypeptide folding pathways for structure formation the same for soluble proteins and for proteins associated with a surface?, *J. Appl. Biochem. 7*, 33−37.
12. Janolino, V. G., and Swaisgood, H. E. (1987) Sulfhydryl oxidase-catalyzed formation of disulfide bonds in reduced ribonuclease, *Arch. Biochem. Biophys. 258*, 265−271.
13. Janolino, V. G., and Swaisgood, H. E. (1992) A comparison of sulfhydryl oxidase from bovine milk and *Aspergillus niger*, *Milchwissenschaft 47*, 143−146.
14. Schmelzer, C. H., Swaisgood, H. E., and Horton, H. R. (1985) Glycylglycyl-L-cysteine as a substrate for renal sulfhydryl oxidase (glutathione oxidase), *Biochim. Biophys. Acta 827*, 140−143.
15. Sliwkowski, M. X., Swaisgood, H. E., Clare, D. A., and Horton, H. R. (1984) Kinetic mechanism and specificity of bovine milk sulphydryl oxidase, *Biochem. J. 220*, 51−55.
16. Janolino, V. G., and Swaisgood, H. E. (1993) Purification of Reactive Sulfhydryl Enzymes by Bioselective Adsorption on Monomeric Avidin - Purification of Sulfhydryl Oxidase, *J. Food Biochem. 16*, 389−399.
17. Janolino, V. G. (1973) Ph.D. Dissertation, Department of Food Science, North Carolina State University, Raleigh, NC, p 85.
18. Sliwkowski, M. X., Sliwkowski, M. B., Swaisgood, H. E., and Horton, H. R. (1981) Isolation and Characterization of Sulfhydryl Oxidase by Covalent Chromatography on Cysteinylsuccinamidopropyl-Glass, *Fed. Proc. 40*, 1723−1723.
19. Janolino, V. G., and Swaisgood, H. E. (1990) Homogeneity of Sulfhydryl Oxidase Preparations Obtained by Transient Covalent Affinity-Chromatography, *J. Dairy Sci. 73*, 308−313.
20. Swaisgood, H., and Janolino, V. (2003) Mammalian sulfhydryl oxidase, *Food Sci. Technol. 122*, 539−546.
21. Janolino, V. G., Sliwkowski, M. X., Swaisgood, H. E., and Horton, H. R. (1978) Catalytic effect of sulfhydryl oxidase on the formation of three-dimensional structure in chymotrypsinogen A, *Arch. Biochem. Biophys. 191*, 269−277.
22. Ostrowski, M. C., Kistler, W. S., and Williams-Ashman, H. G. (1979) A flavoprotein responsible for the intense sulfhydryl oxidase activity of rat seminal vesicle secretion, *Biochem. Biophy. Res. Commun. 87*, 171−176.
23. Ostrowski, M. C., and Kistler, W. S. (1980) Properties of a flavoprotein sulfhydryl oxidase from rat seminal vesicle secretion, *Biochemistry 19*, 2639−2645.

24. Hoober, K. L., Glynn, N. M., Burnside, J., Coppock, D. L., and Thorpe, C. (1999) Homology between egg white sulfhydryl oxidase and quiescin Q6 defines a new class of flavin-linked sulfhydryl oxidases, *J. Biol. Chem. 274*, 31759−31762.
25. Benayoun, B., Esnard-Fève, A., Castella, S., Courty, Y., and Esnard, F. (2001) Rat seminal vesicle FAD-dependent sulfhydryl oxidase:biochemical characterization and molecular cloning of a member of the new sulfhydryl oxidase/quiescin Q6 gene family, *J. Biol. Chem. 276*, 13830−13837.
26. Coppock, D. L., and Thorpe, C. (2006) Multidomain flavin-dependent sulfhydryl oxidases, *Antioxid. Redox Signal 8*, 300−311.
27. Thorpe, C., and Coppock, D. L. (2007) Generating disulfides in multicellular organisms: Emerging roles for a new flavoprotein family, *J. Biol. Chem. 282*, 13929−13933.
28. Thorpe, C., Hoober, K., Raje, S., Glynn, N., Burnside, J., Turi, G., and Coppock, D. (2002) Sulfhydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes, *Arch. Biochem. Biophys. 405*, 1−12.
29. Wittke, I., Wiedemeyer, R., Pillmann, A., Savelyeva, L., Westermann, F., and Schwab, M. (2003) Neuroblastoma-derived sulfhydryl oxidase, a new member of the sulfhydryl oxidase/Quiescin6 family, regulates sensitization to interferon gamma-induced cell death in human neuroblastoma cells, *Cancer Res. 63*, 7742−7752.
30. Radom, J., Colin, D., Thiebault, F., Dognin-Bergeret, M., Mairet-Coello, G., Esnard-Feve, A., Fellmann, D., and Jouvenot, M. (2006) Identification and expression of a new splicing variant of FAD-sulfhydryl oxidase in adult rat brain, *Biochim. Biophys. Acta 1759*, 225−233.
31. Tury, A., Mairet-Coello, G., Esnard-Feve, A., Benayoun, B., Risold, P. Y., Griffond, B., and Fellmann, D. (2006) Cell-specific localization of the sulphydryl oxidase QSOX in rat peripheral tissues, *Cell Tissue Res. 323*, 91−103.
32. Coppock, D. L., Kopman, C., Scandalis, S., and Gillerman, S. (1993) Preferential gene expression in quiescent human lung fibroblasts, *Cell Growth Differ. 4*, 483−493.
33. Hoober, K. L., Joneja, B., White, H. B., III, and Thorpe, C. (1996) A Sulfhydryl Oxidase from Chicken Egg White, *J. Biol. Chem. 271*, 30510−30516.
34. Zanata, S. M., Luvizon, A. C., Batista, D. F., Ikegami, C. M., Pedrosa, F. O., Souza, E. M., Chaves, D. F., Caron, L. F., Pelizzari, J. V., Laurindo, F. R., and Nakao, L. S. (2005) High levels of active quiescin Q6 sulfhydryl oxidase (QSOX) are selectively present in fetal serum, *Redox Rep. 10*, 319−323.
35. Tury, A., Mairet-Coello, G., Poncet, F., Jacquemard, C., Risold, P. Y., Fellmann, D., and Griffond, B. (2004) QSOX sulfhydryl oxidase in rat adenohypophysis: localization and regulation by estrogens, *J. Endocrinol. 183*, 353−363.
36. Su, A. I., Wiltshire, T., Batalov, S., Lapp, H., Ching, K. A., Block, D., Zhang, J., Soden, R., Hayakawa, M., Kreiman, G., Cooke, M. P., Walker, J. R., and Hogenesch, J. B. (2004) A gene atlas of the mouse and human protein-encoding transcriptomes, *Proc. Natl. Acad. Sci. U.S.A. 101*, 6062−7; http://symatlas.gnf.org/SymAtlas/.
37. The Cancer Genome Anatomy project (2006) http://cgap.nci.nih.gov/SAGE/MEMatrix?ORG=Mm&METHOD=SS10,-LS10&FORMAT=html&TAG=AAGAAAAAGC&STATE=1,1,1,1,1,1,1,1.
38. Hoober, K. L., and Thorpe, C. (1999) Egg white sulfhydryl oxidase: Kinetic mechanism of the catalysis of disulfide bond formation, *Biochemistry 38*, 3211−3217.
39. Hoober, K. L., Sheasley, S. S., Gilbert, H. F., and Thorpe, C. (1999) Sulfhydryl oxidase from egg white: a facile catalyst for disulfide bond formation in proteins and peptides, *J. Biol. Chem. 274*, 22147−22150.
40. Farrell, S. R., and Thorpe, C. (2005) Augmenter of liver regeneration: a flavin dependent sulfhydryl oxidase with cytochrome C reductase activity, *Biochemistry 44*, 1532−1541.
41. Wang, W., Winther, J. R., and Thorpe, C. (2007) Erv2p: characterization of the redox behavior of a yeast sulfhydryl oxidase, *Biochemistry 46*, 3246−3254.
42. Trinder, P. (1969) Determination of Blood Glucose Using an Oxidase-Peroxidase System with a Non-Carcinogenic Chromogen, *J. Clin. Pathol. 22*, 158−161.
43. Rebouche, C. J., Wilcox, C. L., and Widness, J. A. (2004) Microanalysis of non-heme iron in animal tissues, *J. Biochem. Biophys. Meth. 58*, 239−251.
44. Swaisgood, H. E., and Horton, H. R. (1980) Sulfhydryl oxidase: oxidation of sulphydryl groups and the formation of three-dimensional structure in proteins., *Ciba Found. Symp. 72*, 205−222.
45. Sliwkowski, M. X., Sliwkowski, M. B., Horton, H. R., and Swaisgood, H. E. (1983) Resolution of sulphydryl oxidase from gamma-glutamyltransferase in bovine milk by covalent chromatography on cysteinylsuccinamidopropyl-glass, *Biochem. J. 209*, 731−739.
46. Olsen, J., and Davis, L. (1976) The oxidation of dithiothreitol by peroxidases and oxygen, *Biochim. Biophys. Acta 445*, 324−329.
47. Swaisgood, H. E., and Abraham, P. (1980) Oxygen Activation by Sulfhydryl Oxidase and the Enzyme's Interation with Peroxidase, *J. Dairy Sci. 63*, 1205−1210.
48. Lonnerdal, B., and Iyer, S. (1995) Lactoferrin: molecular structure and biological function, *Annu. Rev. Nutr. 15*, 93−110.
49. Ward, P. P., Paz, E., and Conneely, O. M. (2005) Multifunctional roles of lactoferrin: a critical overview, *Cell Mol. Life Sci. 62*, 2540−2548.
50. Levay, P. F., and Viljoen, M. (1995) Lactoferrin: a general review, *Haematologica 80*, 252−267.
51. Stumm, W., and Morgan, J. J. (1981) *Aquatic Chemistry*, 2nd ed., John Wiley & Sons Inc., New York.
52. Cotton, F. A., Wilkinson, G. M., Carlos, A., and Bochmann, M. (1999) *Advanced Inorganic Chemistry*, 6th ed., Wiley, New York.
53. Hagihara, B., Ishibashi, F., Sasaki, K., and Kamigawara, Y. (1978) Cellulose-Acetate Coatings for Polarographic Oxygen-Electrode, *Anal. Biochem. 86*, 417−431.
54. Raje, S., and Thorpe, C. (2003) Inter-domain redox communication in flavoenzymes of the quiescin/sulfhydryl oxidase family: role of a thioredoxin domain in disulfide bond formation, *Biochemistry 42*, 4560−4568.
55. Wilcox, C. P., Janolino, V. G., and Swaisgood, H. E. (2002) Isolation and partial characterization of CD36 from skim milk, *J. Dairy Sci. 85*, 1903−1908.
56. Mather, I. H. (2000) A review and proposed nomenclature for major proteins of the milk-fat globule membrane, *J. Dairy Sci. 83*, 203−247.
57. Kitchen, B. J. (1974) A comparison of the properties of membranes isolated from bovine skim milk and cream, *Biochim. Biophys. Acta 356*, 257−269.
58. Stewart, P. S., Puppione, D. L., and Patton, S. (1972) The presence of microvilli and other membrane fragments in the non-fat phase of bovine milk, *Z. Zellforsch. Mikrosk. Anat. 123*, 161−167.
59. Plantz, P. E., Patton, S., and Keenan, T. W. (1973) Further evidence of plasma membrane material in skim milk, *J. Dairy Sci. 56*, 978−983.
60. Isaacs, C. (1984) in *Methods in Human Lactation* (Jensen, R. G., and Neville, M. C., Eds.) pp 277−282, Plenum Press, Winter Park, CO.
61. Mairet-Coello, G., Tury, A., Fellmann, D., Risold, P. Y., and Griffond, B. (2005) Ontogenesis of the sulfhydryl oxidase QSOX expression in rat brain, *J. Comp. Neurol. 484*, 403−417.
62. Chakravarthi, S., Jessop, C. E., Willer, M., Stirling, C. J., and Bulleid, N. J. (2007) Intracellular catalysis of disulphide bond formation by the human sulphydryl oxidase, QSOX1, *Biochem. J. 404*, 403−411.
63. Kelly, V. C., Kuy, S., Palmer, D. J., Xu, Z., Davis, S. R., and Cooper, G. J. (2006) Characterization of bovine seminal plasma by proteomics, *Proteomics 6*, 5826−5833.
64. Mairet-Coello, G., Tury, A., Esnard-Feve, A., Fellmann, D., Risold, P. Y., and Griffond, B. (2004) FAD-linked sulfhydryl oxidase QSOX: topographic, cellular, and subcellular immunolocalization in adult rat central nervous system, *J. Comp. Neurol. 473*, 334−363.
65. Isaacs, C. E., Pascal, T., Wright, C. E., and Gaull, G. E. (1984) Sulfhydryl oxidase in human milk: stability of milk enzymes in the gastrointestinal tract, *Pediatr. Res. 18*, 532−535.
66. Matsuba, S., Suga, Y., Ishidoh, K., Hashimoto, Y., Takamori, K., Kominami, E., Wilhelm, B., Seitz, J., and Ogawa, H. (2002) Sulfhydryl oxidase (SOx) from mouse epidermis: molecular cloning, nucleotide sequence, and expression of recombinant protein in the cultured cells, *J. Dermatol. Sci. 30*, 50−62.
67. Kall, L., Krogh, A., and Sonnhammer, E. L. (2007) Advantages of combined transmembrane topology and signal peptide prediction−the Phobius web server, *Nucleic Acids Res.* W429−432.
68. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Clustal-W - Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice, *Nucleic Acids Res. 22*, 4673−4680.

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