Sulfhydryl Oxidase from Egg White

A FACILE CATALYST FOR DISULFIDE BOND FORMATION IN PROTEINS AND PEPTIDES*  

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Both metalloprotein and flavin-linked sulfhydryl oxidases catalyze the oxidation of thiols to disulfides with the reduction of oxygen to hydrogen peroxide. Despite earlier suggestions for a role in protein disulfide bond formation, these enzymes have received comparatively little general attention. Chicken eggwhite sulfhydryl oxidase utilizes an internal redox-active cystine bridge and a FAD moiety in the oxidation of a range of small molecular weight thiols such as glutathione, cysteine, and dithiothreitol. The oxidase is shown here to exhibit a high catalytic activity toward a range of reduced peptides and proteins including insulin A and B chains, lysozyme, ovalbumin, riboflavin-binding protein, and RNase. Catalytic efficiencies are up to 100-fold higher than for reduced glutathione, with typical $K_m$ values of about 110–330 μM/protein thiol, compared with 20 mM for glutathione. RNase activity is not significantly recovered when the cysteine residues are rapidly oxidized by sulfhydryl oxidase, but activity is efficiently restored when protein disulfide isomerase is also present. Sulfhydryl oxidase can also oxidize reduced protein disulphide isomerase directly. These data show that sulfhydryl oxidase and protein disulfide isomerase can cooperate in vitro in the generation and rearrangement of native disulfide pairings. A possible role for the oxidase in the protein secretory pathway in vivo is discussed.

The mode by which disulfide bonds are introduced during protein secretion in prokaryotes and eukaryotes is under active investigation (see recent reviews in Refs. 1–9). Whereas the role of eukaryotic protein disulfide isomerase (PDI) in shuffling preformed disulfide bridges has been extensively investigated, the mechanism of the net formation of disulfides is less clear. The disulfide bridge of PDI is easily reduced (3, 10, 11) and so could serve as an oxidant in the ER, but the ultimate electron acceptor returning PDI to its oxidized state is not yet apparent. The selective transport of oxidized glutathione (GSSG) into the lumen of the mammalian ER may contribute to protein disulfide biosynthesis (12). However, the ultimate oxidant for this process has yet to be identified, and recent studies have shown that glutathione is not an obligatory participant in disulfide bond formation in yeast (13). Other investigators have suggested that cystamine, generated by a NADPH-dependent microsomal flavoprotein, monooxygenase (14), and vitamin K epoxide (15) could serve as oxidants for protein cysteine residues. Neither of these proposals has as yet gained wide acceptance. Elegant yeast selection schemes have identified a redox-active protein from the ER, ERO1 (13, 16), that appears to be involved in some as yet unidentified aspect of protein disulfide bond formation. Recently, modulation of the redox potential of the yeast ER has been suggested to involve a flavin monooxygenase that may generate GSSG and other small molecular weight disulfides at the outer face of the ER for transport into the lumen (17). Clearly, the issue of the ultimate oxidant(s) for disulfide bridge formation in eukaryotes is receiving renewed attention.

This communication addresses a class of enzymes whose possible role in the protein secretory pathway merits renewed scrutiny. Metalloprotein (18–22) and FAD-linked sulfhydryl oxidases (23–26) are found in a range of tissues and catalyze the oxidation of a variety of small monothiol and dithiol substrates such as cysteine, glutathione, β-mercaptoethanol, and DTT, according to the following stoichiometry: 2 R-SH + O$_2$ → R-S-S-R + H$_2$O$_2$. A role for these enzymes in protein disulfide bond formation has been suggested for many years (18, 22, 23, 25–29) starting with the pioneering work of Swaisgood and co-workers. However, the activities observed in these studies seemed to be rather modest, which may have discouraged the widespread adoption of these proposals.

The present work deals with a sulfhydryl oxidase (26, 29) that is secreted, together with a number of other disulfide-bridged proteins, into the egg white of the chicken. The oxidase is a dimeric glycoprotein of 80-kDa monomers each bearing a noncovalently bound FAD and a redox-active disulfide bridge (26). Here we show, for the first time, that a sulfhydryl oxidase can rapidly and directly introduce disulfide bonds into a wide range of proteins and peptides with catalytic efficiencies about 100-fold higher than for free cysteine or glutathione. Further, these oxidized, misfolded proteins are good substrates for PDI.

EXPERIMENTAL PROCEDURES

Materials—Egg white sulfhydryl oxidase and recombinant rat liver protein disulfide isomerase were purified and assayed as described previously (4, 26). Bovine pancreatic RNase and insulin, egg white lysozyme and ovalbumin, rabbit muscle aldolase and pyruvate kinase, and DTT, DTNB, ultra-pure urea, and guanidine hydrochloride were purchased from Sigma. TCEP was from Pierce. Egg white riboflavin binding protein was a generous gift from Dr. Harold B. White III. The ovalbumin heptapeptide N-(acetyl)-EAQCQTS-carboxyl (83% pure by high pressure liquid chromatography) was from Genosys Biotechnologies, Inc.

Preparation of Reduced Protein—Proteins (20 mg of RNase, lysozyme, ovalbumin, and riboflavin-binding protein) were dissolved in 1-mL aliquots of degassed 100 mM Tris buffer containing 6 M guanidine hydrochloride and 0.3 mM EDTA, followed by the addition of at least a 5-fold excess of DTT over total protein thiols. The solution was incubated at pH 8 for 1 h at 37 °C and then adjusted to pH 3.5 with glacial
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Table I

Comparison of several substrates for the egg white sulfhydryl oxidase

| Protein          | M<sub>r</sub> | pI  | -SH<sup>+</sup> initial | -SH<sup>+</sup> final | T<sub>Nmax</sub><sup>a</sup> | K<sub>m</sub><sup>b</sup> | T<sub>Nmax</sub>/K<sub>m</sub><sup>c</sup> |
|------------------|--------------|-----|-------------------------|----------------------|------------------|----------------|-------------------|
| RNase            | 12,700       | 7.8 | 0                       | 0                    | 8                | 610            | 115 (14)         |
| Lysozyme         | 14,300       | 10.7| 0                       | 0                    | 8                | 860            | 110 (14)         |
| Riboflavin-binding protein | 34,000       | 4.2 | 18                      | 18                   | 1100             | 230 (13)       | 8.0 × 10<sup>4</sup> |
| Ovalbumin        | 45,000       | 4.5 | 0                       | 0                    | 8                | 860            | 110 (14)         |
| Aldolase         | 40,200       | 6.1 | 8                       | 8                    | 475              | 1,250 (140)    | 6.3 × 10<sup>3</sup> |
| Pyruvate kinase<sup>f</sup> | 59,250       | 6.6 | 9                       | 9                    | 475              | 1,250 (140)    | 6.3 × 10<sup>3</sup> |
| Insulin A chain  | 2,340        | 3.8 | 0                       | 0                    | 2                | 700            | 300 (150)        |
| Insulin B chain<sup>f</sup> | 3,400        | 6.9 | 8                       | 8                    | 4                | 700            | 300 (150)        |
| N-acetyl-EAQCGTS | 740          | 4.0 | 1                       | 1                    | 1                | 1420           | 1,720 (14)       |
| GSH<sup>e</sup>  | 300          | 2.8 | 1                       | 1                    | 1                | 1385           | 20,000           |

<sup>a</sup> Number of thiols before reduction.
<sup>b</sup> Total thiols after reduction of any disulfide bonds.
<sup>c</sup> T<sub>Nmax</sub> values are disulfide bonds formed/minute.
<sup>d</sup> K<sub>m</sub> values are expressed both on a per thiol basis and per substrate molecule (shown in parentheses).
<sup>e</sup> TN<sub>max</sub>/K<sub>m</sub> uses thiol (not protein) concentrations.
<sup>f</sup> In 3 M urea to maintain solubility at higher substrate concentrations.

<sup>e</sup> See Ref. 26.

RESULTS

Table I summarizes the physical and kinetic properties for a number of reduced proteins and peptides used as substrates in this study. Although RNase solutions are easily handled after denaturation and reduction, many other reduced proteins aggregate severely when the denaturant is removed. For consistency, a standard procedure was adopted to reduce each protein substrate. The denatured, reduced protein was rigorously freed of DTT by gel filtration in 8 M urea containing 0.1% v/v acetic acid and 3 mM EDTA. Reduced denatured proteins were stored under nitrogen and were standardized for thiol content by DTNB.

Insulin (30 mg) was suspended in 3 ml of 50 mM Tris buffer, pH 7.6, containing 1 mM EDTA and dissolved upon the addition of a minimal volume of 1 M HCl. After adding 15 mM TCEP, the pH was adjusted to 3.8 with KOH, and the clear solution was flushed with nitrogen. Reduced A and B chains precipitated during overnight incubation at 20 °C and were recovered by centrifugation (4 min at 12,000 × g). The pellet was resuspended in 3 ml of 8 M urea containing 25 mM Tris buffer, 1 mM EDTA and dissolved after the pH was adjusted to 8.0 with KOH. The solution was applied to a 0.5 × 5-cm DE-52 column equilibrated with this same buffer. The reduced insulin B chain emerged unretained in the wash. The A chain was eluted with the same buffer containing 0.08 M sodium acetate well separated from excess phosphate reductant.

Aldolase and pyruvate kinase were dissolved in 8 M urea containing 100 mM potassium phosphate, 0.3 mM EDTA, pH 7.5, prior to use.

Lysozyme, a highly basic protein containing 84% of the total (see “Experimental Procedures”), was oxidized by sulfhydryl oxidase. Reduced lysozyme in 8 M urea (see “Experimental Procedures”) was diluted to a final concentration of 12.2 μM in 1.2 ml of 2 M urea in 100 mM potassium phosphate, pH 7.5, containing 1 mM EDTA at 25 °C in the absence (○) or presence of 34 mM sulfhydryl oxidase (●). Samples were withdrawn and assayed in duplicate for thiol content under denaturing conditions as described under “Experimental Procedures.” The inset plots turnover numbers obtained from tangents to progress curves obtained using up to 460 μM total lysozyme thiols. The solid line is fit to a maximal turnover number of 860 disulfide bonds formed/minute with a K<sub>m</sub> of 110 μM protein thiol. The K<sub>m</sub> expressed in terms of lysozyme molecules would be 14 μM.

In view of the refractility of ovalbumin to complete oxidation, we tested the oxidase using proteins without naturally occurring disulfide bridges. Aldolase and pyruvate kinase are abundant cytoplasmic proteins with eight and nine cysteine residues, respectively, in approximately 30% of the total, and may reflect the progressive difficulties of inserting additional disulfides into an ever more constrained substrate.

The inset in Fig. 1 plots initial turnover numbers (expressed as disulfide bonds generated per minute) as the lysozyme thiol concentration is raised. The K<sub>m</sub>/thiol is 110 μM (14 μM/lysozyme molecule), markedly lower than the corresponding values for glutathione (20 mM) (26)). Riboflavin-binding protein, a strongly acidic protein with nine disulfide bridges, accounts for about 0.8% of the egg white. The reduced denatured riboflavin-binding protein was freed from both excess DTT and bound riboflavin by gel filtration and was found to be an excellent substrate of the oxidase. Oxidation of cysteine residues was essentially complete (less than 2 of the 18 cysteine residues in 10 μM reduced aporiboflavin-binding protein remain after 16 min using 100 nM oxidase; not shown).

Initial rates yield a turnover number of 1100/min with an apparent K<sub>m</sub> of 290 μM/thiol. Ovalbumin, the dominant egg white protein (54% of the total), is unusual in that one native disulfide coexists with four free cysteine residues (most secreted proteins do not have free cysteine residues (Table I)). Prolonged incubation of the oxidase with reduced ovalbumin consistently led to the decrease of 1.8 of 6 total thiols. This failure to oxidize the approximately four remaining thiols is consistent with a native structure for reoxidized ovalbumin.

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The amount of GSSG directly produced by the oxidase in the absence of GSH (30) leads to a marked increase in the regeneration of active RNase. The amount of GSSG directly produced by the oxidase in the initial stages of the assay (<0.02 mM) is insufficient to support significant nonenzymatic RNase oxidation. In any event, Table I shows that RNase thiols would be considerably better substrates of the oxidase than GSH under these conditions.

Fig. 2B shows that sulfhydryl oxidase can oxidize reduced PDI directly. Reduced PDI is assayed by the ability of its redox-active dithiols to isomerize scrambled RNase in the absence of GSH (30). Hence, the decline in PDI activity observed in Fig. 2B, when reduced PDI is incubated with increasing concentration of the oxidase in the absence of GSH, reflects the conversion of PDI into its inactive oxidized form.

**Discussion**

Egg white sulfhydryl oxidase is a facile, versatile, and direct catalyst for the in vitro formation of disulfide bridges within peptides and proteins. There seem to be no obvious restrictions as to molecular weight or pI for the substrates tested (Table I). The oxidase can even oxidize unfolded cytoplasmic proteins without native disulfide bridges. Secreted proteins with two or more thiols seem to be the best substrates of the oxidase, with typical Km values of 110–330 μM/thiol (Table I) compared with values of 1.7 mM for the monothiol ovalbumin heptapeptide, 20 mM for GSH, and 50 mM for β-mercaptoethanol (26). The high Km and the low catalytic efficiency of glutathione (e.g., 100-fold lower than for lysozyme thiols; Table I) suggest that this abundant cellular reductant is not likely to be a primary substrate of the enzyme. The GSH concentration in the ER has been estimated as 0.5–1 mM (12), a value far lower than the Km of 20 mM for this sulfhydryl oxidase. However, GSH might serve as a co-substrate of the oxidase, with the generation of mixed disulfide bridges as a first step in the oxidative process in vivo.

The conditions of these in vitro experiments, with dilute solutions of reduced proteins in 2 or 3 M urea, are far from the crowded environment of the ER, in which co- and post-translational disulfide bridge formations (31–35) are supported by a wealth of folding factors and ancillary proteins (1, 3). Fig. 2 supports a potential cooperation between sulfhydryl oxidase and PDI in the ER. Oxidase-treated reduced RNase is not only a good substrate of PDI, but the oxidase can also oxidize reduced PDI directly. Thus, the sulfhydryl oxidase-catalyzed oxidation of nascent chains in the ER could occur via direct interaction or through the mediation of PDI.

Clearly, the current experiments do not definitively prove that this FAD-linked sulfhydryl oxidase is directly involved in the maturation of secreted proteins. Indeed disulfide bond formation in eukaryotes may involve multiple pathways and redundancies. However, the present experiments do show, apparently for the first time, that this FAD-linked enzyme can oxidize a wide range of protein and peptide substrates with catalytic efficiencies up to 100-fold higher than glutathione. It is important to note that oxidation is direct and does not require the mediation of small molecular disulfides such as GSSG. If sulfhydryl oxidases are not involved in the generation of protein disulfide bridges, what is the physiological role of these widely distributed metalloprotein and flavin-linked catalysts (18–26)?

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