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SKIN SULFHYDRYL OXIDASE

PURIFICATION AND SOME PROPERTIES *

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Summary

A sulfhydryl-oxidizing enzyme has been found in skin of young rats and a method for purifying the enzyme over 600-fold has been developed. Enzymatic activity was assayed either by its ability to oxidize dithiothreitol or by measuring its ability to renature reductively denatured ribonuclease A.

Skin sulfhydryl oxidase catalyzed the oxidation of various thiols: dithiothreitol, dithioerythritol, D-penicillamine, and L-cysteine. Glutathione and 2-mercaptoethanol were very poor substrates for the enzyme. The enzyme also reactivated reductively denatured ribonuclease A, with neither the presence of a thiol nor prior reduction of the enzyme being necessary.

The molecular weight of the enzyme was estimated to be 66 000 ± 2000, and the isoelectric point was determined to be at pH 4.65. Alkylating reagents alone had some inhibiting effect on skin sulfhydryl oxidase; when the enzyme was preincubated with thiols which were substrates, inhibition by alkylating reagents was greatly increased. After preincubation with dithiothreitol, treatment of the enzyme with alkylating reagents or *N*-ethylmaleimide caused significant inhibition; preincubation with a poor substrate, reduced glutathione, did not enhance inhibition by alkylating reagents or *N*-ethylmaleimide.

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Introduction

Disulfide bonds are present in most classes of extracellular proteins including degradative enzymes, polypeptide hormones, immunoglobulins, milk proteins and in the intracellular keratins. Of particular interest to our laboratory is the fact that disulfide formation is critical to normal hair structure, epidermal structure and function. Hair is composed of cortical cells which contain two groups of proteins, matrix proteins and filamentous proteins, both of which are covalently linked via their disulfide bonds. Sulfhydryl abnormalities of hair in copper deficient sheep [1], decreased sulfhydryl bond formation in Menkes disease [2], and the loss of curl and sulfur-rich high cystine proteins in human dietary protein deficiency [3] have led us to study keratinized tissue for disulfide bond-forming enzymes.

A variety of sulfhydryl-oxidizing and disulfide bond-forming enzymes have been reported. Freedman and Hawkins [4] discussed the similarities and differences of two groups of enzymes, known as the protein disulfide-isomerases and the glutathione-insulin transhydrogenases (glutathione:protein-disulfide oxidoreductase, EC 1.8.4.2), which can catalyze intramolecular disulfide interchange. These enzymes required either prior reduction of the enzyme or the presence of a thiol substrate before disulfide interchange of another cosubstrate (generally a protein such as denatured ribonuclease A or insulin) could take place.

Sulfhydryl-oxidizing enzymes have been described which are specific for sulfhydryl compounds, and which do not require cosubstrates for activity [5—8]. These enzymes have many substrates in common with the sulfhydryl-disulfide interchange enzymes, especially among the small thiols such as glutathione, L-cysteine, 2-mercaptoethanol and others. Reductively denatured ribonuclease A is another protein-thiol substrate common to both interchange and sulfhydryl oxidizing enzymes. Whereas the interchange enzymes require the presence of a small thiol or prior reduction of the enzyme in order to react with the protein substrates, the sulfhydryl-oxidizing enzymes can act independently on either small thiol or thiol-protein substrates.

This paper describes a sulfhydryl oxidase distinct in its catalytic properties, its reactivity and its substrate specificity from previously reported sulfhydryl-oxidizing enzymes.

Materials

Male Lewis rats (150—200 g) were obtained from Microbiological Associates (Bethesda, MD). DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (Clifton, NJ); CM Sephadex C-50 and Sephadex G-75 (Superfine) from Pharmacia (Uppsala, Sweden); bovine ribonuclease A (64 Kunitz units/mg), 5,5′-dithiobis-(2-nitrobenzoic acid), bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c*, iodoacetamide and iodoacetic acid were obtained from Sigma (St. Louis, MO); *N*-ethylmaleimide from Aldrich (Milwaukee, WI); dithiothreitol from Bachman Feinchemikalien (Liestal, Switzerland); 2-mercaptoethanol from Eastman Kodak (Rochester, NY); ethylene diamine tetraacetic acid from MCB (Norwood, OH); and yeast

ribonucleic acid was purchased from Worthington (Freehold, NJ). All other reagents were of the highest quality commercially available.

Methods

Preparation of enzyme. Rats were killed with ether, hair was plucked from the lower back and the skin was removed. Subcutaneous tissues were scraped away, the skin was weighed and minced finely with scissors. The minced skin was suspended in nine parts (w:v) 50 mM sodium phosphate/1 mM EDTA, pH 7.6, and the suspension was homogenized with a Polytron (Brinkmann Instruments) for three 30 s bursts, with a 30 s cooling interval between bursts. The homogenate was centrifuged at 27 000 × *g* for 30 min at 4°C. The supernatant was decanted, defatted and recentrifuged as above. The final supernatant was stored frozen at −20°C. These and all further procedures were done at 4°C unless otherwise stated.

Assay of enzymatic activity. Enzymatic activity was determined by measuring the loss of sulfhydryl groups of a substrate by the method of Ellman [9]. In a typical assay, the reaction mixture contained 0.1 ml of 2 mM dithiothreitol, 0.7 ml of 50 mM sodium phosphate with 1 mM EDTA, pH 7.6, and 0.4 ml of enzyme solution. Control incubations replaced the enzyme with an equal volume of sodium phosphate buffer. Reaction mixtures were incubated at 37°C in a shaking water bath and 0.3 ml samples were removed at 0, 30 and 60 min. The 0.3 ml samples were added to tubes containing 3.0 ml of 0.17 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 50 mM sodium phosphate and 1 mM EDTA, pH 7.6. The absorbance of these solutions was measured at 412 nm using a Gilford spectrophotometer model 250. The differences in $A_{412\text{nm}}$ between 0 and 30 min, and 0 and 60 min were taken as the rate of sulfhydryl oxidation. The concentration of remaining sulfhydryl groups was calculated using a molar absorptivity of 5,5′-dithiobis(2-nitrobenzoic acid) of 13 000 $M^{-1} \cdot cm^{-1}$. The activity was expressed as nmol reduced 5,5′-dithiobis(2-nitrobenzoic acid) produced per μg protein per 30 and 60 min.

Assay of ribonuclease A activity. 0.1 mg ribonuclease A (RNAase A) was dissolved in 2.0 ml water, then further diluted to a concentration of 20 μg/ml with 0.1 M sodium acetate buffer, pH 5.0. The substrate used in this assay was 1% yeast RNA in 0.1 M sodium acetate buffer, pH 5.0. Various concentrations of RNAase A, between 0–12 μg/ml in acetate buffer, were preincubated at 37°C for 3 min. Then, 1.0 ml 1% yeast RNA was added to each tube, and the incubation continued at 37°C. After 4 min, the reaction was stopped by the addition of 1.0 ml ice-cold 0.75% uranyl acetate in 25% perchloric acid. The tubes were vortexed and centrifuged at 2000 rev./min in a Sorval GLC tabletop centrifuge for 5 min at 4°C. 0.1 ml of each resulting supernatant was mixed with 3.0 ml deionized water and absorbance at 260 nm was measured using a Gilford model 250 spectrophotometer. Regular assay controls included a uranyl acetate blank, 2 μg RNAase A alone, 2 μg RNAase A plus yeast RNA, and tubes with yeast RNA alone [10].

Preparation of denatured ribonuclease A. RNAase A was reduced with 2-mercaptoethanol in the presence of 8 M urea using a modification of a procedure described by Anfinsen and Haber [11]. 30 mg RNAase A were dissolved

in 3.0 ml 8 M urea which had been filtered through a Whatman No. 1 filter and passed through a mixed bed ion exchange column. This solution was adjusted to pH 8.6 with 5% methylamine and 1 μl of 2-mercaptoethanol per mg RNAase A was added. The tube containing RNAase A, urea and 2-mercaptoethanol was then flushed with nitrogen, sealed with parafilm and incubated at room temperature for 4.5 h. At that time, the pH was adjusted to 3.5 with glacial acetic acid and the solution was chromatographed on a Sephadex G-10 column (3 X 25 cm) equilibrated in 0.1 M acetic acid. The tubes containing RNAase A were pooled and diluted with 0.1 M acetic acid, if necessary, to an A_{280} of 0.35—0.40 (approx. 600—660 μg/ml) and frozen in 3.0 ml fractions.

Reactivation of denatured ribonuclease A. Reactivation of denatured RNAase A was measured using the method of Haber and Anfinsen [12]. Frozen-denatured RNAase A (600—660 μg/ml) was thawed at room temperature and just before assay the pH was adjusted to 7.0 with a saturated solution of Tris-HCl. Each assay tube contained 1.0 ml denatured RNAase A and various amounts of sulfhydryl oxidase, with the volume balanced with buffer to a final volume of 2.0 ml. Controls were denatured RNAase A alone and sulfhydryl oxidase alone. These tubes were incubated at room temperature (23—25°C). At 0, 30 and 60 min, 0.1 ml aliquots from each tube were removed for the RNAase A activity assay. RNAase A activity measuring the digestion of yeast RNA was expressed in terms of acid-soluble oligonucleotides liberated per min, measured at 260 nm. Renaturation of RNAase A, alone or in the presence of sulfhydryl oxidase, was assessed by comparing the RNAase A activity measured at the above sampling intervals. RNAase A reactivation was also followed by determining the decrease of the free sulfhydryl groups which accompanied reactivation; the free sulfhydryl groups were measured using Ellman's reagent as described above.

Preliminary characterization studies. Experiments were done with crude skin sulfhydryl oxidase, using the assay methods described above, in order to determine the conditions under which the enzyme most effectively operated. Enzymatic activity was measured via the oxidation of dithiothreitol at 10 pH values between 6.0 and 10.0. pH values were controlled using phosphate or sodium glycinate and all pH values were corrected to pH 9.0 in the Ellman's reaction portion of the assay. In temperature studies, aliquots of a crude supernatant were studied in quadruplicate for activity at 4, 20, 25, 37, 45, and 50°C. Enzyme localization studies were done by separating epidermis from dermis by standard means (heating to 56°C for 30 s, then chilling on ice, or immersion in 2 M KBr for 15 min at 37°C). Cell fractionation studies were done using a variation of the method of Goldberger et al. [13] in order to locate the enzyme within the cell.

Purification of enzyme. The frozen 27 000 X g supernatant was thawed at room temperature and centrifuged at 27 000 X g for 30 min. This supernatant was concentrated 8.3-fold by the use of an Amicon PM-10 filter and was again frozen, thawed and centrifuged as above. Solid $(NH_4)_2SO_4$ was added over 1 h to 30% saturation (0.164 g/ml). Stirring continued for another hour, and the suspension was centrifuged at 12 000 X g for 10 min. The $(NH_4)_2SO_4$ concentration of the supernatant was increased to 60% saturation (0.181 g/ml) and the suspension was centrifuged at 27 000 X g for 30 min. The pellet from the

second centrifugation was dissolved in 1 mM sodium phosphate buffer, pH 7.6, and dialyzed against several changes of the same buffer for 2 days. A small precipitate was removed by centrifugation at 27 000 × *g* for 30 min.

The supernatant was chromatographed on a DEAE-cellulose column (2.5 × 14 cm) previously equilibrated in 1 mM sodium phosphate buffer, pH 7.6, and eluted in the same buffer. Tubes with enzymatic activity were pooled and concentrated by ultrafiltration using an Amicon PM-10 filter. The enzyme concentrate was dialyzed overnight against 5 mM sodium phosphate buffer containing 1 mM EDTA, pH 7.0, and a small precipitate was removed by centrifugation at 27 000 × *g* for 30 min. The supernatant was applied to a CM-Sephadex C-50 column (1.0 × 15 cm) equilibrated with the above buffer. The column was washed with equilibrating buffer and was eluted with a 400 ml linear gradient of sodium phosphate (5—200 mM) containing 1 mM EDTA, pH 7.0 (Fig. 1). The tubes with enzymatic activity were concentrated on an Amicon PM-10 and the solution was dialyzed overnight against 5 mM sodium phosphate containing 1 mM EDTA, pH 6.0, and centrifuged at 27 000 × *g* for 30 min. This supernatant was applied to a CM-cellulose column (1.0 × 20 cm) equilibrated with 5 mM sodium phosphate/1 mM EDTA, pH 6.0. The column was washed with equilibrating buffer and then eluted with a 400 ml linear gradient of sodium phosphate (5—200 mM) with 1 mM EDTA, pH 6.0. The peak tubes were concentrated to 2.0 ml and centrifuged at 27 000 × *g* for 30 min. The enzyme solution was chromatographed on a column (2.5 × 80 cm) of Sephadex G-75 in 50 mM sodium phosphate/1 mM EDTA, pH 7.6. The typical elution pattern obtained is shown in Fig. 2.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed with a 7.5% gel at pH 8.9 according to the method of Davis [14]. Polyacrylamide gel electrophoresis with SDS was performed to estimate the molecular weight and purity of the enzyme sample by the method of Laemli [15]. Samples were incubated at 100°C for 2 min in 2% SDS and 5% 2-mercaptoethanol before electrophoresis. Electrophoresis was performed with Tris-glycine buffer using a 7.5% polyacrylamide gel containing 0.1% SDS and gels were stained with 0.25% Coomassie brilliant blue. Bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome *c* were used as molecular weight standards.

Protein determination. Protein was measured according to the method of Lowry et al. [16] with bovine serum albumin as a standard.

Isoelectric focusing. Isoelectric focusing was done in an LKB 8101 column (110 ml capacity) according to the manufacturer's instructions using 1% carrier ampholine (pH 3.5—10.0). Electrophoresis was started at 1200 volts and was continued for 22 h at 1620 V. The solution in the column was then fractionated with a fraction size of 2 ml, followed by determination of pH and enzymatic activity.

Heat stability studies. Aliquots of pure enzyme in 50 mM sodium phosphate buffer, pH 7.6, were incubated in sealed tubes for 0, 15, 30 and 60 min at 60°C and rapidly cooled in an ice bath. Similar incubations at 60°C were performed in the presence of 1 mM dithiothreitol or reduced glutathione. Then, each sample was dialyzed overnight at 4°C against 50 mM sodium phosphate buffer, pH 7.6, and assayed for enzyme activity.

Thiol substrates. A variety of thiols were tested using the basic low molecular weight thiol oxidation assay described above: dithiothreitol was replaced by 2 mM concentrations of dithioerythritol, penicillamine, cysteine, 2-mercaptoethanol, reduced glutathione, α-methylcysteine and *N*-acetylcysteine, and reactions were run at 37°C in 50 mM sodium phosphate/1 mM EDTA, pH 7.6.

Effect of sulfhydryl inhibitors. Purified enzyme was preincubated in 50 mM sodium phosphate, pH 7.6, with or without 1 mM dithiothreitol or reduced glutathione, in sealed tubes at 37°C for 15 min. Then, *N*-ethylmaleimide, iodoacetamide, or iodoacetic acid was added to a final concentration of 4 mM and the reaction was incubated for 60 min at 37°C. At that time, reduced glutathione (20 mM final concentration) was added to stop the reaction. These reaction mixtures were dialyzed exhaustively overnight at 4°C against 50 mM sodium phosphate buffer, pH 7.6. The resulting solutions were assayed for enzymatic activity.

Results

Assay of enzymatic activity. Enzymatic activity was shown to be linear with respect to the time of incubation and the amount of enzyme added to the assay medium.

Characterization studies. Early studies done with crude sulfhydryl oxidase showed a sharp pH optimum at pH 8.2. Comparable results were obtained at overlapping pH values of the two buffer systems. Denatured RNAase A reactivation was not catalyzed at pH 6.5 or 9.5, but was catalyzed at pH 8.0. Temperature studies using the lower molecular weight thiol oxidation assay (dithiothreitol/5,5′-dithiobis(2-nitrobenzoic acid)) showed a linear increase in activity up to 45°C with enzyme activity decreasing rapidly thereafter; there was no activity at 4°C. After separating dermis from epidermis, significant enzymatic activity was found in both layers of skin.

Heat separation of new born epidermal skin produced epidermis free of dermis as determined histologically. The dermis contained many hair follicles. The sulfhydryl oxidation (A_{412}/30 min per mg protein) was 0.073 and 0.1080 for the epidermis and dermis, respectively. The RNAase renaturation (A_{260}/30 min per mg protein) was 0.009 and 0.012 for epidermis and dermis, respectively. When adult-rat whole skin was separated using KBr the levels of sulfhydryl oxidation (A_{412}/30 min per mg protein) were 0.102, 0.120 and 0.114 for untreated whole skin, KBr-separated epidermis and KBr-separated dermis, respectively. The RNAase renaturation (A_{260}/30 min per mg protein) was 0.024, 0.021 and 0.033 for whole skin, KBr-separated epidermis and KBr-separated dermis, respectively. The KBr treatment produced complete dermal-epidermal separation by histological examination. The specific activity of the enzyme was higher in dermis than epidermis, using both the dithiothreitol/5,5′-dithiobis(2-nitrobenzoic acid) assay and the denatured RNAase A renaturation system. Cell fractionation studies showed the enzyme to be located almost exclusively in the cytosol. In a sample experiment in the 17 000 × *g* supernatant and in the 105 000 × *g* supernatant there was enzymatic activity and none in the 17 000 × *g* pellet or the 105 000 × *g* pellet. Activity in the RNAase

renaturation assay (A_{260}/mg per 30 min) and in the 5,5′-dithiobis(2-nitrobenzoic acid) assay (A_{412}/mg per 30 min) was in the 17 000 × *g* supernatant 0.010 and 0.111, respectively, and in the 100 000 × *g* supernatant 0.009 and 0.095, respectively.

Enzyme isolation. The purification steps and yields are summarized in Table I. The preparation in the final step showed an overall 590-fold purification with a recovery of 6% of the original activity based on the 5,5′-dithiobis-(2-nitrobenzoic acid) assay. There was a 90-fold purification in terms of RNAase reactivation: initial activity 0.010 A_{260}/30 min per mg, final 0.905 A_{260}/30 min per mg. Although more than 50% of enzymatic activity was lost with the 30—60% $(NH_4)_2SO_4$ precipitation procedure, it was an essential step in order to get the highest specific activity and a single band on SDS-electrophoresis.

The enzyme was not bound to DEAE-cellulose at pH 7.6, and was eluted with the equilibrating buffer, 1 mM sodium phosphate, pH 7.6. More than 50% of the enzyme activity eluted from CM Sephadex A-50 between 40—60 mM sodium phosphate (Fig. 1) in a single peak, resulting in about a 5-fold purification. A typical Sephadex G-75 gel filtration pattern of the final step in enzyme purification and a polyacrylamide gel electrophoresis pattern of purified skin sulfhydryl oxidase in SDS are shown in Fig. 2. The purified enzyme was stable for at least 2 weeks at 4°C. The molecular weight of the enzyme was estimated to be 66 000 ± 2000, by comparison with several marker proteins, using SDS electrophoresis (Fig. 2). This value was consistent with the molecular weight of skin sulfhydryl oxidase estimated by gel filtration. The isoelectric point was determined to be pH 4.65 by isoelectric focusing and the measuring of enzymatic activity. There was no obvious color associated with the purified protein.

Reactivation of denatured ribonuclease A by skin sulfhydryl oxidase. Reactivation of denatured RNAase A by this enzyme was studied in order to determine whether this enzyme can form disulfide bonds in a protein. As illustrated

TABLE I

PURIFICATION OF SULFHYDRYL OXIDASE FROM RAT SKIN

Enzyme activity: nmol reduced 5,5′-dithiobis(2-nitrobenzoic acid) per 1 ml for 60 min, at 37°C. The homogenated supernatant for step 1 was obtained from 300 g rat skin.

Purification step	Volume (ml)	Total protein (ml)	Total enzymatic activity	Specific activity	Purification	Yield
1. PM-10 Amicon concentrated homogenate supernatant	360	4705	76 320	16.2	1.0	100
2. $(NH_4)_2SO_4$ fractionation	40	1500	36 640	24.4	1.5	48
3. DEAE-cellulose chromatography	15	92.3	27 475	291.4	18.0	36
4. CM Sephadex chromatography	12.5	8.8	15 264	1535.4	94.8	20
5. CM cellulose chromatography	8.0	3.2	9 158	2577.1	159.0	12
6. Sephadex G-75 gel	6.2	0.4	4 579	9515.2	587.4	6

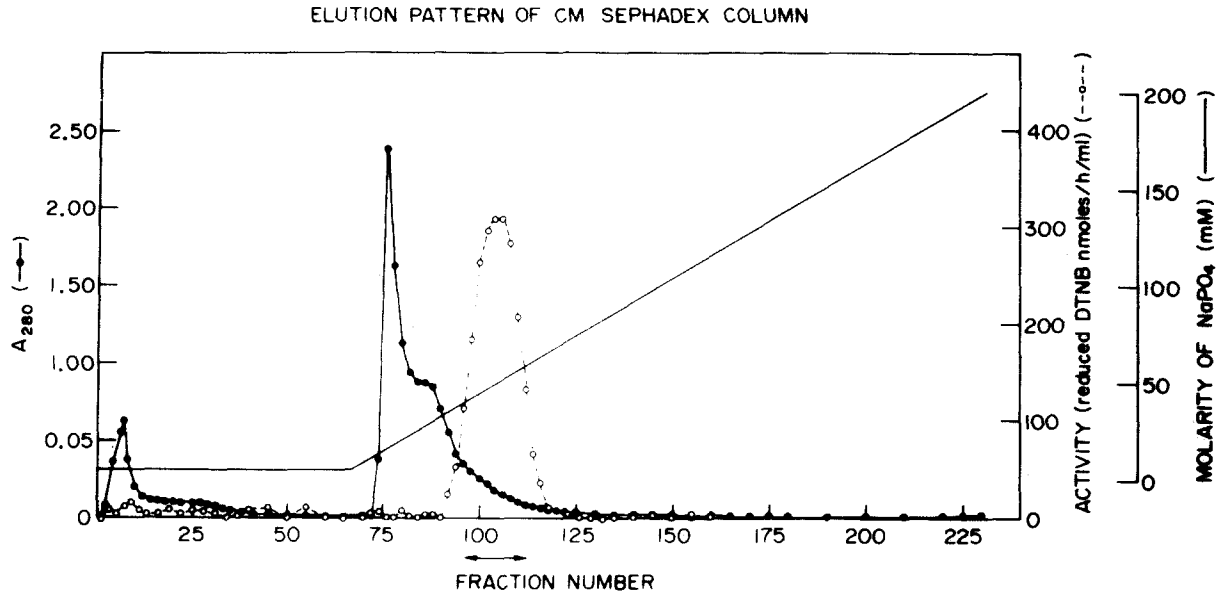

Fig. 1. Elution profile on CM Sephadex C-50 of a skin sulfhydryl oxidase fraction from a DEAE-cellulose column. The column (1.0 X 15 cm) was equilibrated with 5 mM sodium phosphate containing 1 mM EDTA, pH 7.0, at 4°C. 92.3 mg protein in 15 ml was applied and eluted with a linear gradient of 5—200 mM sodium phosphate containing 1 mM EDTA, pH 7.0. Fractions of 2.5 ml were collected at a rate of 15 ml/h and 0.1 ml of each fraction was assayed for enzyme activity at pH 7.6 as described under Methods. Fractions, 96—113, were combined for subsequent treatment. DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

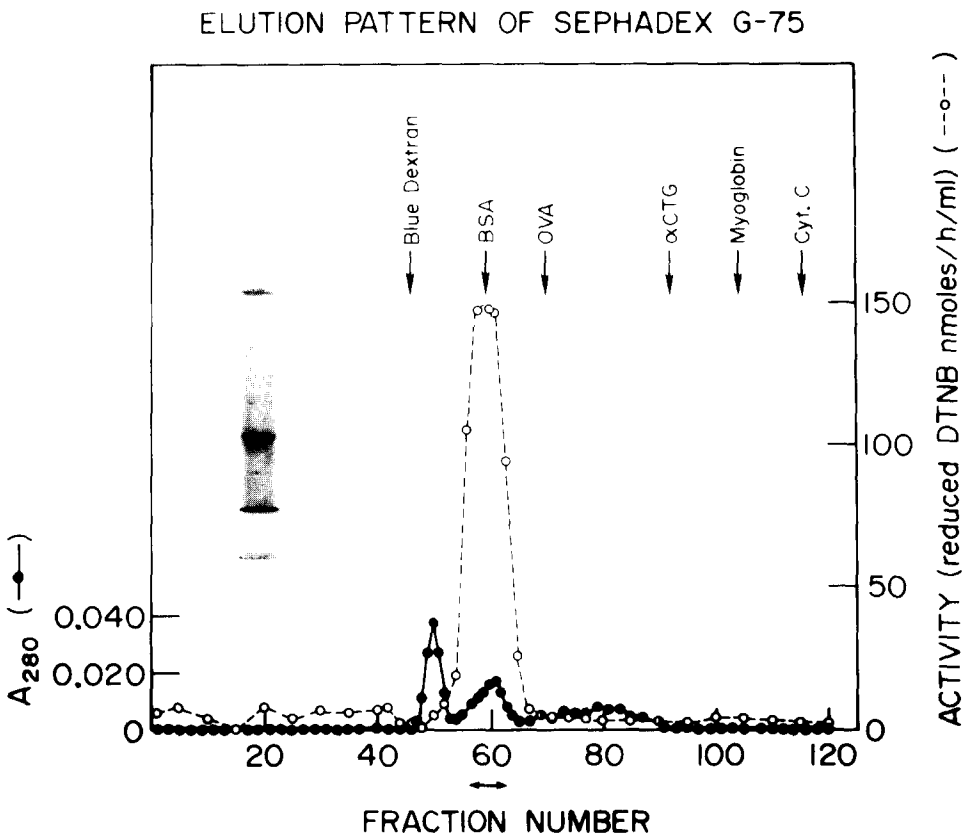

Fig. 2. Gel filtration of skin sulfhydryl oxidase on Sephadex G-75 and polyacrylamide gel electrophoresis of purified skin sulfhydryl oxidase. The column (2.5 X 80 cm) was equilibrated with 50 mM sodium phosphate buffer containing 1 mM EDTA, pH 7.6. After purification by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose, CM Sephadex C-50 and CM-cellulose, the sample was applied. Fractions of 2.5 ml were collected and 0.10 ml of each fraction was assayed for enzyme activity as described under Methods. Purified enzyme samples were incubated at 100°C for 2 min in 0.07 M Tris-HCl buffer (pH 6.8), 2% SDS, 5% 2-mercaptoethanol and run in SDS-polyacrylamide (7.5%) gels as described under Methods. The gels were stained for protein with Coomassie brilliant blue.

in Fig. 3, the reactivation of denatured RNAase A by skin sulfhydryl oxidase was proportional to the time of incubation and the degree of reactivation was dependent on the amounts of the skin enzyme added.

Thiol substrate specificity. In an effort to determine the substrate specificity of skin sulfhydryl oxidase, various thiol-containing compounds were examined with the dithiothreitol/5,5'-dithiobis(2-nitrobenzoic acid) assay method described in Methods, using various substrates. Dithiothreitol and its isomer dithioerythritol were the substrates most efficiently oxidized; D-penicillamine, L-cysteine, *N*-acetyl cysteine, 2-mercaptoethanol and reduced glutathione all had less than 33% relative efficiency at 0.17 mM (Table II). Glutathione, which is a cosubstrate of protein disulfide-isomerase and glutathione-insulin transhydrogenase [17], was a very poor substrate for skin sulfhydryl oxidase, as was 2-mercaptoethanol.

Effect of thiols on skin sulfhydryl oxidase. Purified enzyme was preincu-

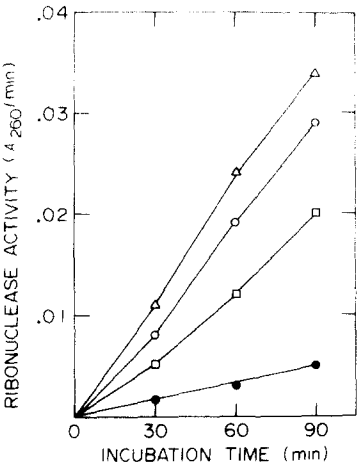

Fig. 3. Reactivation of denatured ribonuclease by skin sulfhydryl oxidase. 20 μg inactive, denatured ribonuclease was incubated in assay buffer at 25°C in the absence of skin sulfhydryl oxidase (●———●) and also in the presence of 2.8 μg (□———□), 5.7 μg (○———○) or 8.5 μg (△———△) purified enzyme. Aliquots were removed at the times indicated and assayed for ribonuclease activity as described in Methods.

bated with 1 mM concentrations of various thiols in the presence of 1 mM EDTA and, after dialysis, enzyme activities were measured using dithiothreitol as substrate as described in Methods. The results are shown in Table III. Dithiothreitol and dithioerythritol, both good substrates of skin sulfhydryl oxidase, caused a 34 and 31% decrease, respectively, in enzyme activity; 2-mercaptoethanol and reduced glutathione did not inhibit enzyme activity at all. The potency of inhibition was in this order: dithiothreitol = dithioerythritol > D-penicillamine > *N*-acetyl cysteine. The most potent inhibitors were the best substrates under assay conditions.

Reaction of essential groups of enzyme with various reagents. To determine if the enzyme had residue(s) essential for enzymatic activity, purified sulfhydryl oxidase was treated with 1 mM dithiothreitol or 1 mM reduced gluta-

TABLE II

THIOL SUBSTRATE SPECIFICITY OF SKIN SULFHYDRYL OXIDASE

Thiol concentration was 0.17 mM in each case. Enzymatic activities were measured in 50 mM sodium phosphate with 1 mM EDTA (pH 7.6) and 0.17 mM various thiol compounds at 37°C for 60 min as described in Methods.

Thiol	Enzymatic activity (reduced 5,5′-dithiobis(2-nitrobenzoic acid) nmol/h per μg protein)	Relative activity (%)
Dithiothreitol	132	100
Dithioerythritol	132.7	101
D-Penicillamine	43.6	33
L-Cysteine	22.3	17
N-Acetylcysteine	6.1	4.6
2-Mercaptoethanol	4.8	3.7
Glutathione	0.98	0.7

TABLE III

EFFECT OF THIOL ON SKIN SULFHYDRYL OXIDASE

Purified skin sulfhydryl oxidase was incubated with 1 mM various thiol containing compounds in 50 mM sodium phosphate buffer with 1 mM EDTA, pH 7.6, at 37°C. After 15 min, samples were cooled in an ice-bath and the reagents were removed by dialysis overnight against 50 mM sodium phosphate containing 1 mM EDTA, pH 7.6, at 4°C. The resulting solutions were assyed for enzymatic activity as described in Methods.

Thiol (1 mM)	Enzymatic activity (reduced 5,5′-dithiobis(2-nitrobenzoic acid) nmol/h per μg protein)	Relative activity (%)
None	33.0	100
Dithiothreitol	21.7	66
Dithioerythritol	22.6	69
D-Penicillamine	25.6	78
L-Cysteine	29.6	90
N-Acetylcysteine	31.7	96
2-Mercaptoethanol	33.3	101
Glutathione	33.2	100

thione prior to incubation with iodoacetamide, iodoacetic acid and *N*-ethylmaleimide. The results are summarized in Table IV. EDTA was not present during the preincubations. Enzyme activity was unchanged after treatment with dithiothreitol or reduced glutathione alone. Iodoacetamide and iodoacetic acid moderately decreased enzyme activity, while *N*-ethylmaleimide had almost no effect. When enzyme was preincubated with dithiothreitol and then treated with these same reagents, inhibition was increased; pretreatment with reduced glutathione did not affect the reactivity of the group(s) of the enzyme with these reagents. These results suggest that there is an essential residue(s), possibly at the enzyme's active site and the reactivity of the group(s) is increased by a substrate induced change in this residue (Table V).

Effect of EDTA on skin sulfhydryl oxidase. Experiments were performed to determine if the enzyme has an EDTA-chelatable metal necessary for its activity. The enzyme used in this experiment was prepared using 50 mM sodium phosphate buffer, pH 7.6, without 1 mM EDTA in the final step of the purification. The enzyme was treated with or without 1 mM dithiothreitol prior to treatment with EDTA and was then dialyzed; activity was assayed as described under Methods. In both cases, skin sulfhydryl oxidase was unaffected by 20 mM EDTA. This finding suggests that the enzyme does not require a loosely bound EDTA-chelatable metal for activity.

Effect of detergent on skin sulfhydryl oxidase. In order to determine the differences between other disulfide forming enzymes and skin sulfhydryl oxidase, the effects of Triton X-100 and deoxycholate were investigated. Enzyme activity was assayed in the presence of various concentrations of Triton X-100 or deoxycholate. The results showed that skin sulfhydryl oxidase was activated by both detergents: with Triton X-100, at 0—0.1% concentration there was at most 30% activation; with deoxycholate, at 0—0.1% concentration, there was at most 20% activation above control levels.

Heat stability of the enzyme. Heat stability of skin sulfhydryl oxidase at 60°C was determined as function of the time of heat treatment. The crude

TABLE IV

EFFECT OF THIOL ON THE REACTIVITY OF SKIN SULFHYDRYL OXIDASE WITH ALKYLATING REAGENTS

The final concentrations of thiol and alkylating reagents were 1 and 4 mM, respectively. Purified skin sulfhydryl oxidase was incubated alone, in Expt. I with 1 mM dithiothreitol, and in Expt. II with 1 mM glutathione in 50 mM sodium phosphate buffer, pH 7.6, and a total volume of 0.6 ml in sealed tubes. After 15 min, 0.20 ml buffer or 0.20 ml of the indicated alkylating reagent solution (16 mM) was added and the incubation was continued for another 30 min period. The reactions were stopped by adding 0.20 ml 100 mM glutathione. Reagents were removed by dialysis overnight against 50 mM sodium phosphate buffer, pH 7.6, at 4°C. The resulting solutions were assayed for enzymatic activity as described in Methods. All incubations were carried out at 37°C.

Expt.	Condition	Addition of reagents		Remaining enzymatic activity (reduced 5,5'-dithiobis(2-nitrobenzoic acid) nmol/h per μg per protein)	Relative activity (%)
		Preincubation (37°C 15 min)	Incubation (37°C 60 min)		
I	Control	None	None	5.10	100
	Dithiothreitol	Dithiothreitol	None	4.95	97
	N-ethylmaleimide	None	*N*-ethylmaleimide	4.96	97.2
	Dithiothreitol and *N*-ethylmaleimide	Dithiothreitol	*N*-ethylmaleimide	3.82	74.8
	+ Iodoacetamide	None	Iodoacetamide	3.75	73.5
	Dithiothreitol and iodoacetamide	Dithiothreitol	Iodoacetamide	0.58	11.5
	+ Iodoacetic acid	None	Iodoacetic acid	2.62	51.4
	Dithiothreitol + iodoacetic acid	Dithiothreitol	Iodoacetic acid	0.70	13.7
II	Control	None	None	5.08	100
	Glutathione	Glutathione	None	5.08	100
	N-ethylmaleimide	None	*N*-ethylmaleimide	4.97	98
	Glutathione and *N*-ethylmaleimide	Glutathione	*N*-ethylmaleimide	4.97	98
	+ Iodoacetamide	None	Iodoacetamide	3.50	69.1
	Glutathione + iodoacetamide	Glutathione	Iodoacetamide	3.51	69.1
	+ Iodoacetic acid	None	Iodoacetic acid	2.70	53.2
	Glutathione + iodoacetic acid	Glutathione	Iodoacetic acid	2.92	57.5

enzyme was remarkably heat stable (unpublished results), but the purified enzyme was quite heat labile. Enzyme activity decreased linearly during incubation at 60°C; the enzyme retained about 15% of initial activity following a 15 min incubation, but was completely inactivated after 30 min. The heat stability of the enzyme proved to be enhanced by the addition of 1 mM dithiothreitol, but 1 mM reduced glutathione had no effect.

Discussion

We have purified an enzyme from rat skin which is distinct from previously reported sulfhydryl-oxidizing enzymes in several characteristics. Skin sulfhydryl oxidase catalyzed the oxidation of various thiols, among them dithiothreitol, dithioerythritol, D-penicillamine and L-cysteine. Glutathione and 2-mercaptoethanol were very poor substrates for skin sulfhydryl oxidase. The

skin enzyme also reactivated reductively-denatured RNAase A without the necessity of a thiol cosubstrate or prior reduction of the skin enzyme. The activity of the enzyme increased with purification. There was not an exact correspondance in the degree of purification when expressed in terms of thiol reduction and RNAase renaturing activity. This may reflect different but similar enzymes, or different inhibitory factors removed at different stages of the purifications. The molecular weight of the enzyme was estimated to be 66 000 ± 2000, and the isoelectric point determined to be pH 4.65. Iodoacetic acid and iodoacetamide alone had some inhibiting effect on the enzyme; after preincubation with thiols which were substrates, enzyme inhibition was greatly enhanced. The enzyme was present in both epidermis and dermis. Since hair follicles are a significant cellular constituent of the dermis they may represent the source of dermal enzyme. Both the crude epidermal and dermal enzyme oxidized low molecular weight thiols and renatured reduced RNAase but the enzymes from the separate skin layers were not characterized individually any further.

Hawkins and Freedman [17] copurified glutathione-insulin transhydrogenase and protein disulfide-isomerase and found that, although their apparent isoelectric focusing points (pH 4.65) and molecular weights were the same, the transhydrogenase activity was demonstrably more sensitive to heat denaturation than the isomerase, while isomerase was more sensitive to deoxycholate inactivation than transhydrogenase. Ohba et al. [18] discovered that their microsomal protein disulfide-isomerase was inhibited by low concentrations of Triton X-100, and was also significantly activated by high concentrations of the same detergent. After a variety of experiments involving an antibody to the purified low-concentration detergent-labile enzyme, the authors concluded that they were working with two antigenically distinct enzymes: strong detergent treatment inhibited one enzyme (probably located on the outside of the microsome) while releasing latent enzyme activity inside the intact microsomes. The authors speculated that this detergent-resistant enzyme might be the same as glutathione-insulin transhydrogenase, which some investigators have routinely extracted from acetone powdered tissue by detergent-containing buffers [19,20].

Skin sulfhydryl oxidase resembles both of these possible enzyme groups in that it was somewhat activated by deoxycholate and Triton X-100, and it was quite heat labile. The skin enzyme also has the same p*I* as the enzymes of Hawkins and Freedman (4.65). Skin sulfhydryl oxidase also resembles the sulfhydryl-disulfide interchange enzymes in its enhanced susceptibility to the effects of alkylating reagents after pretreatment with thiols which are substrates. The possible inhibiting effect of alkylating reagents on bovine-milk sulfhydryl oxidase and epididymal sulfhydryl oxidase after pretreatment with thiol substrates was not mentioned in those papers [5—8]. Unlike the sulfhydryl-disulfide interchange enzymes, skin sulfhydryl oxidase catalyzes the formation of enzymatically active RNAase from non-enzymatically active reductively denatured RNAase A without an additional reducing agent present or prior reduction of the skin enzyme. Since disulfide bond reformation is necessary for enzymatic activity of RNAase the skin enzyme is responsible for this activity.

Like epididymal sulfhydryl oxidase, the skin enzyme in pure form is heat

labile. Epididymal enzyme was inhibited 50% after heating at 60°C for 3.5 min [6]. After 15 min of incubation at 60°C, the skin enzyme retained only about 15% of its initial activity; after 30 min at 60°C, the skin enzyme was completely inactivated. The heat stability of skin sulfhydryl oxidase proved to be enhanced by the addition of 1 mM dithiothreitol, but the addition of 1 mM reduced glutathione had no effect. The heat stability of glutathione-insulin transhydrogenase was similarly increased by one of its substrates, glutathione, but was decreased by its other major substrate, insulin [21].

Skin sulfhydryl oxidase differs from the sulfhydryl oxidase found in milk in several respects. Skin sulfhydryl oxidase does not have an easily chelatable metal cofactor necessary for activity, whereas milk sulfhydryl oxidase required iron which was easily removed by EDTA and restored by dialysis in 1 μM ferrous sulfate. Bovine-milk enzyme oxidized glutathione and restored activity to reduced RNAase A; skin sulfhydryl oxidase shares the ability of the milk enzyme to reactivate reduced RNAase A, but is unable to oxidize glutathione. The skin and milk enzymes also differ with respect to molecular weight, optimum pH and optimum temperature: 66 000 ± 3000 vs. 89 000 ± 900, pH 8.2 vs. 7.0, and 45 vs. 35°C, respectively [4].

The epididymal sulfhydryl oxidase of Chang and Morton [5] closely resembled skin enzyme in that it required no loosely bound metal cofactors for activity, and was little affected by metal chelators. One seminal vesicle enzyme purified by Ostrowski et al. [6], probably the same enzyme considered by Chang and Morton, was found to have a molecular weight of 66 000, which is the same as that of skin sulfhydryl oxidase. With many of the small thiol substrates common to both the epididymal vesicle and skin enzymes, the major differences between the two enzymes are their source (skin vs. genitourinary tract) and the greater general reactivity with sulfhydryl compounds possessed by the seminal vesicle enzymes. Seminal vesicle enzyme oxidized dithiothreitol, glutathione, L-cysteine, 2-mercaptoethanol and thioglycolate. Skin sulfhydryl oxidase was virtually ineffective with glutatione and 2-mercaptoethanol as substrates. The seminal vesicle enzyme after purification was found to be yellow and to contain a flavoprotein component. The skin enzyme showed no yellow color even after concentration in pure form during gel chromatography and during SDS-polyacrylamide gel electrophoresis.

Investigators have attributed different physiological roles to the sulfhydryl-oxidizing and reducing enzymes, most commonly the degradation of disulfide-containing peptide hormones [22—24] by glutathione-insulin transhydrogenase or involvement in crosslinking of nascent chains following protein synthesis [25,26] by protein disulfide isomerase.

Chang and Morton [5] hypothesized that their sulfhydryl oxidase might act as a protective agent in the reproductive tract, preserving sperm structure and function. Ostrowski et al. [6], who probably purified the same seminal-vesicle enzyme, disagreed but proposed no alternative role.

The observed oxidation and reactivation of reduced RNAase A suggest a possible biological role for sulfhydryl oxidase in the cellular synthesis of protein. Janolino and Swaisgood [7], DeLorenzo et al. [27] and Fuchs et al. [28] have hypothesized similar involvements in protein synthesis for the sulfhydryl oxidase and sulfhydryl-disulfide interchange enzymes they have described.

The varied forms of these sulfhydryl-modifying enzymes suggest the existence of a large family of isoenzymes responsible for the post-translational changes in proteins, including disulfide bond formation. The skin sulfhydryl oxidase described here might be one such enzyme responsible for the disulfide cross-linking of the structural proteins of hair and skin.

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