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# LENS METHIONINE SULFOXIDE REDUCTASE[+]

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SUMMARY: Investigation of human and bovine lenses has demonstrated the presence of a methionine sulfoxide (Met(O)) peptide reductase activity. The reductase can use either dithiothreitol or thioredoxin but not glutathione as a reducing agent. The enzyme is present primarily in the water soluble fraction. The highest specific activity is in the outer epithelial layer with decreasing activity in the inner layers of the tissue. The known high level of methionine sulfoxide residues in cataractous lens protein is not due to a decreased level of Met (O)-peptide reductase itself since a comparison of normal and cataractous human lenses showed no statistically significant decrease in reductase activity in the cataract population. However, it is not known whether the reducing system for Met (O)-peptide reductase (probably the thioredoxin system) is deficient in cataractous lenses.

INTRODUCTION: Lens protein is particularly susceptible to the effects of oxidation since protein synthesis is confined to the outer region of the tissue and most protein macromolecules must remain for the life of the tissue (1,2). It is therefore surprising that not only is the young normal human lens devoid of oxidized protein methionine and cysteine residues but even in old normal human lenses only slight protein oxidation is found and primarily in the membrane fraction (3). However, with the development of cataract, extensive oxidation of methionine and cysteine residues in lens protein is observed (3,6). Examination of some lens proteins isolated from cataract indicates that more than two-thirds of the methionine has been converted to methionine sulfoxide (3). It has been suggested that the oxidation commences at the cell membrane and extends into the cytoplasm (7). This viewpoint is supported by the elevated levels of $H_2O_2$ found in the aqueous fluids surrounding the lenses of cataract patients (8), since $H_2O_2$ is capable of causing the observed oxidative damage in the lens protein (9,10).

The lens contains a number of defense mechanisms to protect against oxidation or reverse oxidative damage. The tissue contains a high concentration of glutathione

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(11,12) as well as, glutathione reductase (12), catalase (13), glutathione peroxidase (14) and superoxide dismutase (15). However, no mechanism for repairing methionine oxidation in the lens has been reported previously. Recently Brot et al (16) reported the presence of a methionine sulfoxide (Met(O)) peptide reductase in mammalian tissue. It was therefore of interest to determine if the lens contained this enzyme. In this communication, the presence of the enzyme in normal bovine lenses and normal and cataractous human lenses are reported.

MATERIALS AND METHODS: Bovine eyes were obtained from the slaughter house dissected and the lenses were used either within 3 hours of death or frozen at -20°C until use. Normal human eyes were obtained from the New York Eye Bank for Sight Restoration within 48 hours of death, dissected and the lenses were used immediately or stored at -80°C. Cataracts were obtained from the operating room immediately after surgery. The enzyme activity was measured by following the reduction of N-acetyl-[$^3$H]Met(O) with slight modification of the procedure of Brot et al (17). In most cases 2 or 3 lenses were used. The capsule epithelium was homogenized at 0°C in a polytron (Brinkmann) in a concentration of 1 gm wet weight in 6 ml. in 0.05 M Tris, pH7.4, 1 mM mercaptoethanol. The decapsulated lens was weighed, frozen on dry ice and the central region removed with a trephine. The sections were weighed and the ends of the central core shaven to give the reported separations. The cortex, the outer 60% of the decapsulated lens, was prepared in a similar manner to the capsule epithelium as was the remainder of the lens, the nucleus, but a hand homogenizer was utilized. The preparations were centrifuged in a Beckman L-2 preparative ultracentrifuge at 28,000 rpm with a Ti50 rotor for 20 minutes at 0°C. The precipitate was suspended in the original buffer in a volume equal to the supernatant. The enzymatic reaction mixture, total volume 30 µl, contained 0.025 M Tris-Cl, pH 7.4, 0.01 M $MgCl_2$, 0.016 M dithiothreitol (DTT), 170 pmol N-acetyl-$^3$H-Met(O), ~4000 cpm/pmol and enzyme preparation (bovine or human lens). Incubations were for 60 min at 37°. The reaction was stopped by the addition of 1.0 ml of 0.1 N HCl. Extraction of N-acetyl-[$^3$H] Met was accomplished with 3 ml of ethyl acetate equilibrated with 0.1 N HCl utilizing vortexing for 30 sec followed by low speed centrifugation for 2 min. 2.5 ml of the ethyl acetate layer were removed and dried with anhydrous sodium sulfate. After shaking and allowing the material to settle, 2.0 ml were analyzed for radioactivity in 5 ml of Liquiscent (National Diagnostic). The enzyme assay gave a linear response with protein concentrations up to 450 µg in the 30 µl reaction volume. A unit of activity is defined as pmol of product formed under the conditions used. The labeled N-acetyl-Met(O) was synthesized from [$^3$H] methionine essentially as described by Brot et al. (17). Thioredoxin and thioredoxin reductase were obtained from Dr. A. Holmgren, Karolinska Institute, Sweden.

Enzymatic reduction of oxidized E. coli ribosomal protein L12 (Met(O)-L12) followed by acetylation with [$^3$H] acetyl-CoA was performed as previously described using a two-step procedure (16). For these experiments, the dissection of the lens and preparation of the extract were modified slightly. The lens extracts containing Met(O)-peptide reductase were prepared by homogenization in a buffer containing 10 mM Tris-HCl, pH 7.4; 10 mM $NH_4Cl$, and 10 mM $MgCl_2$. Epithelium-outer cortex was prepared from approximately the outer 10% of the bovine lens. The inner cortex represented approximately the next 50% of the tissue and the remaining material was defined as the nucleus.

RESULTS: The assay to detect Met(O)-peptide reductase activity is based on the observation that the N-acetyl-Met(O) in acid solution is not extracted into ethyl acetate whereas the reduced component is readily extracted (17). Crude preparations of both bovine and human lenses were found to reduce N-acetyl-Met(O). The requirements of the system are shown in Table I. An essentially complete requirement for a dithiol was demonstrated. Almost no activity was observed when DTT was omitted. Substitution of comparable concentrations of mercaptoethanol or glutathione

TABLE I

Met(O)-Peptide Reductase Activity in Bovine Lens extracts

| System | cpm |
|---|---|
| Complete | 6750 |
| - enzyme | 0 |
| - DTT | 150 |
| - DTT + mercaptoethanol | 373 |
| - DTT + glutathione | 180 |
| - DTT + thioredoxin system | 4476 |
| - enzyme + thioredoxin system | 250 |

The complete system is defined in the Materials and Methods section. Components were added as indicated in the Table to give the following composition: 0.016 M mercaptoethanol; 0.016 M glutathione; thioredoxin, 120 pmol; thioredoxin reductase, 3.5 µg; 0.0016 M NADPH.

had little effect. The thioredoxin system containing thioredoxin, thioredoxin reductase and NADPH (16) was found to effectively substitute for DTT, although under the present conditions only about two-thirds of the activity obtained with DTT was observed.

Studies were performed to determine whether the enzyme was present in the soluble or particulate fraction of the extract. As shown in Table II, when the initial bovine lens homogenate was centrifuged and the precipitate suspended in a final volume approximately equal to the original homogenate, comparable amounts of activity were found in both fractions. However, the specific activity usually was

TABLE II

Distribution of Met(O)-Peptide Reductase Activity in Lens Extracts

| Fraction | Total Units | | Met(O)-Peptide Reductase Activity units/mg |
|---|---|---|---|
| Supernatant | 160 | | 8 |
| Precipitate | 193 | | 55 |
| Wash 1 | 88 | 354 | 43 |
| Wash 2 | 118 | | 60 |
| Wash 3 | 95 | | 1,150 |
| Wash 4 | 53 | | 1,000 |
| Washed precipitate | 25 | | 8 |

The results were obtained with bovine capsule epithelium plus the outer 10% of the lens. The homogenate was centrifuged at 28,000 rpm for 20 min in a ti50 Spinco rotor. The washes were made with the homogenizing buffer in volumes equal to that of the supernatant. A unit is defined as the pmoles of N-acetyl-[$^3$H] Met(O) reduced at 37° in 60 min under the described conditions. See text for additional details.

TABLE III

Localization of Met(0)-Peptide Reductase in Lens

| Species | Fraction | Substrates | |
|---|---|---|---|
| | | N-Acetyl-Met(0) | Met(0)-L12 |
| | | units/mg | units/mg |
| Human | Epithelium | 20.5 | 93 |
| | Cortex | 10.9 | 23 |
| | Nucleus | 7.9 | -- |
| Bovine | Epithelium | 18.4 | 145 |
| | Cortex | 4.8 | 60 |
| | Nucleus | 1.3 | 18 |

See text for details of the dissection and incubation. The bovine lenses were from 3 to 6 months of age and the human lenses ranged from 58 to 68 years. No activity with either substrate was observed in the absence of lens extracts. With N-acetyl-Met(0) as substrate, the human lens and bovine lens had 561 and 3000 total units of activity, respectively.

markedly higher in the precipitate. Repeated washings of the precipitate removed most of the activity. After such washings, little more than 10% of the activity remained in the precipitate. It was interesting to note that the activity extracted with the four washes represented almost twice the activity initially observed with the precipitate. This observation suggests that there are inhibitors of the activity in the suspended precipitate. The increased specific activity in the final washes shows that a significant purification was obtained by this procedure. $NaDodSO_4$ polyacrylamide gel electrophoresis in the presence of DTT indicates that washes three and four contain a number of polypeptide bands in the 22 to 26 x $10^3$ dalton range. At the present level of purification it is not possible to delineate the size of the Met(0)-peptide reductase, although it is of interest to note the E. coli Met(0)-petide reductase has an apparent molecular weight of ∿20,000 (unpublished results).

Whereas the reduction of N-acetyl-Met(0) suggests that the enzyme is capable of reducing Met(0) residues in proteins, demonstration of direct reduction of such groups is desirable. Utilizing the observation that the E. coli ribosomal protein L12 cannot be acetylated if the methionine residues are oxidized it is possible to follow the reduction of Met(0)-L12 by acetylating the reduced protein with ($^3$H) acetyl-CoA (16) Typical results (Table III) on the distribution of the enzyme using both N-acetyl-Met(0) and Met(0)-L12 as substrates show that in approximately ten preparations of both human and bovine lenses the epithelium has a 2 to 4 fold greater specific activity than the cortex. The inner nuclear region had the least activity. Although the specific activity is much higher in the epithelium, it represents only a few percent of the mass of the tissue and most of the activity is present in the cortical region. Because of the different conditions used in the Met(0)-L12 reduction system, it is not possible to compare specific activities from this assay with those based on the reduction of N-acetyl-Met(0), except in terms of location of activity in the lens.

TABLE IV

Comparison of Met(O)-Peptide Reductase Activity in Normal and Cataractous Lens

| Normal | | Cataract | | |
|---|---|---|---|---|
| Age | units/lens[++] | Age | units/lens[++] | Classification[#] |
| 54 | 456 | 64 | 614 | $HM\text{-}CXA_4CXP_4N_4SCA_{100}SCP_{100}NS_Y$ |
| 57 | 596 | 64 | 260 | $M\text{-}CXA_4CXP_4N_3SCA_{100}SCP_{90}NS_Y$ |
| 62 | 1,303 | 66 | 382 | $HM\text{-}N_3SCA_{100}SCP_{80}NS_B$ |
| 62 | 273 | 67 | 125 | M |
| 62 | 408 | 69 | 697 | $I\text{-}CXA_3CXP_3N_4NS_B$ |
| 69 | 565 | 69 | 341 | $I\text{-}N_2SCA_{10}SCP_{80}NS_{VDY}$ |
| 70 | 164 | 78 | 415 | M |
| 81 | 560 | 88 | 947 | $I\text{-}CXA_4CXP_4N_4NS_B$ |
| | Mean 541[+++] | | Mean 472[+++] | |
| | SEM[+] 121 | | SEM[+] 94 | |

[+]Standard error of the mean.

[++]pmoles N-acetyl[$^3$H]Met(O) reduced/hr/lens.

[+++]Comparison of the difference of the mean indicates no significant difference ($p>0.05$).

[#]See Chylack (19) for description of classification.

A comparison of Met(O)-peptide reductase activity in normal and cataractous human lenses is shown in Table IV. No correlation could be made with respect to activity, age or type of cataract. While the mean activity for the population of cataractous lenses was somewhat lower than the normal population, no significant difference was found between the two groups.

DISCUSSION: It is clear from this investigation that the outer region of the lens contains an active Met(O)-peptide reductase capable of reducing Met(O) residues in proteins. This enzyme may contribute to the oxidative defenses of the tissue and may explain the absence of Met(O) residues in proteins in the cytoplasm of the normal human lens. Since the enzyme appears to be present primarily in the soluble fraction, it is not surprising to detect in older lenses some oxidation of methionine in membrane polypeptides (3).

While it would appear that there is no marked decrease in Met(O)-peptide reductase activity in human cataractous lenses, such results do not eliminate the possibility that the reducing system is inoperative. In cataract, almost all of the thiol groups of the lens proteins and glutathione are oxidized. It is therefore possible that the natural cofactor, e.g. thioredoxin, utilized by the enzyme is also oxidized. The lens is now being examined to ascertain whether the thioredoxin system is present and, if so, what is the oxidation state of the thioredoxin in the cataract.

In the lens the major source of reducing potential of the tissue arises from the production of NADPH formed during the metabolism of glucose via the hexose monophosphate shunt. This reducing potential could be used to reduce Met(O) via the thioredoxin system. In this system NADPH reduces thioredoxin utilizing thioredoxin reductase. A similar system involving glutathione reductase maintains glutathione in the reducted state. Since glutathione reductase appears to remain active in cataract (18), the rapid disappearance of reduced glutathione during cataract development suggests that NADPH production has not kept pace with the reducing requirements of the tissue.

The distribution of enzyme activity appears to be similar using either oxidized ribosomal protein L12 or N-acetyl-Met(O) as substrate, supporting the view that the same enzyme is involved. At present, it is not known whether all methionine in lens protein that is accessible to oxidative attack by $H_2O_2$ or other oxidizing agents can be repaired by the Met(O)-peptide reductase.

It can be argued that although lens Met(O)-peptide reductase reduces N-acetyl-Met(O) and Met(O)-L12, lens protein is not a substrate. However, very preliminary experiments utilizing lens protein oxidized with $H_2O_2$ suggest that the enzyme maybe capable of reducing oxidized lens protein.

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