Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 156-162

# THIOREDOXIN FRAGMENT 31-36 IS REDUCED BY DIHYDROLIPOAMIDE AND REDUCES OXIDIZED PROTEIN

Abraham Spector, Ruey-Ruey C. Huang, Guo-Zai Yan and Ren-Rong Wang

Biochemistry and Molecular Biology Laboratory
Department of Ophthalmology, College of Physicians and Surgeons
Columbia University, New York, NY 10032

Received December 1, 1987

SUMMARY: The thioredoxin peptide Trp-Cys-Gly-Pro-Cys-Lys, which contains the redox active dithiol, was found to be reduced by lipoamide in a coupled reaction with lipoamide dehydrogenase and NADH. The reduced peptide in turn was shown to reduce insulin, oxidized lens protein and glyceraldehyde-3-phosphate dehydrogenase. While the peptide is not as effective a catalyst for utilizing pyridine nucleotides to reduce protein disulfides as thioredoxin, it offers a system which may be developed to provide more efficient disulfide reduction. This is particularly relevant since no thioredoxin peptides have been found to be active with thioredoxin reductase.



While it is now generally accepted that oxidative insult is involved with the development of maturity onset cataract, there have been relatively few reports of attempts to increase the ability of the lens to resist or repair oxidative damage (1). Some communications have suggested that compounds, such as vitamin E and ascorbic acid, may provide some help (2,3). Recently, it was shown that elevating the glutathione level does not protect lens epithelial cells from oxidative damage and may, in certain situations, be detrimental (4). The latter observation emphasizes the difficulty in protecting the tissue by appreciably increasing the concentration of even a physiological reductant.

An attractive approach to the problem may be the exploitation of the thioredoxin system. Thioredoxin (Tx) is a polypeptide of approximately 12,000 daltons which contains a dithiol that is active in oxidation reduction reactions (5). It is reduced by a flavoprotein Tx reductase which utilizes NADPH. Tx has been shown to reduce protein disulfides and acts as a co-factor for the reduction of protein methionine sulfoxide (5,6). The system appears to be ubiquitous. Tx is usually present at $\mu$molar concentrations in contrast to glutathione which is present in tissue in the mmolar range.

Recent work has shown that while the readily available E. coli Tx is capable of reducing oxidized proteins, it is not very effective in mammalian systems since it is a poor substrate for mammalian Tx reductase (5,7). Furthermore, mammalian Tx contains additional thiol groups and is somewhat

0006-291X/88 $1.50

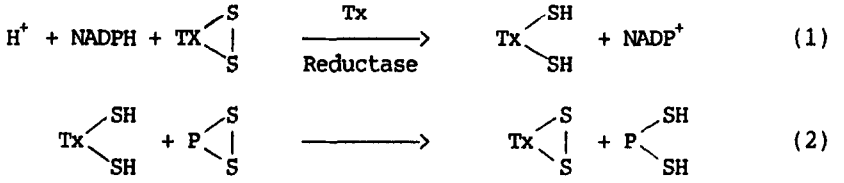
unstable (5). An additional disadvantage of Tx is the size of the polypeptide which retards passage into the tissue.

The primary structure of E. coli Tx and the active dithiol have been delineated by x-ray diffraction (5). Unfortunately, all peptides containing the active dithiol derived from Tx have been found to be unreactive with Tx reductase. Thus, it would appear that the entire polypeptide is required for reduction via Tx reductase. Recently, it has been reported that Tx can be reduced by dihydrolipoamide (8). This observation, thus, suggests that the lipoamide system, i.e., lipoamide dehydrogenase and lipoamide with NADH may be able to reduce not only Tx but Tx peptides containing the active center.

In this report, it is demonstrated that the E. coli Tx fragment 31-36 with the structure Trp-Cys-Gly-Pro-Cys-Lys can be reduced by dihydrolipoamide and linked to the NADH reservoir via lipoamide dehydrogenase and that the peptide can reduce oxidized protein.

MATERIALS AND METHODS: E. coli Tx-(S-S), E. coli Tx reductase and E. coli Tx peptide Txp-$(SH)_2$ were obtained from Repligen Corporation, Boston, MA. Bovine insulin and yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) were purchashed from Sigma Chemical Company, St. Louis, MO. All other compounds were reagent grade. All experiments were conducted as described in the text. The methods used to isolate and oxidize the lens proteins, to oxidize GPD and measure its activity and to determine the thiol content with DTNB have previously been described (7). Txp-$(SH)_2$ was oxidized at pH 8.5, Tris, 100 mM at 37° in air for 40 hours.

RESULTS AND DISCUSSION: Thioredoxin (Tx) reduction of a protein disulfide involves first the reduction of the Tx and then the reduction of the disulfide. The reactions are depicted below:




Insulin, which contains two disulfides which hold the A and B chains together, has been shown to be an ideal protein substrate since the reduced polypeptides are insoluble, particularly the B chain (9). Thus, the turbidity measured at 650 nm can be used to follow reaction 2. The first reaction can be monitored at 340 nm. Figure 1a demonstrates that very shortly after the reduction of Tx has begun, protein turbidity can be detected. The Tx, although present at a low concentration, is rapidly reduced and, in turn, rapidly reduces the insulin. In this process, the Tx returns to the oxidized state. The initial rate of NADH utilization reflects this process with 7.2 nmoles/minute being oxidized. This represents a reduction of more Tx than was originally present indicating the rapid subsequent oxidation of Tx in the insulin reduction step. After a little more than five minutes, insulin precipitation is observed and by

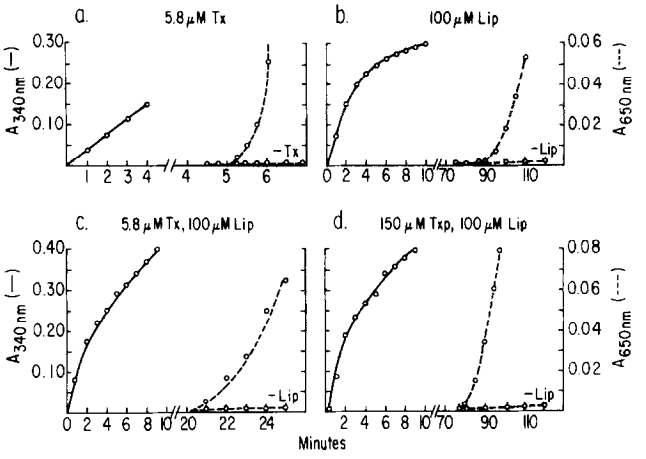
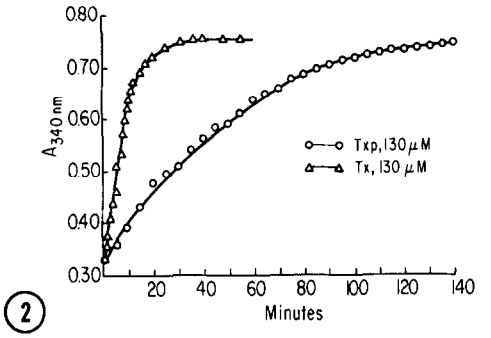



Figure 1. NADPH and NADH oxidation and insulin reduction with thioredoxin (Tx), lipoamide (Lip), and thioredoxin peptide (Txp). NADH and NADPH oxidation was followed at 340 nm and insulin reduction by increase in turbidity at 650 nm. For each experiment, two cuvettes contained in 1 ml, 100 mM potassium phosphate, pH 7.0; 2 mM EDTA, 250 μM NADPH or NADH, 150 μM insulin. (a) 5.8 μM E. coli Tx-(S-S) was added to the reference cuvette and the reaction was initiated by the addition of 1.5 μg of E. coli thioredoxin reductase to both cuvettes. (b) to the reference cuvette, 100 μM Lip-(S-S) was added, the reaction was initiated by the addition of 1.0 μg of lipoamide dehydrogenase to both cuvettes. (c) to the reference cuvette, 5.8 μM E. coli Tx-(S-S) and 100 μM Lip-(S-S) were added. The reaction was initiated as in (b). (d) 150 μM Txp-(S-S) and 100 μM Lip-(S-S) were added to the reference cuvette. The reaction was initiated as in (b). After a given period, as shown in the figures, the cuvettes were reversed and the insulin precipitation followed at 650 nm.

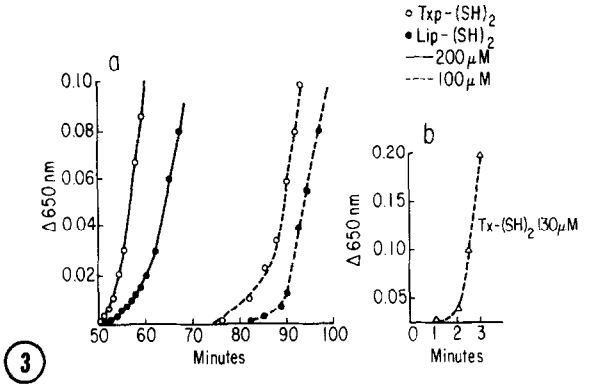
approximately six minutes, significant turbidity can be detected. In the absence of Tx, no turbidity is generated.

The ability of lipoamide to reduce insulin was examined next. Oxidized lipoamide Lip-(S-S) was reduced by NADH in the presence of lipoamide dehydrogenase and then the reduced lipoamide Lip-$(SH)_2$, in turn, reduced the insulin. As shown in Figure 1b, even at concentrations of 100 uM where the Lip-(S-S) is reduced at an initial rate of 12 nmoles/minute and 48% has been reduced in 10 minutes, it is not until ninety minutes that insulin turbidity is observed. Thus, the lipoamide is not as effective a reductant as Tx. Now, if the lipoamide system is used to reduce Tx, the results shown in Figure 1c are observed. The initial rate of NADH utilization of 14 nmoles/minute is somewhat faster than the initial utilization of the lipoamide alone. Insulin precipitation is detected in approximately 20 minutes, again showing the greater effectiveness of Tx in reducing insulin. It is apparent from this experiment that the lipoamide system can substitute for Tx reductase in driving the reaction. The difference in the onset of insulin precipitation is due to the slower rate of Tx reduction by the lipoamide.

The ability of the Tx peptide (Txp) Trp-Cys-Gly-Pro-Cys-Lys to substitute for Tx was now examined. It was found, as previously reported, that the peptide is not reduced by Tx reductase in the presence of NADPH (5). The lipoamide system was, therefore, investigated as a substitute for Tx reductase.




(2)




(3)

Figure 2. Reduction of Tx-(S-S) and Txp-(S-S) by Lip-$(SH)_2$. Lip-(S-S) was first reduced with 250 μM NADH. Both cuvettes contained 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 250 μM NADH. 80 μM Lip-(S-S) was added to the reference cuvette and the reaction was initiated by addition of 1.5 μg lipoamide dehydrogenase to both cuvettes. When no further change in 340 nm absorption was observed, either 130 μM Tx-(S-S) or Txp-(S-S) was added to the reference cuvette and the $A_{340\ nm}$ followed until no further changes were observed.

Figure 3. Reduction of insulin by Lip-$(SH)_2$, Txp-$(SH)_2$ and Tx-$(SH)_2$. Lip-(S-S) was reduced with 350 μM NADH as described in Figure 2 and then used either to reduce insulin or in certain experiments to reduce Txp-(S-S) and Tx-(S-S). The reduced components were then used to reduce insulin. (a) 100 μM and 200 μM of both Txp-$(SH)_2$ and Lip-$(SH)_2$ and (b) 130 μM Tx-$(SH)_2$ were used with 300 μM insulin. See Figure 1 legend for further information.

As shown in Figure 1d, when Txp-(S-S) was used with Lip-(S-S) and NADH, the initial rate of utilization of NADH is almost the same as was observed with the Tx and the lipoamide system, approximately 14 nmoles/minute, although there is approximately 25 fold more Txp than Tx present. The initial rate is approximately 2 nmoles/min faster than with lipoamide alone. In contrast to reduction with Tx, the insulin precipitation was not detected until 80 minutes. This is approximately 10 minutes earlier than is observed with lipoamide alone and the rate of precipitation (change in $A_{650nm}$ from 0.05 to 0.1) is approximately 20% faster.

The effectiveness of the Txp in reducing insulin is based on two reactions, the lipoamide reduction of Txp-(S-S), and Txp-$(SH)_2$ reduction of insulin. The reduction of Txp-(S-S) was examined by first reducing the lipoamide in the absence of Txp and then adding Txp and following the rate of Txp reduction via utilization of NADH. A similar experiment was performed with Tx. The results are shown in Figures 2 and 3. It is again apparent that Tx is a better substrate for the lipoamide system than Txp (Figure 2). The Tx is reduced at an initial rate of 5 nmoles/minute in contrast to the Txp where an initial rate of 1.1 nmoles/minute was observed. In some experiments, the reduction of Lip-(S-S) or Txp-(S-S) was followed by detection of the thiol groups with DTNB. Similar results to those observed by monitoring the $\Delta_{340\ nm}$ absorption were obtained.

The relative effectiveness of Txp-$(SH)_2$, Tx-$(SH)_2$ and lipoamide-$(SH)_2$ to reduce insulin was examined next (Figure 3). Precipitation was observed in

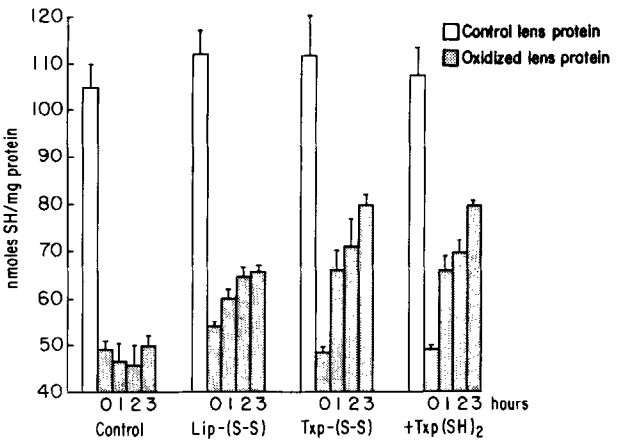



Figure 4. Reduction of lens proteins exposed to $H_2O_2$ by lipoamide and thioredoxin peptide. $H_2O_2$ treated lens soluble protein 2 mg/ml was incubated with a control solution: 100 mM potassium phosphate, pH 7.0, 20 mM EDTA or the former solution plus the lipoamide system: 50 $\mu$M Lip-(S-S), 350 $\mu$M NADH, 1 $\mu$g lipoamide dehydrogenase or the lipoamide system plus 265 $\mu$M Txp-(S-S) or 265 $\mu$M Txp-$(SH)_2$. Aliquots were taken at 0, 1, 2 and 3 hours, precipitated with an equal volume of 20% TCA held in an ice bath 10 minutes and then centrifuged. The pellet was washed with 0.5 ml 10% TCA, followed by 1 ml ethanol and then 1 ml of ether. The thiol content of the precipitate was then determined by DTNB in 7 M guanidine, 0.1 M Tris, pH 8.2 and 2 mM EDTA.

approximately fifty minutes for both lipoamide and Txp when 200 $\mu$M concentrations were used (Figure 3a). However, the Txp reduced the insulin at a significantly more rapid rate, 0.05 vs 0.02 $A_{650\ nm}$/minute (based on $\Delta$650 nm from 0.05 to 0.10). With 100 $\mu$M levels, insulin precipitation was observed at 76 minutes with Txp and 82 minutes with lipoamide. In contrast to these results, it was found that 130 $\mu$M Tx-$(SH)_2$ reduced the insulin with great rapidity with a rate of 0.12 $\Delta$650 nm/minute being observed after only two minutes (Figure 3b).

The ability of Txp-$(SH)_2$ to reduce lens protein which had been oxidized with $H_2O_2$ was now examined (Figure 4). Following oxidation, protein thiol decreased in the control samples from approximately 105 nmoles SH/mg protein to about 50 nmoles/mg protein. Lipoamide reduction, utilizing NADH and lipoamide dehydrogenase, brought the thiol level slowly back from about 50% of the thiols in a reduced state to approximately 65% in three hours. Utilizing either Txp-(S-S) with the lipoamide system or Txp-$(SH)_2$ without lipoamide, similar results were obtained with 80 nmoles SH/mg being detected after three hours of reduction. It is apparent that Txp is more effective than lipoamide in the reduction of lens proteins under these conditions. However, where comparable concentrations of Txp and lipoamide are used, Txp appears only somewhat more effective. Previous work with Tx has demonstrated that under similar conditons, Tx at concentrations of 5.8 $\mu$M produces more rapid reduction with a complete recovery of thiol being observed after two hours.

Glyceraldehyde phosphate dehydrogenase (GPD) is a key enzyme involved in glycolysis. In the lens, this metabolic pathway is the major route of glucose

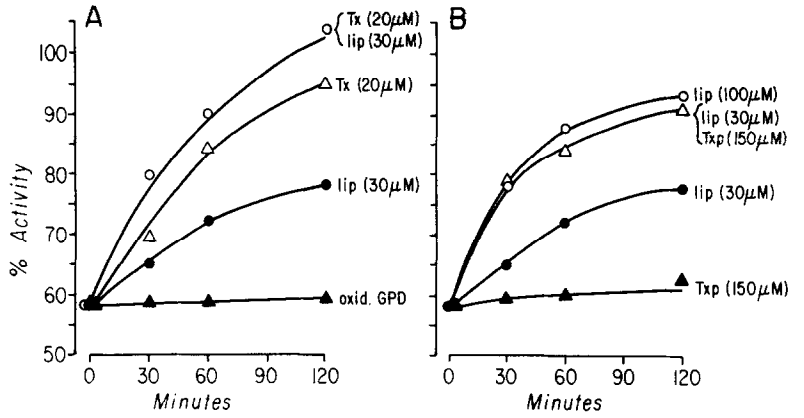



Figure 5. Regeneration of GPD activity. GPD (500 μg/ml) was oxidized with 0.3 mM $H_2O_2$ for five minutes. Catalase was added to stop the reaction (25 μg per ml). The oxidized enzyme at a concentration of 250 μg/ml was then incubated at 37° as follows: A: ▲, solution (a) containing 50 mM potassium phosphate, pH 7.5, 25 mM EDTA; ●, solution (a) plus solution (b) consisting of 30 μM Lip-(S-S), 250 μM NADH, 2 μg lipoamide dehydrogenase; Δ, solution (a) plus 20 μM Tx, 250 μM NADPH, 1.5 μg thioredoxin reductase; O, solution (a) plus 20 μM Tx and solution (b). B: ▲, 150 μM Txp-(S-S) plus solution (a); ●, solution (a) plus solution (b); Δ, solution (a) plus solution (b) plus 150 μM Txp-(S-S); O, 100 μM Lip-(S-S), 250 μM NADH, 2 μg lipoamide dehydrogenase plus solution (a).

metabolism. The enzyme is susceptible to oxidative insult. Following oxidation of the GPD with $H_2O_2$, enzyme activity was reduced to approximately 58%. The oxidized GPD was subjected to reduction under a number of conditions to determine if activity could be regained. With the Tx system using 20 μM Tx-(S-S), the activity was increased to 95% of control values in a two hour period (Figure 5A). In contrast, with 30 μM lipoamide and lipoamide dehydrogenase, less than 80% of the original activity was obtained. When the lipoamide system was substituted for Tx reductase, complete recovery was observed in two hours (Figure 5A). With 30 μM lipoamide and 150 μM Txp, approximately 90% recovery was obtained in two hours (Figure 5B). Increasing lipoamide to 100 μM with lipoamide dehydrogenase resulted in a similar recovery of enzymatic activity.

These experiments indicate that Txp-(S-S) can be reduced by the lipoamide system and that Txp-$(SH)_2$ can reduce protein disulfides but not as effectively as Tx. Thus, there is an opportunity to measure the effect of peptide modification to enhance reduction of the modified peptide by lipoamide and disulfide reduction by the modified peptide. It has been shown that the Tx active center thiols contain an atypical thiol with a pk of 6.7, approximately 2 pH units lower than normal (10). Disulfide exchange reactions which initiate the reduction process require nucleophilic attack. Preliminary experiments suggest that the pk's of the Txp thiols are in the normal range and cannot initiate as effective nucleophilic attack.

It has been suggested that lipoamide may be involved in reactions independent of the pyruvate and α-ketoglutarate dehydrogenase multienzyme

systems. Possibly 50% of the lipoamide may have an unidentified electron acceptor which may be Tx (11). While it is not known if Txp can pass into mitochondria, Tx has been found in these structures.

ACKNOWLEDGMENTS: This work was supported by grants from the National Eye Institute, National Institutes of Health.

REFERENCES

1. Spector, A. (1984) Invest. Ophthal. & Vis. Sci. 25, 130-146.
2. Varma, S. B., Srivastava, V. K. and Richards, R. D. (1982) Ophthal. Res. 14, 167-175.
3. Creighton, M. O. and Trevithick, J. R. (1979) Exp. Eye Res. 29, 689-693.
4. Spector, A., Huang, R-R. C., Wang, G-M., Schmidt, C., Yan, G-Z. and Chifflet, S. (1987) Exp. Eye Res. 45, 453-465.
5. Holmgren, A. (1985) Ann. Rev. Biochem. 54, 237-271.
6. Brot, N., Weissbach, L., Werth, J. and Weissbach, H. (1981) Proc. Natl. Acad. Sci. 78, 2155-2158.
7. Spector, A., Yan, G-Z., Huang, R-R. C., McDermott, M. J., Gascoyne, P. R. C. and Pigiet, V. (1987) J. Biol. Chem., submitted.
8. Holmgren, A. (1979) J. Biol. Chem. 254, 9627-9632.
9. Sanger, F. (1949) Biochem. J. 44, 126-128.
10. Kallis, G-B. and Holmgren, A. (1980) J. Biol. Chem. 255, 10261-10265.
11. Grey, P. A., Ikeda, B. H., Ramos, G., Speckhard, D. C. and Wong, S-S. (1978) J. Biol. Chem. 253, 7234-7241.