Thioredoxin Catalyzes the Reduction of Insulin Disulfides by Dithiothreitol and Dihydrolipoamide*

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Thioredoxin from *Escherichia coli* was shown to catalyze the reduction of insulin disulfides by dihydrolipoamide. A quantitative assay was developed which measures the rate of insulin reduction spectrophotometrically at 650 nm as turbidity formation from the precipitation of the free insulin B chain. Thioredoxin, at 5 mM concentration, accelerated the reaction between 0.130 mM insulin and 1.0 mM dihydrolipoamide at pH 7 around 20-fold. The pH optimum of the reaction was 7.5. Thioredoxins from *E. coli* and calf liver showed similar specific activities.Stopped flow fluorescence measurements of the rate of reduction of thioredoxin-S2 by dihydrolipoamide showed a second order rate constant of 1647 M⁻¹ s⁻¹ at pH 7.2. This is between 10⁵ to 10⁶ times larger than the reaction between insulin or linear model disulfides and dihydrolipoamide. This is consistent with a ping-pong mechanism of thioredoxin catalysis since reduced thioredoxin is known to react very fast with insulin.

Thioredoxin also catalyzed lipoamide-dependent reduction of the insulin disulfides in a coupled system with NADH, lipoamide, and lipoamide dehydrogenase. The fast spontaneous reaction between dihydrolipoamide and thioredoxin-S2 provides a mechanism for NADH or pyruvate-dependent disulfide reduction. The implication of the di-thiol-disulfide oxidoreductase activity of thioredoxin for the regulation of enzyme activities by thiol oxidation-reduction control is discussed.

Thioredoxin-S²⁻ from *Escherichia coli* or mammalian cells contains an oxidation-reduction active cystine disulfide (1, 2) that is reduced to the di-thiol form by NADPH and thioredoxin reductase (Reaction 1) (3, 4). Thioredoxin-(SH)₂ is an effective protein disulfide reductase (Reaction 2) (4, 5). Thus, together, thioredoxin and thioredoxin reductase are a powerful NADPH-protein disulfide reductase system (Reaction 3).

Thioredoxin-S₂⁻ + NADPH

\[ \text{thioredoxin reductase} \]

\[ + II^+ \rightarrow \text{thioredoxin-(SH)₂} + \text{NADP}^+ \]  

Thioredoxin-(SH)₂ + protein-S₂⁻ → thioredoxin-S₂⁻ + protein-(SH)₂

Sum: NADPH + H⁺ + protein-S₂⁻ → NADP⁺ + protein-(SH)₂ (3)

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1. The abbreviations used are: thioredoxin-S₂⁻ and thioredoxin-(SH)₂, the oxidized and reduced form of thioredoxin, respectively; lip-S₂⁻ and lip-(SH)₂, the oxidized and reduced form of lipoamide, respectively.

Recently, thioredoxin was implicated in the regulation of photosynthetic fructose 1,6-bisphosphatase and other enzymes (6, 7). Activation of chloroplast fructose 1,6-bisphosphatase has been assumed to occur by thioredoxin-mediated reduction of a critical disulfide bond in the enzyme (8) and to depend on a reduction mechanism different from NADPH and thioredoxin reductase. It requires reduced ferredoxin, an iron-sulfur protein that functions as acceptor in photosynthetic electron transport, and a chloroplast enzyme called ferredoxin-thioredoxin reductase (6). Alternatively the nonphysiological dithiol reagent dithiothreitol (6, 9) could replace the ferredoxin-linked enzyme.

In this paper, the reaction mechanism of thioredoxin with dithiolo has been examined. This required a method that directly measures protein disulfide reduction. We have developed a rapid spectrophotometric assay that records the turbidity of precipitation of the free A and B chain that are produced by reduction of the two interchain disulfide bonds of insulin. These are rapidly cleaved by reduced thioredoxin at pH 7.0, with an apparent second order rate constant that is around 10⁶ times larger than the corresponding rate constant for the reaction with dihydrolipoamide (5).

The oxidized form of *E. coli* thioredoxin is characterized by a very low quantum yield of tryptophan fluorescence (10, 11). Reduction of the thioredoxin by dihydrolipoamide at pH 7.0 is known to result in a 3-fold increase in tryptophan fluorescence (11). In this study this was used to determine, by stopped flow fluorescence, the rate constant for the reduction of thioredoxin-S₂⁻ by dihydrolipoamide.

Thioredoxin was found to have dithiol-disulfide-oxidoreductase activity and to catalyze the reduction of insulin by dihydrolipoamide. It was also active with dihydrolipoamide. The possible physiological implications of the rapid reaction between dihydrolipoamide and thioredoxin-S₂⁻ are discussed.

**EXPERIMENTAL PROCEDURES**

Materials—Thioredoxin was a homogeneous preparation from *E. coli* B obtained by the method of Holmgren and Reichard (12). Thioredoxins from calf liver and thymus were preparations of better than 95% purity obtained by the procedure of Engstrom et al. (2). Thioredoxin reductase from *E. coli* was a homogeneous preparation obtained by the method of Thelander (13). Bovine insulin, 26.1 units/mg, was purchased from Vitrum, Stockholm, Sweden. Lipoamide dehydrogenase, from pig heart, was type III, 167 units/mg of protein, from Sigma. Dithiothreitol was from Calbiochem. DL-Lipoamide, NADH, NADPH, and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Sigma. Urea was an ultrapure preparation from Mann. All other chemicals were of highest purity commercially available.

Insulin Solutions—Stock solutions were prepared at 10 mg/ml (1.67 mM) by suspending 50 mg in 4 ml of 0.05 M Tris-Cl, pH 8.0, and adjusting to pH 2 to 3 by addition of 1.0 M HCl and rapidly titrating the solution back to 8.0 with 1.0 M NaOH with a pH meter. Finally the volume was adjusted to 5.00 ml with water. The solution of insulin was perfectly clear and was stored at -20°C.

Spectrophotometric Determinations—All measurements were car-
ried out at room temperature, 23° ± 1°C, in 0.10 M potassium phosphate or Tris-C1 buffers containing 2 mM EDTA. A Zeiss PMQ 3 spectrophotometer equipped with an automatic multiple sample exchanger and a Servogor 541 recorder was used with semimicro cuvettes containing a final volume of 0.6 ml. The times between measurements were 0.2, 0.5, or 1 min.

Turbidimetric Assay of Insulin Disulfide Reduction—Freshly prepared solutions of insulin, 1 mg/ml in 0.1 M potassium phosphate (pH 6.5 or 7.0), 2 mM EDTA, and 100 mM dithiothreitol were stored in an ice bath.

The assay mixture was prepared in cuvettes by addition of 500 μl of insulin plus thioredoxin and water to give a final volume of 0.60 ml. The reaction was started by pipetting dithiothreitol (2 to 10 μl) in all cuvettes, except the blank. The cuvettes then were thoroughly mixed and placed in the spectrophotometer. The measurements were performed at 650 nm using either 1.0- or 0.5-min recordings. Generally, no further mixing of the cuvettes was done for assays lasting up to 60 min. In all experiments, the nonenzymatic reduction of insulin by dithiothreitol was recorded in a control cuvette without thioredoxin.

The time for the start of precipitation, defined as an increase by 0.02 at A450 over a stable base-line recording, was determined. The second parameter calculated was the rate of precipitation at 650 nm, defined as the maximal increase ΔA450 × min⁻¹ in the interval between 0 and 1.0 in absorbance at 650 nm.

NADPH-dependent Reduction of Insulin—The conditions for following the rate of disulfide reduction at 340 nm have been described previously (5).

RESULTS

Thioredoxin Catalyzes Reduction of Insulin by Dithiothreitol—Reduction of insulin by NADPH and the thioredoxin system leads to cleavage of the two interchain disulfide bridges at similar overall rates (5). As reduction proceeds a white precipitate is formed mainly from the free B chain of insulin which is insoluble (14). This phenomenon was used in the present experiments to devise a rapid and simple assay for measurements of dithiothreitol reduction, where the rate of precipitation was recorded at 650 nm.

The reduction of insulin disulfides by dithiothreitol at pH 7.0 was determined in the presence and absence of E. coli thioredoxin-S1. The assay mixtures contained a final concentration of 0.13 mM insulin (0.75 mg/ml) and 0.33 mM dithiothreitol. In the control cuvette, containing only dithiothreitol, no precipitation was observed until after 70 min. The addition of 7.8 μM thioredoxin-S1 resulted in rapid precipitation appearing after 9 min, demonstrating a catalytic effect of thioredoxin (Fig. 1). A lower concentration of thioredoxin-S2, 3.9 μM resulted in a longer delay before turbidity appeared and a corresponding slower rate of precipitation (Fig. 1). After leaving the assay cuvettes overnight, the same amount of final precipitate was obtained irrespective of thioredoxin addition. This is expected since all cuvettes contained the same amount of dithiothreitol and only the time to reach equilibrium was different in the presence of thioredoxin. Thus, thioredoxin catalyzed the dithiothreitol reduction of insulin disulfides (Reaction 4).

\[
\text{SH} \quad \text{Dithiothreitol} \quad + \text{insulin-S} \quad \text{thioredoxin} \quad \rightarrow \quad \text{SH} \quad \text{S} \quad \text{dithiothreitol} \quad + \text{insulin-(SH)} \quad (4)
\]

Quantitation of the Turbidimetric Assay—A quantitative analysis of the precipitation rate assay was required for this method to be useful. Apparently both the time before visible precipitation was observed, and the rate of precipitation measured as ΔA450 × min⁻¹ seemed to be useful quantitative parameters (Fig. 1). A quantitative relationship between the rate of insulin disulfide reduction and the rate of precipitation could be obtained by extrapolating the results from the NADPH-dependent reduction of thioredoxin by thioredoxin reductase (Reactions 1 and 2). The rate of NADPH consumption was first established at 340 nm before precipitation appeared and the reaction then was followed at 650 nm. The results at pH 6.5 to 8.0 are summarized in Table I. The rate of precipitation of insulin was much higher at pH 6.5 than at pH 8.0. Also the time for precipitation to appear was shorter at pH 6.5, consistent with a lower solubility for insulin and free peptide chains at this pH. Measurements below pH 6.5 were not possible due to the insolubility of insulin at low pH.

The relationship between insulin S-S bond reduction and precipitation is illustrated in Fig. 2. It is clear that a linear relationship is only observed at low ΔA450. Furthermore, the precipitation rate was a nonlinear function of the absolute amounts of insulin disulfides reduced and varied not only with the rate of reduction but also with pH. These results are explained by the large variations in solubility of insulin and the free peptide chains at different pH. Furthermore, the time for complete precipitation of the free insulin B chain aggregates may be long compared with the rate of production of free chain resulting in a delay of precipitation. At high ΔA450 × min⁻¹ values, the actual disulfide reduction rate is thus higher than at lower ΔA450 × min⁻¹ rates (see Fig. 2). The alternative explanation that the order of reduction of the three insulin disulfides might vary with rate of reduction was not ruled out, but it seemed very unlikely.

pH Dependence—The pH dependence of insulin reduction with 0.33 mM dithiothreitol (Reaction 4) was investigated with 3.9 and 7.9 μM thioredoxin. Rapid precipitation was found at pH 6.5 and 7.5, whereas the insulin was too soluble at pH 8.5 and 9.0 to give any precipitation. The thioredoxin-catalyzed reaction had a pH optimum at 7.5 similar to the thioredoxin reductase-catalyzed reaction (see Table I). The total concentration of thiols in dithiothreitol (0.33 mM) was similar to the
of the precipitation assay of insulin reduction was highest at the thioredoxin concentrations were varied from 2 to 5.0 μM. The reaction was started by addition of 1 to 20 μl of E. coli thioredoxin reductase to both cuvettes and the NADPH oxidation was followed by recording the increase in absorbance at 340 nm each 1 min in the reference cuvette, until turbidity appeared. The cuvettes then were shifted and the increase in absorbance at 650 nm was followed every 1/2 min to obtain the maximal rate of precipitation.

The rate of precipitation against thioredoxin concentration (Fig. 3) was observed between 1 to 8 μM. At least 75-fold. To what extent the nonlinear rate of precipitation at high dithiothreitol and thioredoxin concentrations seen in Fig. 3 represent saturation kinetics or simply the nonlinear relationship between rate of precipitation and dithiothreitol reduction (Fig. 2) cannot be safely concluded from the data. The rate of insulin reduction at 5 mM dithiothreitol and high thioredoxin concentration was also too rapid to permit accurate measurements.

Comparison of E. coli and Calf Thioredoxin in Insulin Reduction—Thioredoxin from calf liver and calf thymus also catalyzed the dithiothreitol-disulfide reduction of insulin (Reaction 4). When compared on a weight basis, the mammalian thioredoxin in fact showed somewhat higher specific activity than E. coli thioredoxin (Table III). It was noted previously (4) that in vitro oxidized preparations of liver thioredoxin determined at steady state.

<table>
<thead>
<tr>
<th>pH</th>
<th>Thioredoxin reduction</th>
<th>Rate of NADPH oxidation</th>
<th>S-S bond reduction</th>
<th>Rate of precipitation</th>
<th>S-S bonds reduced before precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>1.7 x 10^-8</td>
<td>0.019</td>
<td>3.1</td>
<td>0.080</td>
<td>21</td>
</tr>
<tr>
<td>6.6</td>
<td>1.7 x 10^-8</td>
<td>0.048</td>
<td>7.7</td>
<td>0.150</td>
<td>32</td>
</tr>
<tr>
<td>7.0</td>
<td>1.7 x 10^-8</td>
<td>0.190</td>
<td>19.4</td>
<td>0.295</td>
<td>45</td>
</tr>
<tr>
<td>6.6</td>
<td>1.7 x 10^-7</td>
<td>0.073</td>
<td>11.8</td>
<td>0.120</td>
<td>48</td>
</tr>
<tr>
<td>7.5</td>
<td>6.6 x 10^-8</td>
<td>0.100</td>
<td>16.1</td>
<td>0.100</td>
<td>74</td>
</tr>
<tr>
<td>8.0</td>
<td>3.4 x 10^-8</td>
<td>0.270</td>
<td>43.6</td>
<td>0.140</td>
<td>113</td>
</tr>
<tr>
<td>8.5</td>
<td>3.4 x 10^-8</td>
<td>0.110</td>
<td>10</td>
<td>0.040</td>
<td>235</td>
</tr>
</tbody>
</table>

* Determined at steady state.

** Experimental Procedures.**

Fig. 2. Relation between disulfide reduction and precipitation of insulin at pH 6.5, 7.0, and 7.5. For details see Table I and "Experimental Procedures."

Total disulfide concentration in insulin (0.39 mM) in these experiments. From the level of precipitation an apparent equilibrium constant, for Reaction 4, of 2.75 was calculated.

Effect of Thioredoxin and Dithiothreitol—The sensitivity of the precipitation assay of insulin reduction was highest at pH 6.5 and this pH was chosen for further experiments. Thioredoxin concentrations were varied from 2 x 10^-7 to 2 x 10^-5 M and dithiothreitol concentrations used were 0.33, 1.0, and 5.0 mM (Table II). A catalytic effect of thioredoxin was clear already at 2 x 10^-7 M and an apparently linear relationship of the rate of precipitation against thioredoxin concentration (Fig. 3) was observed between 1 to 8 μM with 1 mM dithiothreitol. In this concentration range, the rate of precipitation may be used as a very simple assay for thioredoxin. At 1 x 10^-5 M thioredoxin the catalytic effect of thioredoxin is to accelerate the reaction compared with 1 mM dithiothreitol at least 75-fold. To what extent the nonlinear rate of precipitation...
contained inactive disulfide-bonded aggregates formed by oxidation of the structural thiol groups. Preincubation with dithiothreitol activates thioredoxin (4). When oxidized calf liver thioredoxin (4) was added directly in the insulin turbidity assay, somewhat lower values for AAm x min⁻¹ were observed initially, followed by an increase in activity with time consistent with activation by dithiothreitol. Preincubation with dithiothreitol removed this lag phase. In fact, preincubation of concentrated oxidized calf liver with 1 mM dithiothreitol in 8 M urea followed by 100-fold dilution and assay gave the highest specific activities. This suggests that complete activation of aggregated oxidized thioredoxin to thioredoxin-(SH)₂ was more efficient under the strongly dissociating conditions at 8 M urea. Furthermore, the results showed that calf liver thioredoxin may be fully renatured from solutions of 8 M urea upon subsequent dilution.

Mechanism of the Thioredoxin-catalyzed Reaction—The mechanism of the thioredoxin-catalyzed reduction of insulin with dithiothreitol (Reaction 4) is suggested to be analogous with the mechanism for thioredoxin reductase-catalyzed disulfide reduction (Reactions 5 and 6):

\[
\text{thioredoxin-SH} + \text{dithiothreitol} \rightarrow \text{thioredoxin-(SH)₂ + dithiothreitol} \]

\[
\text{thioredoxin-(SH)₂ + insulin-S₂} \rightarrow \text{thioredoxin-S₁ + insulin-(SH)₂} \]

These two partial reactions correspond to a ping-pong (15) mechanism with dithiothreitol and insulin as two enzyme substrates.

The mechanism proposed in Reactions 5 and 6 is consistent with a rapid reduction of thioredoxin-S₂ by dithiothreitol. A spectrophotometric method based on measurements of the increase in absorbance at 310 nm from the product, oxidized insulin, was tried to estimate this rate. However, the reduction of thioredoxin-S₂ (100 μM) with 100 μM dithiothreitol was complete in less than 20 s.

Stopped flow fluorescence (17) proved to be the technique which allowed the rate to be determined. The tryptophan fluorescence of thioredoxin increases 9-fold at pH 7.0 when the protein is reduced enzymatically or chemically (11, 18). As shown in Fig. 4, rapid mixing of dithiothreitol and thioredoxin was recorded after 1 min with a AAm of 0.130 x min⁻¹. The thioredoxin-containing cuvette showed precipitation after 7 min with a AAm of 0.013 x min⁻¹. The “dead time” of the instrument (17) was around 2 ms and the time scale covered in the experiment is 100 ms. The fluorescence of the fully reduced thioredoxin was recorded after 1 min as the upper line. The fluorescence change could be fitted to a single first order process. The rate constant (Kₘ) and the second order rate constant (k) were calculated (17).

Since dihydrolipoamide is a thiol similar to dithiothreitol, the effect at pH 7.0 of addition of insulin and thioredoxin was investigated. Thioredoxin, 7.8 μM, accelerated the precipitation of insulin (0.13 mM) in the presence of NADH (0.5 mM), lipoamide (0.6 mM) and lipoamide dehydrogenase. The control without thioredoxin showed precipitation after 58 min and a ΔA₄₅₀ of 0.013 × min⁻¹. The thioredoxin-containing cuvette showed precipitation after 7 min with a ΔA₄₅₀ of 0.130 × min⁻¹. This result is consistent with the following Reactions (Reactions 7 to 9):

\[
\text{NADH + H}^+ + \text{lipo-S₂} \rightarrow \text{lipoamide dehydrogenase} \rightarrow \text{NAD}^+ + \text{lip-(SH)₂} \]

\[
\text{Lip-(SH)₂ + insulin-S₂} \rightarrow \text{thioredoxin} \rightarrow \text{lip-S₁ + insulin-(SH)₂} \]

Net: \text{NADH + H}^+ + \text{insulin-S₂} \rightarrow \text{NAD}^+ + \text{insulin-(SH)₂} \]

The dependence of the reaction on lipoamide at a fixed concentration of thioredoxin (5.8 μM) is shown in Fig. 5. Since the Kₘ value of lipoamide for lipoamide dehydrogenase is around 0.3 mM (3), the reaction rate is proportional to the amount of lipo-S₂ reduced at 0.2, 0.4, and 0.6 mM lipo-S₂. Controls with only 0.6 mM lipo-S₂ and no thioredoxin or thioredoxin...
Dithiol-Disulfide Oxidoreductase Activity of Thioredoxin

FIG. 5. Turbidimetric assay of insulin reduction by dihydro-lipoamide and thioredoxin. The incubation mixture contained, in a final volume of 0.60 ml: 0.10 M potassium phosphate, 0.13 mM insulin, 0.5 mM NADH, 5.8 pM thioredoxin-S2, and the indicated concentration of oxidized lipoamide. The reaction was started by addition of 1 µg of lipoamide dehydrogenase at zero min. Controls without lipoamide (Lip-) or with 0.60 mM lipoamide but without thioredoxin (T) are also shown.

Fig. 6. Rate of NADH-oxidation and insulin reduction by thioredoxin and lipoamide and lipoamide dehydrogenase. Initially, two cuvettes each contained, in 0.600 ml of 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA: 0.13 mM insulin and 0.5 mM NADH. To one cuvette was added 0.6 mM oxidized lipoamide (Lip-S2) and the reaction was started by addition of 1 µg of lipoamide dehydrogenase (Lip-S2) to both cuvettes. The reduction of lip-S2 was followed at 340 nm (angi). After 17 min, thioredoxin-S2 (T-S2), 5.0 µM was added to both cuvettes and the rate of insulin precipitation was followed at 650 nm (×--×). Without lip-S2 showed that both lip-S2 and thioredoxin were required for activity.

The quantitative relationship between lipoamide (0.6 mM) reduction by NADPH and insulin reduction mediated by thioredoxin was studied as shown in Fig. 7. In this case the low lipoamide dehydrogenase gave saturation kinetics. A K_m value for thioredoxin of 1.3 µM was calculated under this set of conditions.

DISCUSSION

When the two interchain disulfides of insulin are split by reduction, the free B chain will aggregate and precipitate from neutral solutions at low concentration. This well known phenomenon (14) was used in this study to develop a rapid and simple spectrophotometric assay for thiol-mediated protein disulfide reduction. The quantitative relationship between disulfide reduction and the onset of a rate of precipitation was calculated from extrapolation of the rate of NADPH oxidation in the presence of NADPH and thioredoxin reductase as the reducing system for thioredoxin. The two interchain disulfides of insulin are split by thioredoxin at similar rates (5). The K_m value for insulin with thioredoxin is 11 µM (5). The concentration of insulin in the turbidimetric assay (130 pM) is thus sufficiently high to saturate the thioredoxin-(SH)2 reaction.

The main result of this study is the demonstration of thioredoxin as a dithiol-disulfide oxidoreductase that catalyzes the reduction of protein disulfides by dithiothreitol or dihydrolipoamide. Chemically reduced ribonuclease has also been found to be a very efficient dithiol substrate for thioredoxin. This result is consistent with a general function of thioredoxin in catalyzing oxidoreduction between suitable dithiols and exposed protein disulfides. The enzymatic mechanism of thioredoxin involves the reduction and oxidation of an exposed active site disulfide bridge (1) with the structure:

\[
\text{S-S} \rightarrow 2\text{H} \quad \text{SH} \quad \text{SH}
\]

The disulfide in thioredoxin-S2 and the dithiol in thioredoxin-(SH)2 both have unusual reactivities in thiol-disulfide interchange reactions. The second order rate constant for the reduction of thioredoxin by dithiothreitol obtained from the stopped flow fluorescence measurements is compared with measurements by Creighton for model disulfides (19) in Table V. Apparently thioredoxin-S2 reacts between 2 to 3 orders of magnitude faster at physiological pH values than the model disulfides. Since thioredoxin-(SH)2 shows an apparent reactivity with insulin that is around 10^4 times higher at pH 7.0 than dithiothreitol (5), the combined effect of these two rates explains the catalytic action of thioredoxin in reducing insulin

\[ \text{A. Holmgren, unpublished results.} \]
TABLE V
Rate of reduction of disulfides by dithiobitol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH 7.0</th>
<th>pH 8.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystamine</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Dithiodiglycolic acid</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Insulin</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Thioredoxin-S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1647</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Taken from work by Creighton (19).
<sup>b</sup> From Ref. 5.
<sup>c</sup> pH 7.23.

with dithiothreitol. Thioredoxin-S<sub>2</sub> and thioredoxin-(SH)<sub>2</sub> may thus be regarded as two species of an enzyme working in a ping-pong reaction. The local conformational change in thioredoxin upon oxidation observed by tryptophan fluorescence (11) and nuclear magnetic resonance spectroscopy (20) explains the differences in reactivity of the two species of thioredoxin.

Dihydriolipoamide was a very good dithiol substrate for thioredoxin. This provides a mechanism for NADH-dependent reduction of disulfides to sulfhydryl groups through the operation of lipoamide dehydrogenase. Romano and Nickerson (21) have described the existence of a specific NADH-dependent cystine reductase (EC 1.6.4.1) or NADH-cystine transhydrogenase in yeast. This cystine reductase activity has not been purified and characterized but could be identical to the combined effect of NADH, lipoamide, lipoamide dehydrogenase, and thioredoxin.

Eldjarn and Bremer (22) and Skrede (23) have studied disulfide reduction in mitochondria and shown that reduction of cystamine is NADH and lipoic acid-dependent and occurs by the lipoamide dehydrogenase enzyme in the α-oxoacid complexes. The studies of Tietze (24) also suggest mitochondria as the place of NADH-dependent disulfide reduction since only NADPH was observed to support this process in 100,000 X g supernatants of rat liver.

Is there any physiological basis for the rapid reaction between dihydriolipoamide and thioredoxin-S<sub>2</sub>? Some recent results regarding the functional organization of the pyruvate dehydrogenase complex in E. coli suggest a possible mechanism (25, 26). Lipoic acid is bound in an amide linkage to the dihydriolipoate transacetylase component of the pyruvate and α-ketoglutarate dehydrogenase multienzyme complexes in E. coli and mitochondria. Studies on the site coupling in electron and acetyl group transfer in the E. coli pyruvate dehydrogenase complex has suggested (26) that only half of the α-lipoil moieties are coupled to lipoamide dehydrogenase and NADH formation. The other half of the dihydriolipoamide groups may have a yet unidentified electron acceptor (26). Experiments with isolated E. coli pyruvate dehydrogenase complex should decide if this can be thioredoxin. Thioredoxin has been found in mitochondrial preparations from calf liver (27). The coupling between pyruvate decarboxylation and the reduction of thioredoxin may of course represent an unknown substrate cycle. This could operate in cells lacking thioredoxin reductase, as erythrocytes seem to do. It should also be noted that a novel role for lipoic acid in oxidative phosphorylation has been suggested recently (28).

The fast reduction of thioredoxin-S<sub>2</sub> by dihydriolipoamide observed in this study represents the only kinetically favorable reaction between any of the three compounds, lipoic acid, glutathione and thioredoxin. The reduction of the disulfides in these ubiquitous compounds is catalyzed by the three similar but specific dehydrogenases called NADH-lipoamide dehydrogenase, NADPH-glutathione reductase and NADPH-thioredoxin reductase. The electrode potentials (E<sub>a</sub> values), of the Lip-S<sub>2</sub>, GSSG, and thioredoxin-S<sub>2</sub> couples are similar or -0.29, -0.25, and -0.26 V, respectively (3). The small molecules GSH and lipoic acid show no particularly rapid thiol-disulfide interchange rates (3). Thioredoxin-S<sub>2</sub> is only very slowly reduced by the monothiol GSH (29) nor does thioredoxin-(SH)<sub>2</sub> react rapidly with GSSG (5).

Thioredoxin was originally considered to be a substrate cofactor for the two enzymes thioredoxin reductase and ribonucleotide reductase (30). As shown here, it should rather be regarded as a small disulfide reductase enzyme. This applies for the thioredoxins from E. coli, yeast, and mammalian cells which are homologous proteins that all give high activity in the insulin turbidimetric assay.

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