Studies on the Specificity and Mechanism of Action of Hepatic Glutathione-Insulin Transhydrogenase

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SUMMARY

Studies on the specificity and mechanism of action of hepatic glutathione-insulin transhydrogenase, an enzyme capable of catalyzing the reductive cleavage of the disulfide bonds of insulin in the presence of excess reduced glutathione, have been carried out. By coupling reactions catalyzed by glutathione-insulin transhydrogenase to that of glutathione reductase, it was indicated that during the course of enzyme action, transhydrogenase undergoes reduction by GSH and that the reduced enzyme is autoxidizable; it is inferred that this reduced form is an active intermediate during transhydrogenase-catalyzed reductions of disulfide-substrates. Under conditions of limited spontaneous air oxidation of protein sulfhydryls, transhydrogenase was shown to promote also the net oxidation of such thiols as the reduced forms of insulin and RNase.

During the process of reoxidation of reduced forms of insulin and RNase, glutathione-insulin transhydrogenase was capable of enhancing the rates of regeneration of the native proteins from their reduced precursors as determined by their immunologic and enzymic properties, respectively. Furthermore, this transhydrogenase was able to promote the reconstitution of the native activity from a reduced form of RNase already extensively oxidized by dehydroascorbate. Contrary to previous indications, glutathione-insulin transhydrogenase was found to have no observable activity in specifically directing the preferential re-establishment of the native configuration of insulin.

On the basis of evidence presented in this study, a mechanism involving a series of disulfide-interchange reactions has been proposed to explain the aforementioned reactions catalyzed by glutathione-insulin transhydrogenase. The possible relationship or identity of the transhydrogenase to other insulin-reducing and RNase-reactivating enzymes is pointed out.

In substrate specificity studies on a number of chemically modified and proteolytically degraded forms of insulin, it was found that neither biological activity nor native structure is prerequisite for activity as substrate for glutathione-insulin transhydrogenase-catalyzed reduction.

Glutathione-insulin transhydrogenase is the name proposed (1) for a purified protein fraction of bovine liver (2) capable of catalyzing the reductive cleavage of the disulfide bonds of insulin by glutathione (1, 3-5). Other small thiols such as cysteine1 and mercaptoethanol (6) may substitute for GSH in this reaction. By coupling the transhydrogenase-catalyzed reduction

\[(\text{---S--S---)} + 2 \text{GSH} \rightarrow 2 (-SH) + \text{GSSG} \] (1)

\[\text{GSSG + TPNH + H}^+ \rightarrow 2 \text{GSH} + \text{TPN}^+ \] (2)

of insulin by GSH (Reaction 1) with a glutathione reductase system capable of regenerating reduced glutathione (Reaction 2), it has been possible to study the kinetics and substrate specificity of the enzyme by a convenient spectrophotometric procedure utilizing the disappearance of the characteristic absorption of TPNH at 340 mu (1). Because of the demonstrated stoichiometry between the disappearance of TPNH and the total amount of insulin half-cystine residues present in the reaction medium (5), and in view of the accelerated appearance of separated, intact glycyl (1, 6) and phenylalanyl (1) chains during the course of enzyme reaction, the net action of the enzyme has been interpreted as a catalysis of the reductive cleavage of the disulfide bridges of insulin by GSH or similar thiol. In addition to the foregoing observations, preliminary data have been presented (5) which indicate a transhydrogenase-catalyzed formation of insulin-like protein from a mixture of reduced insulin and GSSG.

The present investigation was undertaken to inquire further into the specificity and mode of action of glutathione-insulin transhydrogenase and to clarify its observed role in the formation of insulin-like protein from chemically reduced precursors. In addition, it seemed of interest to examine the possible relationship of the transhydrogenase to other known proteins possessing similar catalytic properties. Preliminary results of this work have been presented (7).

EXPERIMENTAL PROCEDURE

Three times recrystallized ribonuclease A was obtained from Worthington. HMB2 and sodium dehydroascorbate were products of Nutritional Biochemicals and Mann Research Labora-

1 Unpublished observations.

* The abbreviation used is: HMB, sodium p-hydroxymercuro-
benzoate.
Glutathione-Insulin Transhydrogenase

Fig. 1. Representative plots obtained in the Berson-Yalow immunoassay procedure applied to insulin regeneration mixtures. A typical calibration curve is shown on the left. The dilution curves after 4 hours of reoxidation shown in the inset were obtained from paired solutions of reduced insulin which had been subjected to air oxidation at 4°C in the absence of EDTA. Except for the presence of 35 μg per ml of glutathione-insulin transhydrogenase (GIT) in one of the oxidation mixtures, the solutions were identical. Further details of the reduction and reoxidation are given in “Experimental Procedure.”

Protein Reductions—Insulin was purified from beef liver according to the procedure of Tomizawa and Halsey (2) with minor modifications (1); the final product assayed 5,000 units per mg. Crystalline bovine zinc insulin was a gift of Dr. O. K. Behrens of the Eli Lilly Research Laboratories. Zinc-free insulin was prepared from the foregoing material by exhaustive dialysis against 5 mM HCl and by lyophilization. Acetyl insulin was prepared by treatment of a 0.05 M phosphate-buffered (pH 8.0) solution of the zinc-free hormone (1 μmole per ml) with acetic anhydride (90 μmoles per ml) for 2 hours at 0°C; following prolonged dialysis against water, the reaction product was lyophilized. Extensive acetylation of protein amino groups was confirmed by ninhydrin assay. De-alanyl insulin was prepared by treatment of zinc insulin with carboxypeptidase A essentially according to the procedure of Nicol (8); following dialysis against dilute HCl, the product was lyophilized. Deoctapeptide- and dealanyl-deasparaginyl-insulins were prepared from the zinc-free hormone by incubation with trypsin and carboxypeptidase A, respectively, according to the general conditions of Young and Carpenter (9) and Slobin and Carpenter (10). After this treatment, the reaction mixtures were acidified, dialyzed against water, and lyophilized. In the case of the insulin-trypsin mixture, acetylation was preceded by a further incubation with a 10-fold molar excess of diisopropyl phosphofluoridate to inactivate the enzyme. Increases in ninhydrin color yield during the course of the enzymic reactions, as well as subsequent loss of biological activity, were consistent with the established nature of these biologically inactive products.

Except for the experiments utilizing the original transhydrogenase assay procedure (see Table II), the spectrophotometric method employed in the present study of transhydrogenase activity is a modification of the former procedure (1) using the coupled enzyme systems shown in Reactions 1 and 2. In those experiments conducted anaerobiically, the entire procedure was carried out at room temperature in Thunberg type anaerobic cuvettes from which oxygen was excluded by repeated evacuations under reduced pressure followed by replacements with nitrogen gas. Measurements of optical density relative to a control sample containing only buffer were made in the Beckman DU spectrophotometer before and after rapid mixing of the side arm contents with the contents of the main compartment of the cuvette.

Protein sulfhydryl group estimations were carried out according to the method of Ellman (11) in 0.05 M phosphate-5 M guanidine chloride-0.01 M EDTA buffer, pH 8.0.

Protein Reductions—Insulin (2.0 μmoles) or RNase (1.0 μmole) was dissolved in 1.0 ml of phosphate-buffered 8 M urea, pH 8.0, and treated with an excess of 2-mercaptoethanol (thiol-half-cystine molar ratio of 40:1). After 4 hours at 26°C the insoluble reduced proteins were washed four times by suspension in 10 volumes of cold acid-acetone solution (12). The final well drained precipitates of reduced protein were then dissolved in the appropriate aqueous solvent; reduced RNase was maintained in the cold as a stable stock solution in 0.1 M acetic acid while reduced insulin was dissolved immediately in the reoxidizing buffer. Estimations of thiol content of the reduced proteins indicated that under the above reducing conditions approximately 85 to 90% of the maximum possible sulfhydryl residues were formed in both cases.

Insulin Reoxidation-Regeneration—The reoxidation of reduced insulin was carried out at a concentration of 600 μg per ml in 0.1 M Tris buffer, pH 8.3, at 4°C and 26°C. Since earlier studies (5) on the effect of transhydrogenase on insulin regeneration had incorporated EDTA in the oxidizing buffer, this chelating agent was added in a number of experiments in the present study to assess its effect on the rate and extent of oxidation and regeneration. The reduced protein was added to the buffer, and the mixture was divided into two 5-ml portions; to one portion was added transhydrogenase to a concentration of 35 to 70 μg per ml. Reoxidations were carried out in open 50-ml Erlenmeyer flasks with occasional swirling. At various intervals of time, 0.5-ml aliquots of the regeneration mixtures were added to tubes containing 5 mg of HMB to stop further oxidation. The HMB-treated samples were stored at -20°C prior to insulin assay which was performed essentially by the immunoassay procedure of Berson and Yalow (13) as previously described (5). For each pair of aliquots of regeneration mixtures withdrawn at a given time, dilution curves, such as are shown in the inset of Fig. 1, were constructed. By reference to a calibration curve resulting from the immunoassay procedure (Fig. 1) carried out at the same time with a standard insulin solution, the insulin content of that dilution yielding a B:F ratio (13) in the range of greatest accuracy, i.e. 1 to 2, could be readily determined. Control incubations revealed that glutathione-insulin transhydrogenase itself contained no immunologic insulin-like activity.

RNase Reoxidation-Regeneration—Oxidation of reduced RNase was carried out in open vessels at 26°C in 0.1 M Tris buffer, pH 7.8, in the absence and presence of various concentrations of glutathione-insulin transhydrogenase. In those regeneration experiments employing sodium dehydroascorbate, the freshly prepared oxidant solution was added to both the control and enzyme regeneration mixtures immediately prior to the addition.
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-0.1
-E
-0.2
< -0.3
t0
L
-0.4
c0
F -0.5
a_0
<1
-0.6
-0.7

FIG. 2. Oxidation of TPNH resulting from action of glutathione-insulin transhydrogenase in the spectrophotometric assay system in the absence of disulfide-containing substrate. Transhydrogenase (500 µg) was added at zero time to a primary incubation mixture containing 16 µmoles of GSH, 0.38 µmole of TPNH, and excess glutathione reductase (G.R.) in 0.1 M phosphate-EDTA buffer, pH 7.5, at a final volume of 1.4 ml at 25° (Curve C). Insulin (1 µmole) was added to C at 8 min as indicated. In control experiments no additions were made (Curve A), or 1 µmole of insulin was added (Curve B) at zero time to the primary mixture. Further details are given in “Experimental Procedure.”

of transhydrogenase. RNase assays were carried out in 0.1 M acetate buffer, pH 5.0, by RNA digestion (14). Control experiments established that the preparation of transhydrogenase was itself completely devoid of RNase activity at concentrations employed in these regeneration studies.

RESULTS

Spectrophotometric Evidence for Reduction of Transhydrogenase by GSH—In a previous communication (1) it was shown that glutathione-insulin transhydrogenase catalysis of the reduction of insulin by GSH could be followed spectrophotometrically by coupling this reaction with the glutathione reductase system. In this procedure the addition of transhydrogenase or insulin to an otherwise complete system containing insulin (or transhydrogenase, respectively), GSH, TPNH, and glutathione reductase at pH 7.5 resulted in oxidation of TPNH which could be measured at 340 mµ. As was noted at the time, oxidation of TPNH by mechanisms other than those coupled to insulin reduction was obscured because both the reference and reaction cuvettes contained all reagents except the final component (transhydrogenase or insulin) added to the reaction cuvette to initiate the measured reaction (1). In the present study, in order to investigate the possibility that highly purified transhydrogenase could itself promote the oxidation of GSH in the absence of added insulin as substrate, the enzyme was added to a primary incubation mixture containing only GSH, TPNH, and glutathione reductase in phosphate-EDTA buffer, and the resultant change in optical density at 340 mµ was measured (Fig. 2). It can be seen that a small but significant oxidation of TPNH occurred even when no additions were made to the primary incubation mixture (Fig. 2, Curve A), indicating a small amount of baseline oxidation of GSH under these circumstances. The addition of insulin to this incubation mixture resulted in a substantial increase in the rate of TPNH oxidation (Fig. 2, Curve B) reflecting the sum of the rate of nonenzymic reduction of insulin by GSH plus the base-line rate of GSH oxidation. In contrast, a far more rapid rate of TPNH oxidation ensued following the addition of transhydrogenase to the primary mixture in the absence of insulin (Fig. 2, Curve C). It is noteworthy that after the initial burst of TPNH oxidation (Phase 1) the final rate of decline of optical density in the presence of transhydrogenase was linear (Phase 2) and still greater than the base-line rate seen in Curve A. This behavior indicated that after the initial rapid reaction with transhydrogenase (presumably to form reduced enzyme), a steady state rate of accelerated GSH oxidation had been reached, possibly involving the cyclic reduction-reoxidation of the enzyme (see below, Fig. 5). The addition of insulin after establishment of this steady state (vertical arrow at the 8-min point of Curve C, Fig. 2) resulted in an abrupt further decline in optical density followed almost immediately by the appearance of turbidity due to the formation of insoluble products (1).

In order to compare the foregoing kinetic behavior of the transhydrogenase-catalyzed oxidation of GSH with that exhibited by heavy metal ion catalysis, CuCl₂ was substituted for transhydrogenase in a reaction system otherwise identical with
throughout the observed course of the reaction it was, therefore, expected that the separate incubation of GSH with transhydrogenase in the absence of TPNH and glutathione reductase would lead to the accumulation of GSSG. Accordingly, the extent of formation of GSSG arising from a 10-min incubation of transhydrogenase with GSH was determined by adding this mixture to a separate mixture of glutathione reductase and TPNH and subsequently measuring the change in optical density at 340 μm. This entire experiment was carried out under aerobic and anaerobic conditions (Fig. 5). Under both aerobic and anaerobic conditions the base-line oxidation of TPNH in the presence of glutathione reductase was negligible during the preincubation period (0 to 10 min) and continued unchanged thereafter, following the addition of a control incubation solution of transhydrogenase (Curves A, A’). The aerobic addition of a control incubation solution of GSH resulted in an initial sharp decline in optical density due to the rapid utilization of GSSG which had been formed by spontaneous oxidation during the 10-min preincubation period and which had also been present initially as contaminant (Curve B). The continued aerobic oxidation of GSH following this initial phase is evident from the subsequent small but steady decrease in optical density with time (Curve B, 11 to 15 min). Anaerobic conditions virtually eliminated this small secondary oxidation, as seen by the second phase of Curve B’ (see below). Of added interest is the aerobic experiment depicted in Curve C; addition of the separately incubated mixture of GSH and transhydrogenase resulted in an initial extent of TPNH oxidation which exceeded the sum of the individual oxidations from the control additions of transhydrogenase and GSH. Moreover, the steady decline in optical density (second phase of Curve C), following that just described. Fig. 3 shows that although, as expected, copper ions catalyzed the disappearance of TPNH, the kinetic character of this process differed considerably from that seen with transhydrogenase catalysis in that the rate was rectilinear throughout the observed course of the reaction. Further evidence that the increased rate of GSH oxidation brought about by purified transhydrogenase in the absence of disulfide-containing substrate was not due to metal ion contamination may be summarized as follows: (a) transhydrogenase which has been heat-inactivated with respect to its insulin-reducing properties suffers almost total loss of its ability to promote GSH oxidation; (b) the protocol involved in the preparation of purified transhydrogenase (1, 2) makes use of a number of procedures which would be expected to eliminate excess quantities of heavy metal contaminants, e.g. chromatography on cation exchange resin, dialysis against buffers containing EDTA, and as a last step, isolation of anionic protein components after starch block electrophoresis; and (c) catalysis of sulfhydryl oxidation by transhydrogenase is relatively more pronounced in the presence of EDTA than in its absence (see Figs. 6 and 8). Of additional significance were the observations that the specific activity of transhydrogenase with respect to its ability to catalyze the direct oxidation of GSH increased during enzyme purification and was reproducible from preparation to preparation.

The dependence of the initial rate of oxidation of TPNH on the concentration of transhydrogenase, in the absence of added substrate other than GSH, is depicted in Fig. 4. The initial velocity of this reaction is seen to be proportional to the concentration of transhydrogenase.

These findings all pointed to a reduction of transhydrogenase by GSH with concomitant formation of GSSG which, in the presence of excess glutathione reductase, brought about the immediate oxidation of TPNH with consequent reformation of reduced glutathione. It was, therefore, expected that the separate incubation of GSH with transhydrogenase in the absence of TPNH and glutathione reductase would lead to the accumulation of GSSG. Accordingly, the extent of formation of GSSG arising from a 10-min incubation of transhydrogenase with GSH was determined by adding this mixture to a separate mixture of glutathione reductase and TPNH and subsequently measuring the change in optical density at 340 μm. This entire experiment was carried out under aerobic and anaerobic conditions (Fig. 5). Under both aerobic and anaerobic conditions the base-line oxidation of TPNH in the presence of glutathione reductase was negligible during the preincubation period (0 to 10 min) and continued unchanged thereafter, following the addition of a control incubation solution of transhydrogenase (Curves A, A’). The aerobic addition of a control incubation solution of GSH resulted in an initial sharp decline in optical density due to the rapid utilization of GSSG which had been formed by spontaneous oxidation during the 10-min preincubation period and which had also been present initially as contaminant (Curve B). The continued aerobic oxidation of GSH following this initial phase is evident from the subsequent small but steady decrease in optical density with time (Curve B, 11 to 15 min). Anaerobic conditions virtually eliminated this small secondary oxidation, as seen by the second phase of Curve B’ (see below). Of added interest is the aerobic experiment depicted in Curve C; addition of the separately incubated mixture of GSH and transhydrogenase resulted in an initial extent of TPNH oxidation which exceeded the sum of the individual oxidations from the control additions of transhydrogenase and GSH. Moreover, the steady decline in optical density (second phase of Curve C), following

![Fig. 3. Effect of CuCl₂ on the oxidation of GSH. CuCl₂ (2000 μg) was added at zero time to the primary incubation mixture described in Fig. 2.](image)

![Fig. 4. Relationship between the concentration of glutathione-insulin transhydrogenase and initial rate of TPNH oxidation in the spectrophotometric assay system in the absence of disulfide-containing substrate. Various amounts of purified transhydrogenase were added to the primary incubation mixture described in Fig. 2. Each point has been corrected for the base-line rate of TPNH oxidation in the absence of transhydrogenase.](image)
FIG. 5. Oxidation of TPNH under aerobic and anaerobic conditions resulting from the addition of a separately incubated mixture of GSH and glutathione-insulin transhydrogenase to the glutathione reductase system. The main compartment of the cuvette contained excess glutathione reductase together with 0.38 μmole of TPNH in 1.0 ml of 0.1 M phosphate-0.005 M EDTA buffer, pH 7.5. Following separate 10-min incubation periods, the following additions (each to final volume of 1.4 ml with buffer) were made (vertical arrow) from the side arm: Curves A, A', 170 μg of transhydrogenase; Curves B, B', 16 μmoles of GSH; Curves C, C', mixture of transhydrogenase and GSH (170 μg and 16 μmoles, respectively). The experiments were carried out in their entirety either aerobically (closed symbols) or anaerobically (open symbols). All values after the addition of the side arm contents have been corrected for dilution. Further details are given in "Experimental Procedure."

Fig. 6. Effect of glutathione-insulin transhydrogenase on the rate of oxidation of reduced RNase in the absence (A) and presence (B) of 10⁻² M EDTA. Reduced RNase (280 μg per ml) was dissolved in 0.1 M Tris buffer, pH 7.8, and the rate of reoxidation at 26° in the presence (○ — ○) and absence (□ — □) of transhydrogenase, 35 μg per ml, was followed by periodic measurement of thiol content. Further details are given in "Experimental Procedure."

the initial rapid decline, was considerably more rapid than the corresponding rate for GSH alone. These observations confirmed the earlier indication of a reaction between transhydrogenase and GSH with the formation of GSSG, the subsequent rapid utilization of which was brought about by excess amounts of glutathione reductase. To decide further whether this interaction represented merely enzyme catalysis of the air-oxidation of GSH, or whether transhydrogenase itself played a more direct role in this oxidation, e.g. as a reducible substrate, the preceding experiments were repeated under anaerobic conditions. Under
Fig. 7. Effect of glutathione-insulin transhydrogenase on regeneration of insulin-like protein at 26° in the absence (A) and presence (B) of 10^{-3} M EDTA. Reduced insulin was reoxidized in air in the absence (open symbols) and presence (closed symbols) of transhydrogenase (A, 70 μg per ml; B, 35 μg per ml). The dashed curves in A show the progressive declines in sulfhydryl titers. Further details are given in "Experimental Procedure."

These circumstances the initial control drop in optical density following the addition of GSH alone (Fig. 5, Curve B') was less than that arising under aerobic conditions of preincubation (Curve B) and is presumed to be due to the small amount of GSSG which is an invariable contaminant of commercial preparations of GSH. That this amount of GSSG did not, on the contrary, arise during the 10-min anaerobic preincubation by oxidation with residual traces of oxygen was indicated by the occurrence of an identical initial drop in optical density in control anaerobic experiments in which the preincubation period was omitted. The initial drop in optical density following addition of the anaerobically incubated mixture of transhydrogenase and GSH to the glutathione reductase system (Curve C') was significantly greater than that which occurred in the control experiments, indicating that oxidation of GSH by transhydrogenase had taken place, even in the absence of oxygen. Furthermore, the final rate of reaction following the initial rapid utilization of accumulated GSSG was negligible (Curve C', second phase), showing both that anaerobic conditions were being maintained and that the observed reaction of transhydrogenase with GSH had gone to completion. In comparison, the relatively rapid final rate of aerobic TPNH oxidation, as previously seen in the second phase of Curve C, further suggests that under these conditions, the enzyme, following its reduction by GSH, was being continuously reoxidized to permit further reaction with GSH.

On the basis of the absorbance data for the anaerobic experiment of Fig. 5, it can be calculated that the oxidation of 1 mole of TPNH is effected by approximately 10,000 g of transhydrogenase. A lower value, about 5,000, may be derived from the experiment shown in Fig. 2, although in this case the instability of GSH under the aerobic conditions employed may have contributed to an abnormally large absorbance change. Although limitations in the experimental procedure preclude a reliable quantitative estimate of the stoichiometry of this reaction, it would appear from these preliminary experiments that the en-
zyme may either possess a relatively low molecular weight or be composed of more than one reducible group per molecule.

**RNase Reoxidation and Regeneration**—Although previous studies had revealed the inability of transhydrogenase to enhance reduction of native RNase, it was considered desirable to examine the effect of transhydrogenase on the oxidation of the reduced form of RNase. Results of these experiments are shown in Fig. 6, A and B. The absence of EDTA and the rate of disappearance of reduced RNase thiol groups at 26° is not affected by transhydrogenase (Fig. 6A), while in buffer containing $10^{-3}$ M EDTA the rate of oxidation is significantly accelerated by transhydrogenase (Fig. 6B). These data thus confirm and extend the spectrophotometric observations described in the preceding section relative to the influence of the enzyme on the oxidation of reduced glutathione. As was to be expected, the rate of oxidation in the control solution containing EDTA (Fig. 6B) was considerably retarded in comparison with that which occurred in the counterpart solution lacking this agent (Fig. 6A), an observation explained by complexation with trace amounts of metal impurities that are known to catalyze the oxidation of sulfhydryl groups. In Table I are summarized the levels of reconstituted activity attained after the oxidation of reduced RNase in the presence and absence of transhydrogenase. In the experiments represented in this table, the degree of sulfhydryl oxidation had reached, in every instance, well over 90% completion before RNase assay. It can be seen that in all cases the level of RNase regeneration was greater in those mixtures containing glutathione-insulin transhydrogenase than in the control solutions. In the absence of sodium dehydroascorbate there was approximately a 3-fold increase in the level of regenerated RNase catalyzed by transhydrogenase. In order to effect a more rapid rate of oxidation during the course of enzymic regeneration, sodium dehydroascorbate was added to regeneration mixtures in a number of experiments shown in Table I in a manner similar to that previously employed by other workers (15, 16). In the presence of $10^{-3}$ M sodium dehydroascorbate, the regeneration rate was significantly lower than that noted previously in the absence of this oxidant. It is noted (Lines 3 and 4) that increasing the concentration of sodium dehydroascorbate (and therefore the rate of thiol oxidation), while simultaneously decreasing the time available for subsequent disulfide rearrangements (see below) leads to a much greater decrease in the control level of regeneration than that observed in the presence of transhydrogenase. This circumstance, which leads to greatly increased regeneration ratios, is undoubtedly a reflection of the ability of transhydrogenase to catalyze a rapid rate of disulfide rearrangement within an extensively oxidized and inactive form of RNase to permit the establishment of the correct disulfide bridges characteristic of the native molecule (see “Discussion”). Consistent with this concept, a 2-fold increase in the concentration of transhydrogenase under similar conditions led to a considerable increase in the level of RNase regeneration and, consequently, in the regeneration ratio (Table I, Line 5). The ability of transhydrogenase to bring about increased levels of enzymically active RNase following rapid and extensive reoxidation is strikingly similar to that exhibited by other purified fractions of bovine liver (16, 17) and pancreas (18) (see “Discussion”).

**Insulin Regeneration**—Previous evidence indicated an ability of transhydrogenase to enhance the reconstitution of insulin-like protein during reoxidation of the reduced and inactive form of the hormone (5). However, the mechanism of this transhydrogenase-catalyzed activity was not clarified, nor was it established whether this enzyme influenced the rate or ultimate magnitude of regeneration, or both. In the present study, extents of reformation of insulin-like protein during the course of reoxidation in the presence and absence of transhydrogenase were followed by immunoassay. Fig. 1 illustrates some typical data obtained by this procedure, following reoxidation of reduced insulin for a specific period of time. That the curve for the sample containing transhydrogenase lies below the control curve indicates that at a particular period of time following commencement of oxidation the enzyme mixture contained more immunoassayable insulin-like protein than did the control sample. Similar pairs of aliquots obtained during the course of regeneration under a variety of conditions were analyzed in analogous fashion, the results of which are illustrated in Fig. 7, A and B, and Fig. 8, A and B. In Fig. 7A, it may be seen that when the reoxidations were conducted in the absence of EDTA at room temperature, the rate of regeneration was but slightly affected by the presence of transhydrogenase. Concomitantly, there was no appreciable effect of the enzyme on the rate of disappearance of the protein sulfhydryl groups, an observation consistent with that made in the case of the reoxidation of reduced RNase under similar conditions (see Fig. 6). However, in buffer containing $10^{-3}$ M EDTA the rate of reformation of insulin immunologic activity was significantly increased by the presence of transhydrogenase (Fig. 7B). It is of particular interest that in neither experiment did the enzyme have any detectable effect on the final level of regeneration under these conditions. Although sulfhydryl data were not obtained for the experiment in Fig. 7B, it appears likely, in view of the results in the analogous experiment on RNase oxidation (cf. Fig. 6), that the increased rate of regeneration of immunoassayable insulin in the presence of enzyme can be ascribed primarily to the transhydrogenase-accelerated rate of oxidation of reduced insulin. It is clear from the experiment shown in Fig. 7B that such an accelerated rate of thiol oxidation could well have accounted for earlier

**Table I**

<table>
<thead>
<tr>
<th>Transhydrogenase concentration (μg/ml)</th>
<th>Sodium dehydroascorbate concentration (mM)</th>
<th>Regeneration</th>
<th>Regeneration ratio</th>
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<td>90</td>
<td>0</td>
<td>8</td>
<td>28</td>
</tr>
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<td>19</td>
<td>32</td>
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<td>90</td>
<td>10^{-3}</td>
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<td>0.4</td>
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<tr>
<td>180</td>
<td>10^{-3}</td>
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<td>37</td>
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Oxidation of 140 μg per ml of reduced RNase was carried out in the absence or presence of glutathione-insulin transhydrogenase in the amounts indicated. Sodium dehydroascorbate was included as indicated. Following oxidation for the designated periods of time, the reaction mixtures were brought to pH 5 by the addition of 0.1 M acetate buffer containing 0.1% serum albumin, and the RNase contents therein were determined. Further details are given in “Experimental Procedure.”
urea denaturation, have been reported (22, 23). The addition of following structural alteration of the protein by proteolysis or forms of oxidized protein, one of which corresponds to the native rates of disulfide rearrangements, of a limited number of stable generated immunoassayable protein in the mixture containing were identical after 50 hours, at which time the amount of re-

duction than the derivatives of the hormone. Similar increases

in the rate of reduction of insulin and other proteins by GSH, substrates, the native insulins being more resistant to such re-

duction of insulin and some of its modified forms by GSH is

Modified Insulins—The thiol-disulfide interchange (3) has occurred.

dation of the remaining thiol groups (which are necessary for

divation because of the failure to achieve complete oxidation within

time period employed; here, as in the analogous experiment at higher temperature, the increased rate of regeneration in the presence of transhydrogenase is most likely a consequence of the greater rate of oxidation of reduced protein. As with the oxidations carried out at 26° in the absence of EDTA (see Figs. 6A and 7A) the enzyme was found to accelerate only slightly the rate of oxidation of reduced protein (Fig. 8A) in comparison with oxidations conducted in the presence of this chelating agent (see Figs. 6B and 8B); the ultimate levels of oxidation (at least 90%) were identical after 50 hours, at which time the amount of re-

generated immunosassayable protein in the mixture containing enzyme exceeded that in the control mixture by approximately 50%. This increase may be explained as the result of the ability of transhydrogenase to permit the formation, through increased rates of disulfide rearrangements, of a limited number of stable forms of oxidized protein, one of which corresponds to the native molecule (the others being inactive) before nearly complete oxidation of the remaining thiol groups (which are necessary for thiol-disulfide interchange) has occurred.

Action of Glutathione-Insulin Transhydrogenase on Native and Modified Insulins—The effect of transhydrogenase on the reduction of insulin and some of its modified forms by GSH is shown in Table II. A considerable variation in the basal (non-

enzyme) rate of reduction by GSH is seen within this series of substrates, the native insulins being more resistant to such redu-
tion than the derivatives of the hormone. Similar increases in the rate of reduction of insulin and other proteins by GSH, following structural alteration of the protein by proteolysis or urea denaturation, have been reported (22, 23). The addition of transhydrogenase to the reaction mixture results, in every instance, in an increased rate of reduction. The net enzymic rates (calculated as the difference between the total enzymic rates and the corresponding basal rates) were, as found with the basal rate, greater for the modified proteins than for the parent species. This suggests that, to some extent at least, accessibility of the enzyme to the disulfide bonds of the substrate may be a limiting factor in determining the specificity of protein reductions catalyzed by this enzyme. This limitation may well explain the in-
certness of transhydrogenase toward serum albumin and RNase (1), two proteins which, in the native state, are themselves relatively resistant to reduction by small organic thiols (24). Indeed, it is clear from the present studies on the enzymic regeneration of RNase that the randomly oxidized, and presumably unfolded, form of RNase is readily susceptible to reaction with transhydrogenase. In contrast to the increased net enzymic rates observed with the modified forms of insulin, the relative per cent increase effected by transhydrogenase was greatest with the native insulins and tended to decrease with increasing basal rates. This suggests that there exists a relative degree of specificity of transhydrogenase toward native insulin, in that the latter appears to have a greater enzymic requirement for structural alteration than do those substrates more readily reducible non-

enzymically.

Previous studies have also shown that transhydrogenase ex-
hibits a moderate degree of specificity with respect to disulfide-containing substrates. Bovine plasma albumin was slowly reduced by the enzyme in comparison with insulin, oxytocin, and vasopressin, while prolactin, lipoic acid, homocysteine, and cystine were unaffected. The results described here have extended these studies to a number of derivatives of insulin with varied degrees of biological activity and demonstrate that biological integrity is not a prerequisite for the catalytic reduction of the hormone by transhydrogenase.

DISCUSSION

It is now generally accepted that net oxidation-reduction re-

ductions between excess thiol and disulfide proceed by way of the intermediate formation of mixed disulfides in a series of inter-
change reactions such as those shown below (25).

\[
\text{RSH} + \text{R'SSR}'' \rightarrow \text{RSSR} + \text{R''SH} \quad (3)
\]

\[
\text{RSH} + \text{RSSR'} = \text{RSSR} + \text{R'SH} \quad (4)
\]

The results of this paper, as well as those presented previously (1, 5), are consistent with a mechanism of action of transhydrogenase in which the enzyme is pictured as an active participant in exchange reactions of this type. This interpretation is sup-

ported by the present spectrophotometric evidence that transhydrogenase can itself be reduced by GSH as well as by the observation that under suitable conditions it can facilitate the re-

verse reaction, i.e. the oxidation of protein sulphydryl groups to reform disulfide bonds (e.g. insulin and RNase). A related aspect is the demonstrated effectiveness of the enzyme in promoting the re-establishment of the native activities of RNase and insulin, following oxidation of their reduced forms.

To account for the various reactions found to be associated with transhydrogenase, namely the reductive reactions as well as those enhancing net sulphydryl oxidations or reconstitution of biological activity from completely or partially reduced and inactive proteins, or both, it is speculated that the enzyme, present
initially in an oxidized disulfide form, (ES)$_2$, can be converted by interchange reactions with GSH or other thiol to the reduced form ESH with concomitant formation of oxidized thiol (cf. Reactions 3 and 4). The reduced enzyme would be capable of reducing other disulfide-containing proteins to their thiol forms via the intermediate mixed disulfide or of being oxidized by O$_2$ to the initial disulfide form (ES)$_2$. (ES)$_2$ as well as ESH is considered to participate directly in a series of disulfide rearrangements involved in the enzymic reconstitution of active from inactive protein substrates. The spontaneous air oxidation of ESH, a continually regenerated product of a cycle of interchange reactions initiated by (ES)$_2$, with reduced substrates, is presumed to account for the observed transhydrogenase stimulation of the oxidation (net disappearance) of the sulfhydryl groups of the reduced forms of RNase or insulin.

It is to be noted that under aerobic conditions the enzyme-catalyzed oxidation of substrate thiols competes with a simultaneous and spontaneous nonenzymic air oxidation of these same groups. Thus under conditions in which competition by rapid nonenzymic oxidation of sulfhydryl groups would be expected to occur (e.g. room temperature and absence of EDTA) no significant enzyme effect on the rate of thiol disappearance has been observed (Figs. 6A and 7A), whereas under conditions likely to retard such spontaneous oxidations (room temperature and presence of EDTA, or 4°C and absence or presence of EDTA), a stimulatory effect of the enzyme was noted (Fig. 6B and Fig. 8, A and B).

In a previous communication (5) we reported that transhydrogenase was capable of stimulating the reformation of biologically active protein substrates. The spontaneous air oxidation of ESH, a continually regenerated product of a cycle of interchange reactions initiated by (ES)$_2$, with reduced substrates, is presumed to account for the observed transhydrogenase stimulation of the oxidation (net disappearance) of the sulfhydryl groups of the reduced forms of RNase or insulin.

In a previous communication (5) we reported that transhydrogenase was capable of stimulating the reformation of biologically active insulin-like protein from the reduced and inactive precursors. At that time it was considered likely that under the conditions employed, complete thiol reoxidation of the protein had occurred in the absence as well as the presence of enzyme. It was, therefore, suggested that the enzyme-enhanced regeneration of such activity was a consequence of the specific catalysis of the reversal of Reaction 1, i.e. an enzyme-directed reformation of the "correct" disulfide bonds in insulin. However, the present data, which indicate that the chelating agent EDTA reduces the rate of air-oxidation of reduced protein, show that the stimulation of the regeneration of hormone activity by transhydrogenase previously alluded to was very likely a consequence of the enzyme-catalyzed reoxidation of the thiol groups of the reduced hormone. This conclusion follows from the observations that under conditions which limit the nonenzymic air oxidation of protein thiols, as in the presence of EDTA and low temperature (see Fig. 8B), transhydrogenase was observed to have its greatest effect on both the net oxidation of the reduced protein thiol groups and the relative regeneration of immunologic activity, whereas under conditions more favorable to spontaneous oxidation (e.g. higher temperature and absence of EDTA, Fig. 7A), transhydrogenase had an insignificant effect on these parameters. Thus, although transhydrogenase has a significant effect on the rate of insulin reconstitution, the data do not indicate an enzymatic capacity of the enzyme to direct specifically the preferential re-establishment of the native configuration.

Whereas prolonged disulfide rearrangements within an already extensively oxidized form of insulin (with low biological activity) do not lead to significantly greater increases in biological activity under present conditions, such circumstances do lead to a nearly complete reconstitution of ribonuclease (27). Analogously, it was found in the present study that transhydrogenase is capable of catalyzing the regeneration of yields of enzymically active RNase considerably higher than those observed with insulin during reoxidation of the reduced and inactive molecule. Moreover, transhydrogenase was also found to enhance significantly the reconstitution of RNase activity in a system containing a form of RNase extensively oxidized by dehydroascorbate and in which presumably only a small fraction of thiol remained. This may best be explained by the interpretation of Givol et al. (16) and Venetianer and Straub (15) for the similar activities of the "RNase-reactivating enzyme" studied in their laboratories. Accordingly, the transhydrogenase-catalyzed regeneration observed in the present study is considered to be the result of an enzyme-catalyzed series of disulfide interchange reactions among the randomized disulfide bonds of inactive forms of extensively oxidized RNase leading to the formation of what has been represented as the thermodynamically most stable form of the molecule (28) by a process referred to (16) as an "unscrambling" of its "incorrect" disulfide bonds.

In addition to the glutathione-insulin transhydrogenase of liver studied here, a number of other purified enzyme preparations have been reported which are capable either of catalyzing the reductive cleavage of insulin disulfide bonds or of accelerating the oxidative regeneration of native proteins from their reduced forms. Of immediate interest has been the RNase-reactivating enzyme isolated from liver microsomes by Anfinsen and his co-workers (16, 17, 29). This enzyme, of which a similar or identical counterpart has been described in mammalian pancreas (15, 18), accelerates the rate of regeneration of native RNase and other proteins during the oxidation of their fully or partially reduced and "scrambled" forms. Apart from their similarities with respect to RNase regeneration, the reactivating enzyme noted above and the purified transhydrogenase studied here resemble one another in a number of additional respects: (a) both activities have been found in mammalian liver (1, 2, 17, 26) and pancreas (18, 30, 31); (b) the purified liver enzymes have been reported to possess similar sedimentation coefficients (1, 2, 16); (c) both hepatic enzymes can, in the presence of small amounts of thiol, catalyze the transformation of insulin into similar soluble and insoluble products (1, 2, 6, 17); and (d) the RNase-reactivating enzyme, like transhydrogenase, is inactivated by iodoacetate, following pretreatment of the purified enzyme with mercaptoethanol.4 The possibility that these remarkably similar activities do, in fact, represent one and the same enzyme must be considered. Attention should also be directed to the recent isolation from Escherichia coli (32) of a highly purified acidic protein termed thioredoxin, which is capable of being reversibly oxidized and reduced by virtue of a single reactive cystine residue. Reduced thioredoxin, formed chemically or enzymically, can serve as a reactive intermediate in the complete reduction of insulin (33), as well as in a system involved in the reduction of ribonucleotides to deoxyribonucleotides. In addition, evidence

4 C. B. Anfinsen, private communication.
for the presence of thioredoxin in rat hepatoma has been presented (34). What appears to be a completely analogous enzyme system had previously been described in yeast by Black et al. (35).

In view of the foregoing possibilities of similarity or identity among the several insulin-reducing systems found in mammalian and other tissues, the assignment of glutathione-insulin transhydrogenase⁴ to a role in the synthesis, function, or metabolism of insulin must await further study.

REFERENCES


⁴ The term glutathione-insulin transhydrogenase (glutathione: protein disulfide oxidoreductase, EC 1.8.4.2) has been employed throughout this paper primarily as a convenience in designating the specific protein fraction of liver originally isolated by Tomizawa and Halsey (2). In view of the enzymic activity displayed toward other thiol-disulfide pairs it is recognized that a broader designation (e.g. thiol:protein disulfide oxidoreductase) may be more appropriate for this enzyme.
Studies on the Specificity and Mechanism of Action of Hepatic Glutathione-Insulin Transhydrogenase
Howard M. Katzen and Frank Tietze


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