BBA 65801

RAT-LIVER THIOL:PROTEIN-DISULPHIDE OXIDOREDUCTASE

PARI D. SPOLTER AND JOHN M. VOGEL

The Research Department, U.S. Public Health Service Hospital, San Francisco, Calif. 94118 (U.S.A.)

(Received March 29th, 1968)
(Revised manuscript received June 17th, 1968)

SUMMARY

The monothiols: glutathione, 2-mercaptoethanol and cysteine, and the dithiols: dihydrolipoate, dihydrolipoamide and dithiothreitol are substrates for rat-liver glutathione-insulin transhydrogenase (glutathione:protein-disulphide oxidoreductase, EC 1.8.4.2) with essentially identical K_m values, but the maximum rate of reaction with the monothiols is only one-half of that obtained with the dithiols. Cysteine inhibits the enzyme at high substrate concentrations. By mixed substrate experiments it has been shown that a single enzyme and the same active site(s) of the enzyme is involved in the reaction with either glutathione or dihydrolipoate. By isotope dilution experiments it has been shown that the affinity of the enzyme for the mildly iodinated samples of [^{131}I]insulin is identical to that for the unlabeled non-iodinated insulin molecule. Five mM EDTA produced 50% inhibition of the rate of reaction with either glutathione or dihydrolipoate as substrate. The inhibition by EDTA was overcome by 0.5 mM NADPH with glutathione as substrate. NADH or ascorbic acid could not replace NADPH in this respect. EDTA also had a pronounced effect on stability of the enzyme. It is suggested that a metal ion is required for activity and for maintenance of the proper conformation of the enzyme. Activation of the enzyme by Mn^{2+} (1 mM) or by Cs^+ (0.3 M) has been demonstrated.

INTRODUCTION

The presence in mammalian liver of an enzyme which inactivates insulin was reported in 1949 by MIRSKY AND BROH-KAHN[1]. The activity was measured by the effect of the insulin samples incubated in the presence or absence of the extract on rabbit blood sugar levels and the authors proposed the term "insulinase" to designate this enzyme. VAUGHAN[2] used the fall in insulin biological activity as determined by the mouse convulsion assay for measurement of the activity and for purification of this enzyme. With the availability of [^{131}I]insulin, new methods based on the extent of trichloroacetic acid-soluble counts produced from labeled substrate were devised

Abbreviations: lip(SH)$_2$, DL-dihydrolipoate; lipS$_2$, DL-α-lipoic acid; lipNH$_2$(SH)$_2$, DL-dihydrolipoamide; lipNH$_2$S$_2$, DL-lipoamide.

for measurement of the insulin degrading activity of the tissue extracts[3,4]. It was subsequently shown that reduced glutathione (GSH) greatly enhanced the insulin degrading activity of the liver[5,6]. The A chain was identified by TOMIZAWA[7] as the only trichloroacetic acid-soluble product from insulin in a reaction catalyzed by the purified beef-liver enzyme. This suggested that the enzyme promoted the reductive cleavage of disulfide bonds of insulin by low molecular weight sulfhydryl compounds such as GSH. KATZEN AND STETTEN[8] proposed the name "glutathione-insulin transhydrogenase" for the enzyme, identified the B chain of insulin as the other product from insulin in the trichloroacetic acid-precipitate and developed a continuous spectrophotometric assay for the determination of the transhydrogenase activity by coupling the reaction with glutathione reductase.

In the present paper some new information on the properties of the insulin degrading enzyme is described. The concept of dithiol group function in insulin degradation is brought forward here for the first time. It has been shown that the activity with dihydrolipoate as substrate is twice that obtained with reduced GSH as substrate. In view of the broad specificity of this enzyme with respect to the low molecular weight sulfhydryl substrate, "thiol:protein-disulphide oxidoreductase" has been used to designate this enzyme.

EXPERIMENTAL

Materials

Amorphous pork insulin (Lot PJ-6577) and amorphous beef insulin (Lot PJ-6574) were generous gifts from Dr. R. E. CHANCE of the Eli Lilly and Co. Mildly iodinated [^{131}I]insulin (bovine, List 6797), containing less than 1 atom of iodine per molecule of insulin, was purchased from Abbott Laboratories. The specific activities of the samples used varied between 4.22 and 10.1 mC/mg of [^{131}I]insulin. DL-Dihydrolipoate ($Lip(SH)_2$), BAL, DFP, L-ascorbic acid (sodium salt), β-NADH (disodium salt), NADPH (tetrasodium salt) and crystalline yeast glutathione reductase were obtained from Sigma Chemical Co. The specific activities of the samples of glutathione reductase used in these experiments were measured spectrophotometrically by observing the rate of decrease in absorbance of NADPH at 340 mμ (ref. 9) and found to be 35–65 μmoles/min per mg. The stock solution of glutathione reductase was diluted in 0.05 M phosphate buffer (pH 7.8), containing 500 μg of bovine serum albumin per ml as has been suggested by RACKER[9]. GSH, GSSG and dithiothreitol were obtained from Calbiochem. 2-Mercaptoethanol was a product of Eastman Kodak Co., cysteine hydrochloride monohydrate a product of Fisher Scientific Co. and crystalline bovine plasma albumin a product of Armour Pharmaceutical Co. Preparation and assay of dihydrolipoamide has been described in another communication[10]. All other chemicals used were commercial products of highest purity. NADH or NADPH, dissolved in 0.02 M NaOH was held at 100° for 1.5 min to destroy any oxidized pyridine nucleotide originally present and was brought to final dilution with 0.02 M NaOH to increase the stability of the solutions[11]. Solutions of monothiols and dithiols were prepared fresh before each use. In the experiments on the activation of the enzyme by metals all salts used were chlorides. CuCl was dissolved in 6 M HCl and a small aliquot was added to the reaction mixture; the final pH of the mixture was 7.2. Turbidity was observed in the reaction mixtures containing Cr^{3+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Cu^{+}. Silicomolyb-

date and phosphomolybdate solutions were prepared as described by GLENN AND CRANE[12] except that the solutions were brought to a final pH of 7.8 instead of 4.8. Molybdenum concentration of the final reaction mixtures was 1 mM and silicate or phosphate was varied to give the desired ratios.

Methods

Fresh liver from adult rats weighing 280–460 g was cut in small pieces and homogenized with 1 vol. of 0.25 M sucrose for 5 min in a Potter–Elvehjem glass homogenizer immersed in ice. The high speed supernatant fraction was obtained by centrifugation of the homogenate in a Spinco ultracentrifuge at 105 000 × *g* for 90 min at 0°. The high speed supernatant fluid was carefully pipetted from beneath the fatty layer. When dialysis was performed, 3–15 ml of a sample was dialyzed against 4.5 l of cold distilled water for 24 h with constant shaking at 0° in a refrigerator with two changes of bath fluid. Protein concentration was determined by measurement of the absorbances at 280 and 260 mμ (ref. 13) in 0.1 M potassium phosphate buffer (pH 7.8). It was found that in 0.1 M phosphate buffer the solutions remained clear, higher ratios of $A_{280\ m\mu}/A_{260\ m\mu}$ readings were obtained and more accurate protein determinations were possible than when measurements were made in distilled water. The oxidoreductase activity was determined by measurements of trichloroacetic acid-soluble counts produced from [^{131}I]insulin, essentially according to the method described by TOMIZAWA AND HALSEY[14], with the following modifications. A typical reaction mixture contained 0.50 ml of 0.2 M potassium phosphate buffer (pH 7.8); an aliquot of enzyme solution; 0.10 ml of 0.01 M GSH (pH 7.8); 0.10 ml of a substrate solution containing 0–30 μg of carrier amorphous insulin, 1 μg of [^{131}I]insulin and 1 mg bovine plasma albumin in a final volume of 1 ml. Buffer, enzyme and water were preincubated at 37° for 5 min before addition of GSH and [^{131}I]insulin to start the reaction. The albumin was included in order to prevent the adsorption of [^{131}I]insulin to glass tubes[15]. Any variation from the above procedure is noted in the legend of individual tables and figures. The mixtures were incubated in a 37° bath usually for 5 and 10 min. Immediately before terminating the reaction 0.5 ml of 2% bovine plasma albumin was added. The reaction was stopped by the addition of 1.5 ml of 10% trichloroacetic acid; tubes were stirred and allowed to stand in the refrigerator overnight or longer; then centrifuged. The supernatant was brought to 10 ml and a 1-ml aliquot was counted. The precipitate was dissolved in 30% KOH, brought to 5 ml and a 0.20-ml aliquot *plus* 0.80 ml distilled water were counted. Radioactivity was measured in a well-type gamma counter. A blank tube, lacking only the enzyme preparation, was run along with each test assay and appropriate corrections were made. In a few experiments the oxidoreductase activity was also determined by the coupled spectrophotometric procedure of KATZEN AND STETTEN[8], using only 25 μg of glutathione reductase in each tube. This amount of glutathione reductase was found to be adequate for use in the system. The molecular weight of insulin has been taken as 6000.

RESULTS

Dithiols as hydrogen donors in the oxidoreductase reaction

The kinetics of the oxidoreductase reaction with three monothiols (GSH, cysteine and 2-mercaptoethanol) and two dithiols (lip$(SH)_2$) and dithiothreitol (reduced

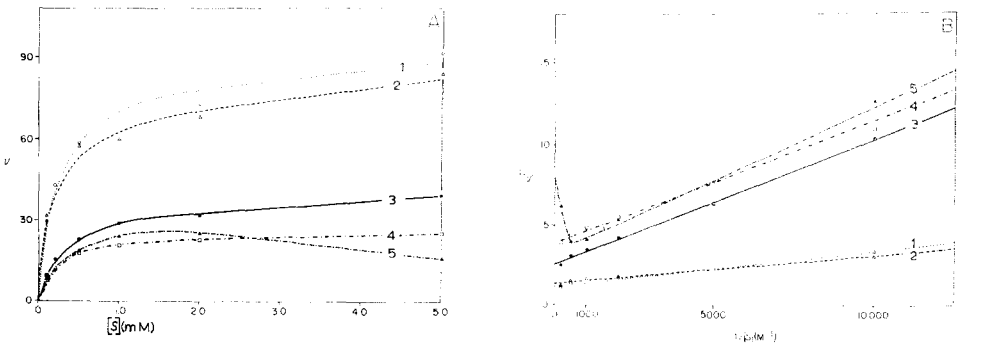


Fig. 1. Kinetics of the oxidoreductase reaction with monothiols and dithiols as substrate. The assay system contained 0.50 ml of 0.2 M triethanolamine (pH 7.8); 0.10 ml of a dialyzed high speed supernatant fraction of liver homogenate containing 6 mg protein; indicated amounts of neutralized substrates; 0.10 ml of a solution containing 14.6 μg of carrier amorphous pork insulin, 0.62 μg of [^{131}I]insulin of specific activity of 6.96 mC/mg and 1 mg bovine plasma albumin in a final volume of 1 ml. A. The effect of substrate concentration on the reaction rate. B. Reciprocal plot of the same data. v is expressed in mμmoles of insulin cleaved in 5 min $\times$ 10^2 (A) or in mμmoles of insulin cleaved in 5 min (B), assuming that both [^{131}I]insulin and non-labeled carrier are acted upon at the same rate. 1, Lip(SH)$_2$; 2, dithiothreitol, reduced form; 3, GSH; 4, 2-mercaptoethanol; 5, cysteine.

form) as substrate is presented in Fig. 1 and Table I. Data obtained with two additional dithiol compounds, *i.e.*, dihydrolipoamide and BAL is also included in Table I. The apparent K_m values for the six substrates were essentially identical, but the maximum rate of reaction with the reactive dithiols was twice that obtained with the monothiols. Cysteine inhibited the enzyme at high substrate concentrations. BAL, having the –SH groups on two adjacent C atoms, was inactive as H donor in the oxidoreductase reaction and preliminary observations indicate that BAL is a powerful inhibitor of this enzyme. Ascorbic acid did not serve as H donor to the oxidoreductase and did

TABLE I

APPARENT K_m AND v_{max} VALUES OF THE OXIDOREDUCTASE REACTION WITH MONOTHIOLS AND DITHIOLS AS SUBSTRATE

For conditions see legend for Fig. 1.

Sulfhydryl substrate	K_m (*M*)	v_{max}
Monothiols		
Glutathione, reduced	$3.1 \cdot 10^{-4}$	40
Cysteine	$2.7 \cdot 10^{-4}$	(30)*
2-Mercaptoethanol	$2.0 \cdot 10^{-4}$	26
Dithiols		
Dihydrolipoate	$2.0 \cdot 10^{-4}$	85
Dihydrolipoamide**	$1.4 \cdot 10^{-4}$	83
Dithiothreitol, reduced	$1.3 \cdot 10^{-4}$	80
2,3-Dimercaptopropanol**	—	00

* Extrapolated value.

** These two determinations were carried out in a separate experiment together with a set of reactions run with various concentrations of GSH and of lip(SH)$_2$. The conditions used were very similar to the ones described above. In the latter experiment the v_{max} obtained for GSH was 42 and that for lip(SH)$_2$ was 86.

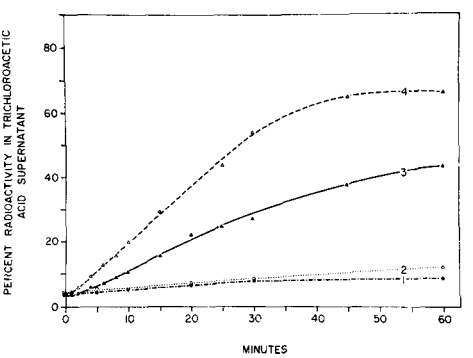


Fig. 2. Progress curve of the oxidoreductase reaction. The complete reaction mixture contained 0.50 ml of 0.2 M potassium phosphate (pH 7.8); 0.03 ml of a dialyzed high speed supernatant fraction of liver containing 1.8 mg protein; 0.10 ml of 0.01 M glutathione (or dihydrolipoate) (pH 7.8); 0.10 ml of a solution containing 15.5 μg of carrier amorphous pork insulin, 0.52 μg of [^{131}I]insulin of specific activity of 10.1 mC/mg and 1 mg bovine plasma albumin in a final volume of 1 ml. 1, no enzyme, + GSH; 2, no enzyme, + lip$(SH)_2$; 3, enzyme, + GSH; 4, enzyme, + lip$(SH)_2$.

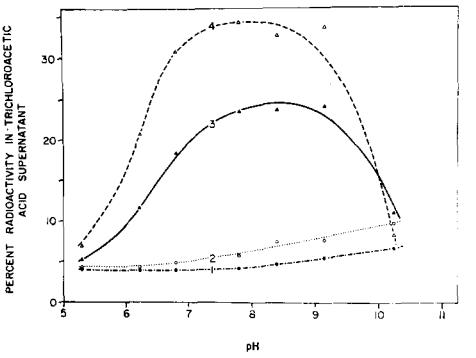


Fig. 3. pH activity curve of the oxidoreductase reaction. The assay conditions are described in the legend for Fig. 2 except that 0.10 ml of a dialyzed extract containing 7 mg protein and buffers of various pH's were used. The reactions were allowed to proceed for 5 min. 1, no enzyme, + GSH; 2, no enzyme, + lip$(SH)_2$; 3, enzyme + GSH; 4, enzyme, + lip$(SH)_2$.

not enhance the activity with either GSH or lip$(SH)_2$ as substrate. The rate of the reaction was constant for about 30 min with either GSH or lip$(SH)_2$ as substrate (Fig. 2) and only a short lag period of about 1 min was observed with either substrate. The enzyme catalyzed reaction had a rather broad pH optimum between 7.5 and 9.0 with either GSH or lip$(SH)_2$ as substrate (Fig. 3).

Mixed substrate experiment

To determine whether rat liver contained two oxidoreductases (or one enzyme with two different sites) one active with GSH and the other with lip$(SH)_2$ as substrate or if a single enzyme and the same active site(s) catalyzed the reaction with either substrate, a mixed substrate experiment was carried out.

In Case I

$$E_1 + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1A \overset{k_2}{\rightarrow} E_1 + P$$

$$E_2 + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_2B \overset{k_4}{\rightarrow} E_2 + P$$

there are two different sites or two different enzymes E_1 and E_2 each of which binds its specific substrate A or B.

In Case II

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \overset{k_2}{\rightarrow} E + P$$

$$E + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EB \overset{k_4}{\rightarrow} E + P$$

the same site(s) or the same enzyme E has a rather broad specificity and combines with both A and B. In the case under consideration here, the rate of the reaction is determined by the extent of trichloroacetic acid-soluble radioactivity produced from [^{131}I]insulin and the same product, P, is measured with either GSH (A) or lip(SH)$_2$ (B) as substrate. Steady-state treatment[16] of the above schemes yields the following expressions for the initial velocities of the forward reactions:

Case I

$$v_{tot} = \frac{V_1 A}{A + K_A} + \frac{V_2 B}{B + K_B}$$

Case II

$$v_{tot} = \frac{V_1 A}{A + K_A\left(1 + \frac{B}{K_B}\right)} + \frac{V_2 B}{B + K_B\left(1 + \frac{A}{K_A}\right)}$$

The values for V_1 and K_A were obtained from a set of reactions run with various concentrations of GSH; similarly the values for V_2 and K_B were determined from reactions carried out with lip(SH)$_2$. Simultaneously, reactions were also run with various known concentrations of both GSH and lip(SH)$_2$. Some representative data from such an experiment is presented in Table II. The results demonstrate that a single enzyme and the same active site(s) of the enzyme catalyzes the reaction with either GSH or lip(SH)$_2$.

Isotope dilution effect

When the rate of the oxidoreductase reaction is measured by the extent of trichloroacetic acid-soluble radioactivity produced from [^{131}I]insulin (S*), the effect

TABLE II

MIXED SUBSTRATE EXPERIMENT WITH GSH AND lip(SH)$_2$

The assay system contained 0.50 ml of 0.2 M triethanolamine (pH 7.8); 0.10 ml of a dialyzed high speed supernatant fraction of liver homogenate containing 7.5 mg protein; indicated amounts of neutralized sulfhydryl compound(s); 0.10 ml of a solution containing 14.6 μg of carrier amorphous pork insulin, 0.67 μg of [^{131}I]insulin of specific activity of 6.30 mC/mg and 1 mg bovine plasma albumin in a final volume of 1 ml. v is expressed in mμmoles of insulin cleaved in 5 min, assuming that both [^{131}I]insulin and unlabeled carrier are acted upon at the same rate.

GSH concn. (mM)	*lip(SH)$_2$ concn. (mM)*	$v_{calc.}$ *Case I**	*Case II***	v_{found}
0.2	0.1	0.647	0.454	0.473
5.0	0.1	0.890	0.463	0.454
0.5	0.2	0.910	0.551	0.551
2.0	0.2	1.018	0.505	0.501
0.2	0.5	0.971	0.737	0.710
0.2	1.0	1.047	0.823	0.859
1.0	1.0	1.215	0.747	0.777
0.5	2.0	1.196	0.856	0.848
0.2	5.0	1.121	0.915	0.911
2.0	5.0	1.333	0.860	0.881

* Two sites or 2 enzymes.
** Same site.

of non-iodinated, unlabeled insulin (S°) on the reaction may be considered by the following equations:

$$E + S^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES^* \overset{k_2}{\rightarrow} E + P^*$$

$$E + S^\circ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES^\circ \overset{k_4}{\rightarrow} E + P$$

Steady-state treatment[16] of this scheme yields the following expression for the reciprocal of the initial velocity of the forward reaction:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_S^*}{V}\left(1 + \frac{S^\circ}{K_S^\circ}\right) \cdot \frac{1}{S^*}$$

in which

$$v = k_2(ES^*),\ V = k_2(E_t),\ K_S^* = \frac{k_{-1} + k_2}{k_1}$$

$$K_S^\circ = \frac{k_{-3} + k_4}{k_3}$$

According to this scheme, addition of carrier insulin should result in a competitive type of inhibition and if S^* is acted upon by the enzyme at the same rate as the unlabeled S° then $k_1 = k_3$, $k_{-1} = k_{-3}$, $k_2 = k_4$ and therefore $K_S^* = K_S^\circ$. Competitive inhibition of the oxidoreductase by non-iodinated, unlabeled insulin with either GSH or lip(SH)$_2$ as substrate is shown in Figs. 4A and B, respectively. The K_S^* value calculated from these data with either GSH or lip(SH)$_2$ was $1 \cdot 10^{-6}$ M. The formula for calculation of the K_S° (or K_i) values at each substrate concentration has been presented in a previous communication[17]. The values obtained for K_S° with 2.5, 5.0, 10 and 20 μM

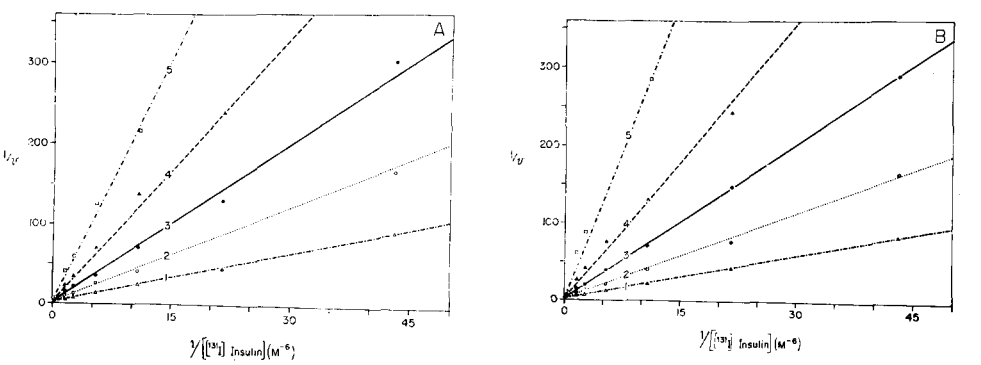


Fig. 4. Competitive inhibition of the oxidoreductase by non-iodinated, unlabeled insulin. The assay system contained 0.50 ml of 0.2 M triethanolamine (pH 7.8); 0.10 ml of a dialyzed high speed supernatant fraction of liver homogenate containing 6 mg protein; 0.10 ml of 0.01 M glutathione (or dihydrolipoate), (pH 7.8); 0.10 ml of a solution containing 0, 15, 30, 60 or 120 μg of carrier amorphous beef insulin, indicated amounts of [^{131}I]insulin (beef) of specific activity of 4.22 mC/mg and 1 mg bovine plasma albumin in a final volume of 1 ml. A. Summary of results obtained with GSH as substrate. B. Results obtained with lip(SH)$_2$ as substrate. v is expressed in mμmoles of [^{131}I]insulin cleaved in 10 min (A) or in 5 min (B). 1, no inhibitor; 2, 15 μg of carrier beef insulin; 3, 30 μg of carrier; 4, 60 μg of carrier; 5, 120 μg of carrier.

unlabeled insulin were 2.5 ± 0.3, 2.2 ± 0.2, 2.1 ± 0.1, and 2.1 ± 0.1 μM with GSH as substrate and 1.9 ± 0.3, 2.0 ± 0.2, 2.0 ± 0.2 and 1.8 ± 0.3 μM with lip$(SH)_2$ as substrate, respectively. These K_S° values are mean ± standard error of the mean for six observations. The differences between the K_S° values obtained at various concentrations of S° were not statistically significant and thus a linear inhibition was observed. The $K_S^\star$ value (1 μM) and the K_S° value (about 2 μM) are of the same order of magnitude and therefore the affinity of the enzyme for the mildly iodinated samples of insulin is within experimental error identical to that for the unlabeled non-iodinated insulin molecule.

Although no kinetic analysis was carried out and the reaction mixtures did not contain a low molecular weight sulfhydryl substrate, a similar competition between unlabeled and labeled insulin has been reported previously by MIRSKY, PERISUTTI AND DIXON[3].

Effects of EDTA

Effect of EDTA on activity of oxidoreductase

In most published procedures for the determination of glutathione-insulin transhydrogenase activity, the recommended method includes the presence of 5 mM EDTA in the reaction mixture[8,14]. The results summarized in Table III show that 5 mM EDTA produced about 50% inhibition of the rate of reaction with either glutathione or dihydrolipoate as substrate when a high speed supernatant fraction of rat-liver homogenate was used as a source of the enzyme. In the experiments summarized in Table III the rate of the reaction was measured by the extent of trichloroacetic acid-soluble counts produced from [^{131}I]insulin. When the coupled spectrophotometric procedure of KATZEN AND STETTEN[8] was used for the determination of the glutathione-insulin transhydrogenase activity, no inhibition by 5 mM EDTA could be detected with an identical source of enzyme preparation. It was therefore decided to test the

TABLE III

EFFECT OF EDTA AND NADPH ON ACTIVITY OF OXIDOREDUCTASE

The assay system contained 0.1 M potassium phosphate (pH 7.8); 0.05 ml of a high speed supernatant fraction of liver homogenate containing 4.4 mg protein; 1 mM GSH (or lip$(SH)_2$) (pH 7.8); 16 μg of carrier amorphous pork insulin, 0.71 μg of [^{131}I]insulin of specific activity of 6.15 mC/mg and 1 mg bovine plasma albumin in 1 ml. Additions were as follows: 5 mM EDTA (pH 7.8); 0.5 mM NADH or NADPH; 1 mM ascorbic acid; yeast glutathione reductase (25 μg/ml). The reactions were allowed to proceed for 10 min.

NADPH	*NADH*	*Ascorbic acid*	*Glutathione reductase*	*% Radioactivity in trichloroacetic acid supernatant*			
				GSH		*lip$(SH)_2$*	
				+EDTA	*No EDTA*	*+EDTA*	*No EDTA*
−	−	−	−	13.6	34.2	25.3	59.2
+	−	−	−	28.9	38.0	24.6	57.8
−	+	−	−	12.8	32.1	25.8	53.3
−	−	+	−	13.1	32.7	24.7	58.4
−	−	−	+	15.0	37.4	26.7	62.7
+	+	+	+	29.7	45.3	27.1	60.2

effect of auxillary components of the spectrophotometric assay, *e.g.* NADPH and yeast glutathione reductase on the oxidoreductase activity as measured by the radioactivity procedure. The results of such an experiment are presented in Table III. It was found that the inhibition by EDTA was overcome by 0.5 mM NADPH with GSH as substrate. NADPH had no effect on the rate of reactions in the absence of EDTA and did not overcome the inhibition by EDTA with dihydrolipoate as substrate. NADH or ascorbic acid could not replace NADPH in this respect.

Effect of EDTA on stability of oxidoreductase

Another remarkable action of EDTA found in the course of these studies was its pronounced effect on stability of the enzyme. Whereas the enzyme retained almost all of its activity upon incubation at 37° in phosphate buffer for 5 h (Figs. 5A and B

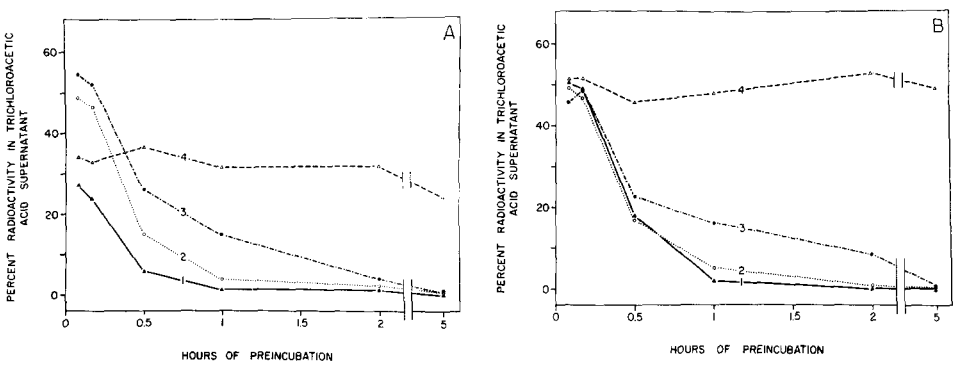


Fig. 5. Effect of EDTA on stability of the oxidoreductase. The preincubation mixtures contained 0.50 ml of 0.2 M potassium phosphate–10 mM EDTA (pH 7.8); 0.10 ml of a high speed supernatant fraction of liver homogenate containing 9.5 mg protein and distilled water in 1 and 2; the same *plus* 0.5 μmole of NADPH in 3; and 0.50 ml of 0.2 M potassium phosphate (pH 7.8); 0.05 ml of the high speed supernatant fraction and distilled water in 4. The mixtures were allowed to stand in a 37° bath with occasional shaking for the indicated periods of time. In the experiment described in A the reactions were then started by addition of 0.10 ml of 0.01 M GSH (pH 7.8) to Tubes 1, 3 and 4; 0.10 ml of 0.01 M GSH (pH 7.8) *plus* 0.5 μmole of NADPH to Tube 2; and 0.10 ml of a solution containing 15 μg of carrier amorphous pork insulin, 0.79 μg of [^{131}I]insulin of specific activity of 6.47 mC/mg and 1 mg bovine plasma albumin to all tubes. In the experiment described in B identical amounts of lip$(SH)_2$ were used instead of GSH. The final volume was 1 ml. The reactions were stopped after 10 min.

No. 4), all activity was lost within 30–60 min if the buffer contained 5 mM EDTA (Figs. 5A and B, No. 1). NADPH in the preincubation mixture reduced the rate of inactivation of the enzyme by EDTA but did not completely prevent it (Fig. 5A and 5B, No. 3).

Activation by metals

Prolonged dialysis of the high speed supernatant fraction of rat-liver homogenate against distilled water does not remove the activating metal ion. The dialyzed extracts are still inhibited by 5 mM EDTA to the extent of 50%. Also, usually between 70 and 100% of the original activity is recovered after dialysis. To demonstrate the metal requirement of the oxidoreductase it was necessary to incubate a sample of the supernatant with an equal volume of 0.2 M potassium phosphate–10 mM EDTA (pH 7.8)

TABLE IV

METAL ION ACTIVATION OF THE OXIDOREDUCTASE

The assay system contained 0.1 M triethanolamine (pH 7.8); 0.10 ml of Prep. A containing 1.3 mg protein or 0.10 ml of Prep. B containing 1.5 mg protein; 1 mM $lipNH_2(SH)_2$; indicated amounts of the cation; 15 μg of carrier amorphous beef insulin, 0.83 μg of [^{131}I]insulin of specific activity of 6.24 mC/mg and 1 mg bovine plasma albumin in 1 ml.

Cation	*Concn. (mM)*	*Prep. A*		*Prep. B*	
		*% Radioactivity**	*Specific activity***	*% Radioactivity**	*Specific activity***
No addition	—	3.0	0.23	29.1	1.94
Mn^{2+}	0.001	2.6	0.20	29.9	2.00
	0.1	26.0	2.04	32.3	2.15
	1.0	55.4	4.36	30.8	2.05
	5.0	40.8	3.21	30.2	2.01
	10.0	28.7	2.26	21.0	1.40
Mg^{2+}	0.001	2.7	0.21	30.2	2.04
	0.1	9.7	0.76	30.4	2.03
	1.0	20.5	1.61	28.9	1.92
	5.0	24.6	1.93	31.9	2.12
	10.0	31.6	2.48	36.6	2.44
Ca^{2+}	1.0	7.2	0.57		
Rb^{+}	300	20.2	1.59		
Cs^{+}	300	31.0	2.44		

* % Radioactivity in trichloroacetic acid supernatant after 10 min of reaction *minus* respective blanks.

** % Radioactivity in trichloroacetic acid supernatant per min per mg protein.

in a 37° bath for 5 min and then dialyze the mixture (Prep. A). Another aliquot of the same sample was treated identically except that EDTA was not included in the incubation mixture (Prep. B). In the absence of added cations Prep. A had very low activity whereas Prep. B was fully active (Table IV). Addition of $1 \cdot 10^{-4}$ M Mn^{2+} to the reaction mixture completely restored the activity of the inactive Prep. A. At $1 \cdot 10^{-3}$ M Mn^{2+} the specific activity of the Prep. A was twice that of the original sample. At still higher concentrations of Mn^{2+} the velocity of the reaction fell off again. Mg^{2+} could also stimulate the rate of the reaction although at higher concentrations than Mn^{2+}. Ca^{2+} at 1 mM was partially active. Rb^{+} and Cs^{+} at 0.3 M were activators of this enzyme. Addition of Mg^{2+} or Mn^{2+} had no effect on the activity of the Prep. B except that Mn^{2+} at 10 mM produced about 30% inhibition.

In similar experiments it has been found that none of the cations Cr^{3+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Cu^{+}, added to a final concentration of 1 mM or of the ions Li^{+}, K^{+}, NH_4^{+} and $Tris^{+}$, added to a final concentration of 0.3 M stimulates the rate of the reaction. MoO_3 (1 mM), Na_2SiO_3 (1 mM), silicomolybdate at Mo:Si ratios of 1:1, 5:1, 10:1, 20:1, 100:1 and phosphomolybdate at Mo:P ratio of 10:1 (Mo at 1 mM) did not stimulate the rate of the reaction.

General properties of the oxidoreductase

Previous finding[18] of the localization of the insulin degrading enzyme in the high speed supernatant fraction of rat-liver homogenate was confirmed using assay

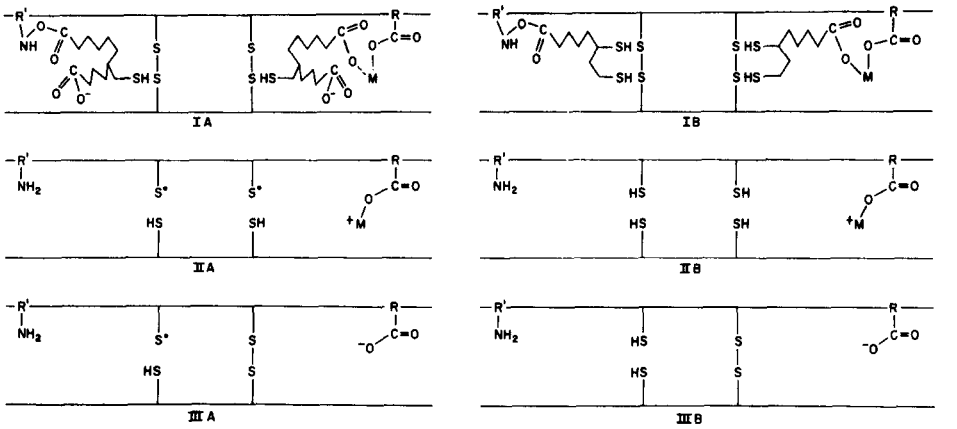


Fig. 6. Schematic diagram of the active site of the oxidoreductase.

systems containing 1 mM GSH. Incubation of a sample of the whole homogenate of that liver with DFP, under the conditions which would have produced complete inactivation of trypsin or chymotrypsin (1 mM DFP; 0.02 M potassium phosphate (pH 7.8); incubated in a refrigerator overnight), did not result in any change in the glutathione-insulin transhydrogenase activity. ATP–Mg^{2+} (6 mM each) in 0.1 M glycylglycine (pH 7.8), had no effect on glutathione-insulin transhydrogenase activity of a dialyzed high speed supernatant fraction of rat-liver homogenate as measured by either radioactivity or coupled spectrophotometric procedure.

TABLE V

EFFECT OF VARIOUS BUFFERS ON ACTIVITY OF OXIDOREDUCTASE

The assay system contained 0.1 M of the indicated buffer anions (pH 7.8); 0.05 ml of a high speed supernatant fraction of liver homogenate containing 4.4 mg protein; 1 mM of GSH (or dihydrolipoate) (pH 7.8); 16 μg of carrier amorphous pork insulin, 0.71 μg of [^{131}I]insulin of specific activity of 6.15 mC/mg and 1 mg bovine serum albumin in 1 ml. The reactions were allowed to proceed for 5 min.

Anions	*% Radioactivity in trichloroacetic acid supernatant*	
	GSH	*lip(SH)$_2$*
Phosphate	17.1	34.2
Glycylglycine	12.0	21.0
Tris	17.2	33.6
Triethanolamine	18.3	36.5
Borate	10.5	25.0

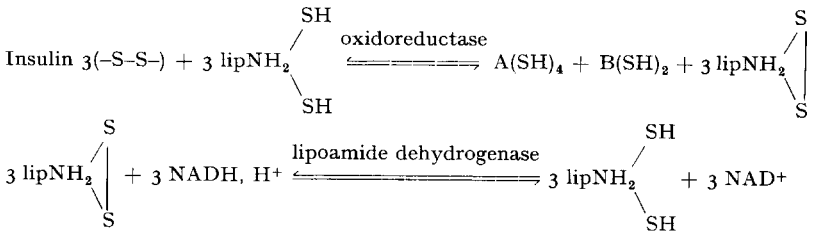
The effect of various buffer anions on the oxidoreductase activity is presented in Table V. The rate of the reaction was the same in phosphate, in Tris or in triethanolamine with either GSH or lip$(SH)_2$ as substrate. The reaction in glycylglycine and borate buffers was partially inhibited; the rates were about 60–70% of that observed in phosphate buffer. Similar results were obtained when dialyzed extracts were used.

DISCUSSION

We have demonstrated that with dithiols as hydrogen donors, the reduction of the insulin disulfide bonds catalyzed by the rat-liver oxidoreductase proceeds at twice the rate found with monothiols. This cannot be explained by differences in the oxidation–reduction potentials of these sulfhydryl compounds. The most reliable values of E'_0 at pH 7 for cystine–cysteine system are approx. −0.33 V (refs. 19, 20), those for GSSG–GSH are approx. −0.32 V (refs. 19, 20), those for $lipS_2$–$lip(SH)_2$ are approx. −0.32 V (refs. 21–24) and that for oxidized–reduced dithiothreitol is −0.33 V (ref. 25). Most likely the steric factors on the enzyme and the enzyme substrate binding are responsible for this differential effect. Fig. 6 is a schematic diagram of the active site of oxidoreductase, which forms a useful working model for future experiments and which explains satisfactorily all of the diverse experimental results obtained so far with this enzyme. It is assumed that the enzyme possesses at least 2 disulfide groups at the active site which are reversibly reduced and oxidized during catalysis with $lip(SH)_2$ as substrate (IB → IIB → IB). With GSH as substrate, it is postulated that one sulfur of each reactive disulfide group accepts one electron and one hydrogen atom leaving the other sulfur of the original disulfide as a free radical (IIA). Alternatively, it may be postulated that each disulfide group forms one –SH and one –SSG (mixed disulfide). In either case this would result in a half-reduced enzyme and in reduction of the catalytic action of the enzyme by one-half as compared to the fully reduced enzyme (IIB) which forms with $lip(SH)_2$ as substrate. A metal ion is necessary for the binding of one of the two molecules of GSH or $lip(SH)_2$ to the enzyme (the molecules represented in the right side of the figures) and its removal by EDTA would result in loss of this portion of the active site (IIIA and IIIB). This would explain a 50% reduction in the rate of reactions by EDTA with either GSH or $lip(SH)_2$ as substrate (Table III). The binding of the other molecule of GSH or $lip(SH)_2$ to the enzyme is less susceptible to inhibition by EDTA probably because either (a) the metal ion is more tightly bound to the enzyme than in the case of the first molecule, (b) the metal ion is less accessible to removal by EDTA because of steric hindrances, or (c) there is no metal ion involved in the binding of the second molecule of substrate. The latter possibility only is represented in Fig. 6. In the presence of EDTA, NADPH reduces the –S· (or –SSG) in Fig. 6 IIIA to –SH resulting in a structure identical to that represented in IIIB. This interpretation of results is consistent with the spectrophotometric observations reported recently by KATZEN AND TIETZE[26]. They observed oxidation of NADPH by purified beef-liver transhydrogenase in the presence of GSH and EDTA. NADPH has no effect on the rate of reaction in the absence of EDTA (Table III), thus, it would seem that a position on the enzyme vacated by a metal ion is required for coenzyme binding. The remarkable effect of EDTA on stability of the enzyme (Fig. 5) suggests that the metal ion besides its function in the binding of the substrate to the enzyme is also responsible to a large degree for maintaining intact the tertiary and quarternary structure of the protein molecule. Thus removal of the metal ion by EDTA results in a progressive change in conformation of the protein structure and loss of total activity.

The presence in rat liver of a soluble lipoamide dehydrogenase has recently been demonstrated in our laboratory[10]. The reduction of insulin by $lipNH_2(SH)_2$ and liver oxidoreductase and the regeneration of $lipNH_2(SH)_2$ from $lipNH_2S_2$ by reduced

pyridine nucleotide and lipoamide dehydrogenase can be represented by the following reactions:



The two activities may be normally coupled in the cell and this considerably widens the possibilities for a physiological role for the cytoplasmic lipoamide dehydrogenase.

ACKNOWLEDGMENT

This work was supported by grant No. AM-7242 of the U.S. Public Health service.

REFERENCES

1 I. A. MIRSKY AND R. H. BROH-KAHN *Arch. Biochem.*, 20 (1949) 1.
2 M. VAUGHAN, *Biochim. Biophys. Acta*, 15 (1954) 342.
3 I. A. MIRSKY, G. PERISUTTI AND F. J. DIXON, *J. Biol. Chem.*, 214 (1955) 397.
4 H. H. TOMIZAWA, M. L. NUTLEY, H. T. NARAHARA AND R. H. WILLIAMS, *J. Biol. Chem.*, 214 (1955) 285.
5 H. T. NARAHARA, H. H. TOMIZAWA AND R. H. WILLIAMS, *Proc. Soc. Exptl. Biol. Med.*, 92 (1956) 718.
6 H. T. NARAHARA AND R. H. WILLIAMS, *J. Biol. Chem.*, 234 (1959) 71.
7 H. H. TOMIZAWA, *J. Biol. Chem.*, 237 (1962) 428.
8 H. M. KATZEN AND D. STETTEN, *Diabetes*, 11 (1962) 271.
9 E. RACKER, *J. Biol. Chem.*, 217 (1955) 855.
10 P. D. SPOLTER AND J. M. VOGEL, in preparation.
11 O. H. LOWRY, J. V. PASSONNEAU AND M. K. ROCK, *J. Biol. Chem.*, 236 (1961) 2756.
12 J. L. GLENN AND F. L. CRANE, *Biochim. Biophys. Acta*, 22 (1956) 111.
13 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 451.
14 H. H. TOMIZAWA AND Y. D. HALSEY, *J. Biol. Chem.*, 234 (1959) 307.
15 R. WISEMAN, JR. AND B. E. BALTZ, *Endocrinology*, 68 (1961) 354.
16 R. A. ALBERTY, *J. Am. Chem. Soc.*, 75 (1953) 1928.
17 P. D. SPOLTER, R. C. ADELMAN AND S. WEINHOUSE, *J. Biol. Chem.*, 240 (1965) 1327.
18 H. T. NARAHARA, H. H. TOMIZAWA, R. MILLER AND R. H. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 675.
19 P. D. BOYER, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 1, Academic Press, New York, 1959, p. 511.
20 W. M. CLARK, *Oxidation–Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, 1960, pp. 471–487.
21 B. KE, *Biochim. Biophys. Acta*, 25 (1957) 650.
22 D. R. SANADI AND R. L. SEARLS, *Biochim. Biophys. Acta*, 24 (1957) 220.
23 D. R. SANADI, M. LANGLEY AND R. L. SEARLS, *J. Biol. Chem.*, 234 (1959) 178.
24 J. ŠPONAR AND M. JIRSA, *Biochim. Biophys. Acta*, 29 (1958) 434.
25 W. W. CLELAND, *Biochemistry*, 3 (1964) 480.
26 H. M. KATZEN AND F. TIETZE, *J. Biol. Chem.*, 241 (1966) 3561.