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Mechanism of action of glutathione–insulin transhydrogenase. Presence of a functional sulfhydryl group for activity

Glutathione–insulin transhydrogenase (glutathione:protein-disulfide oxidoreductase, EC 1.8.4.2) promotes the cleavage of the disulfide bonds of insulin[1–4] and also the formation of insulin from its reduced A and B chains[5,6]. The enzyme carries out this reaction by catalyzing sulfhydryl–disulfide interchange[5,7]. In the present paper, the mechanism of this reaction has been further investigated. The results show that a sulfhydryl residue on the enzyme participates in the reaction. An abstract reporting preliminary data has appeared[8]. The enzyme has been isolated from beef liver[9], human liver[3] and from beef pancreas[4]. The experiments reported in the present communication were carried out with the enzyme obtained from beef pancreas.

Beef pancreatic GSH–insulin transhydrogenase, purified by starch-block electrophoresis[4], was further purified by chromatography on a Sephadex G-100 column (Fig. 1). The details are described in the legend of Fig. 1. The enzyme was present in the second protein peak. The symmetrical protein peak was superimposed upon the peak of enzyme activity. Re-chromatography of this protein peak on the Sephadex G-100 column showed a single protein peak with the same elution volume. The molecular weight of the enzyme was estimated by the molecular sieve technique[10]. 5 mg each of blue dextran (mol. wt. 200 000), bovine serum albumin (mol. wt. 67 000), pepsinogen (mol. wt. 43 000), trypsinogen (mol. wt. 23 700), cytochrome *c* (mol. wt. 12 400) in a total of 1.5 ml of 0.1 M phosphate buffer (pH 7.5) was applied to the same column and eluted as described in Fig. 1. The blue dextran gave the void

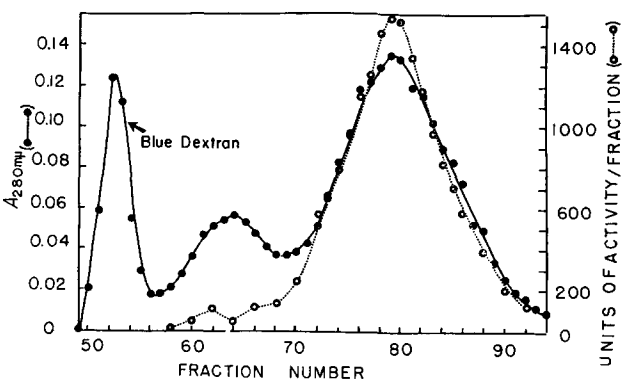

Fig. 1. Fractionation of GSH–insulin transhydrogenase on Sephadex G-100. The lyophilized enzyme (9.75 mg), purified through the step of starch-block electrophoresis[4], was dissolved in 1 ml of H_2O and dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) for 24 h. Blue dextran (1 mg) in 0.1 ml of phosphate buffer was added to the dialyzed enzyme solution and the volume was brought up to 1.5 ml with the same buffer. This solution was applied to a 2.5 cm × 92 cm column of Sephadex G-100 which had been equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) for 10 days. The column was eluted with the same buffer at the rate of 6 min per fraction (2.6 ml). All steps were carried out at 3°. Protein was detected by measuring the absorbance of each fraction at 280 mμ and the transhydrogenase activity of each fraction was determined with the radioactive assay as previously described[4].

TABLE I

EFFECT OF THIOL REAGENTS ON THE ACTIVITY OF GSH–INSULIN TRANSHYDROGENASE

All incubations were carried out at 37°. In Expt. I, the enzyme (9.2 μM) was incubated alone, in Expt. II in 1 mM GSH, and in Expt. III in 1 mM 2-mercaptoethylamine in a total volume of 0.5 ml. After 5 min, 0.1 ml of buffer or 0.1 ml of the indicated thiol reagent solution (10 mM) was added and the incubation was continued for another 5-min period. The protein was freed of reagents by dialysis against the buffer for 72 h in the cold room. During the dialysis the buffer was changed every 8–10 h. The buffer used for all the experiments was 0.1 M potassium phosphate (pH 7.5) containing 5 mM EDTA. The resulting solutions were assayed for enzymatic activity by the radioactive assay (using [^{125}I]insulin as substrate) as previously described[4]. The figures shown in the parentheses indicate the number of experiments performed.

Expt. No.	*Enzyme treatment*	*Relative enzymatic activity*
I	A. Buffer	100 (10)
	B. Iodoacetate	104 (4)
	C. Iodoacetamide	100 (4)
	D. *N*-Ethylmaleimide	94 (1)
II	A. GSH + buffer	98 (4)
	B. GSH + iodoacetate	0 (4)
	C. GSH + iodoacetamide	0 (4)
	D. GSH + *N*-ethylmaleimide	0 (4)
III	A. Mercaptoethylamine	100 (2)
	B. Mercaptoethylamine + iodoacetate	0 (2)
	C. Mercaptoethylamine + *N*-ethylmaleimide	0 (2)

volume of the column. A standard curve of the ratio of elution volumes of the proteins of known molecular weights to the void volume *versus* the logarithm of the molecular weight was constructed. The molecular weight of the enzyme calculated from the standard curve was 58 000. Molar concentrations of the enzyme used in the present paper are based on this value.

The effect on the enzymatic activity of the reaction of three thiol reagents with the enzyme are summarized in Tables I and II. The details of the procedure are described in the legends of the tables. When the enzyme was treated with thiol reagents prior to incubation with GSH and substrate, there was no loss of activity. Even when the enzyme was treated with 0.1 ml of 200 mM thiol reagent (20-fold greater concentration), the enzyme remained completely active. When it was preincubated with GSH and then treated with thiol reagent, there was complete loss of activity. The possibility that the glutamyl or glycine portion of the alkylated GSH may be inhibiting the enzyme activity was ruled out for two reasons. When 2-mercaptoethylamine[4] instead of GSH was used as a thiol in the system, the same type of inhibition was observed. Also when carboxymethylated GSH was included in the enzymatic assay, it did not cause any inhibition of enzymatic activity. The inhibition could not be due to incomplete removal of thiol reagents since when the dialysis period was extended to 5 days, the same results were obtained.

The number of cysteine residues involved in the enzymatic activity were estimated by the use of ^{14}C-labeled thiol reagents (Table II). In the presence of GSH, nearly 1 mole of ^{14}C-labeled thiol reagent was incorporated per mole of enzyme with each thiol reagent and there was complete loss of enzymatic activity. Even when

TABLE II

BINDING OF ^{14}C-LABELED THIOL REAGENTS BY GSH–INSULIN TRANSHYDROGENASE

Details of the procedure were the same as described for Expt. II under Table I except that ^{14}C-labeled thiol reagents were used. Radioactivity in the dialyzed solutions was measured in naphthalene–dioxane-counting fluid[11] with a Packard liquid scintillation spectrometer. The figures in the parentheses indicate the number of experiments performed.

Enzyme treatment	*Moles of [^{14}C]-thiol reagent bound per mole of enzyme*
A. GSH + [^{14}C]iodoacetate	0.77 (3)
B. GSH + [^{14}C]iodoacetamide	1.03 (7)
C. GSH + [^{14}C]*N*-ethylmaleimide	0.99 (1)

incubation of the enzyme with thiol reagents (following the 5-min preincubation with GSH) was increased from 5 min to 30 min, only 1 mole of thiol reagent was bound. In the absence of GSH, only 0.1 mole of [^{14}C]thiol reagent per mole enzyme was incorporated, but there was no loss of enzyme activity.

These data indicate that a sulfhydryl group is present in the active site or near the active site of the enzyme and that this sulfhydryl residue participates in the catalytic reaction of sulfhydryl–disulfide interchange between the enzyme and insulin. The involvement of groups other than SH may be ruled out because of the pH of the reaction mixture (pH 7.5), the rapidity of the reaction (5 min), the known specificity of *N*-ethylmaleimide for SH groups[12] and the requirement of thiol. This cysteine residue must be either "buried" or combined in some form so that it becomes fully available only after a reaction with a thiol. GSH or mercaptoethylamine might produce the active cysteine either by bringing about a conformational change in the enzyme or by reducing a disulfide bond. Other explanations for these findings are possible and further work is being undertaken to establish the mechanism involved.

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