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ACCELERATION OF REGENERATION OF INSULIN ACTIVITY FROM ITS INACTIVE REDUCED A AND B CHAINS BY PANCREATIC GLUTATHIONE-INSULIN TRANSHYDROGENASE

P. T. VARANDANI
The Fels Research Institute, Yellow Springs, Ohio (U.S.A.)

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SUMMARY

1. Data are presented which show that GSH–insulin transhydrogenase (thiol: protein-disulfide oxidoreductase, EC 1.8.4.2) which promotes the cleavage of disulfide bonds of insulin is also capable of promoting the regeneration of insulin activity from inactive reduced A and B chains of insulin by catalyzing sulfhydryl–disulfide interchange. With the conditions used, insulin activity regained was about 15 times as much in the presence as in the absence of transhydrogenase. There was complete agreement in the estimates of regenerated insulin activity between immunoassay and fat pad assay.

2. When the reduced chains were allowed to oxidize individually, it was observed the rate of disappearance of 2 of the 4 SH groups of A chain was faster than that of the other 2. In the case of B chain, there was a rapid loss of 0.3–0.6 sulfhydryl group and thereafter almost no further loss of SH group occurred.

INTRODUCTION

Work from this laboratory has shown that GSH–insulin transhydrogenase (thiol:protein-disulfide oxidoreductase, EC 1.8.4.2) promotes the cleavage of disulfide bonds of insulin in the presence of a simple thiol such as GSH by catalyzing sulfhydryl–disulfide interchange[1]. This enzyme has been isolated from beef pancreas[2],beef liver[3], and human liver[4]. In the present paper, evidence is presented that pancreatic GSH–insulin transhydrogenase is able to catalyze the reverse reaction, *i.e.* the combination of inactive reduced A and B chains to regenerate insulin activity, also by promoting sulfhydryl–disulfide interchange. With the conditions used, insulin activity regained was about 15 times as much in the presence as in the absence of transhydrogenase. There was complete agreement in the estimates of regenerated insulin activity between immunoassay and fat pad assay. Some studies on the rates of oxidation (non-enzymatic) of individual reduced chains are also presented: The rate of disappearance

of 2 of the 4 SH groups of A chain was faster than that of the other 2. In the case of B chain, there was a rapid loss of 0.3–0.6 sulfhydryl group and thereafter almost no further loss of SH group occurred. An abstract reporting part of the data has appeared[5].

EXPERIMENTAL

The preparation of purified GSH–insulin transhydrogenase from beef pancreas[2] and reduced A and B chains of insulin have been described previously. Individual chains possessed only 0.02% of the insulin activity of an equimolar quantity of insulin. Insulin activity was determined by the two antibody immunoassay[7] and by the fat pad assay[8] with minor modifications.

Because of the poor solubility of the B chain of insulin at near physiological pH, it was difficult to prepare it in solution. This was accomplished in the following manner: In the preliminary experiments it was found that the maximum concentration of B chain which remained in solution at pH 7.5 was 25 μM and at pH 8 was 100 μM. For a 100 μM solution, 0.2 μmole of a chain preparation was suspended in 0.4 ml of ice-cold distilled water, dissolved by the addition of 40 μl of 1 M NaOH, and the volume was adjusted rapidly to 2 ml by the addition of ice-cold 0.1 M Tris buffer (pH 7.5); the pH of the resulting solution was 8. For 25 μM solution, the 100 μM solution thus prepared was diluted to 1:4 with 0.1 M Tris (pH 7.45) to yield a solution of pH 7.53. The chain solutions were therefore always prepared in 100 μM concentration; this permitted the addition of the enzyme and other substances (whenever tested) in the added pH 7.45 buffer. Mixtures of A and B chains, 25 μM each, were incubated at pH 7.53 and 37° for 20 min, with and without the pancreatic enzyme, and the amount of regenerated insulin was determined routinely by immunoassay and, in a few selected cases, which are noted appropriately in the legends of the figures, by the fat pad assay.

RESULTS AND DISCUSSION

In Fig. 1 the regeneration of insulin at different enzyme concentrations is shown. The amount regenerated increased with an increasing concentration of enzyme but

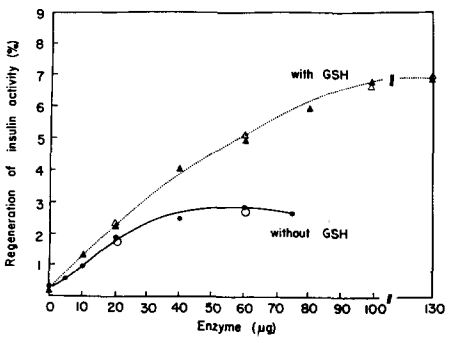

Fig. 1. Regeneration of insulin activity from inactive reduced A and B chains by pancreatic GSH–insulin transhydrogenase in the presence (1 mM) and absence of GSH. The final volume was 0.2 ml in each case. See text for details. Insulin activity was determined by immunoassay, indicated by filled symbols, and by fat pad assay, indicated by open symbols.

TABLE I

EFFECT OF ADDITION OF VARIOUS SUBSTANCES ON THE REGENERATION OF INSULIN FROM A MIXTURE OF A AND B CHAINS (25 μM EACH) BY GSH–INSULIN TRANSHYDROGENASE

The final volume was 0.2 ml in each case.

Addition	*% regeneration*	
	Non-enzymatic	*With enzyme (50 μg)*
None	0.30	2.03
GSH (1 mM)	0.57	4.33
Dehydroascorbic acid (1 mM)	0.33	0.70
Dehydroascorbic acid (1 mM) + GSH (1 mM)	0.66	6.86

reached a limiting value. In the presence of GSH (1 mM), both the percent regeneration at each concentration of enzyme and the maximum yield was higher. When the enzyme was present, the insulin activity regained was about 15 times more than when it was not present. Control experiments, in which the enzyme (50 μg, 100 μg) alone was present, showed that the enzyme did not possess any insulin activity in either assay. The close correspondence between the estimates of the regenerated insulin activity by the immunoassay and by the fat pad assay may be noted; this suggests that the hormonal activity with each assay was due to the same resynthesized material.

The enzyme appears to promote regeneration by sulfhydryl–disulfide interchange rather than by hydrogen transfer. This was indicated by the results of experiments in which dehydroascorbic acid and GSH at a concentration of 1 mM were added to the chain mixture (Table I). GSH alone stimulated whereas dehydroascorbic acid alone inhibited the enzymatic process of regeneration. GSH was able to reverse completely the inhibition caused by dehydroascorbic acid. The higher percent of regeneration observed in the presence of GSH, even though the chains were supplied in the reduced form, probably occurs because nearly 50% of the SH groups of the chains disappear immediately upon the mixing of the two chains. When enzyme was added to a chain mixture which had been previously oxidized during a preincubation period, no more insulin was regenerated than had already been formed by the chemical reaction unless GSH was also added. Likewise, when a chain mixture which had been oxidized in the presence of dehydroascorbic acid was used, a requirement of GSH for the enzyme to regenerate insulin was observed. These results therefore suggest that the enzyme functions by catalyzing sulfhydryl–disulfide interchange. These data thus substantiate the conclusions of our previous studies[1] in which the mode of action of the enzyme from the other direction of the reaction, *i.e.* the cleavage of insulin, was studied.

Reactivation of reduced ribonuclease and reduced lysozyme by a similar mechanism with enzymes obtained from beef liver[9–11] and from pigeon and chicken pancreas[12] have been reported by ANFINSEN's group and by VENETIANER AND STRAUB, respectively. Regeneration of insulin activity from partially reduced insulin with GSH–insulin transhydrogenase derived from beef liver has been reported by KATZEN, TIETZE AND STETTEN[13]. The regeneration values obtained by them were: 1–2% as determined by immunoassay and 11% as determined by fat pad assay.

In Fig. 2 are shown the rates of disappearance of SH groups (determined by ELLMAN's procedure[14]) of reduced A and B chains in air at room temperature. At pH 8.8 and a concentration of 0.2 mM for each chain, 2 of the 4 SH groups of A chain disappeared at a much faster rate (within 4 min) than the other two. In the case of B chain 0.6 SH group disappeared in about 4 min and thereafter further change took place very slowly. It can be seen from the figure that in both chains there is a point of inflection at about 4 min. When the reactions were studied at lowered pH (pH 8) and concentration (0.1 mM) in deoxygenated medium (nitrogen was bubbled through water and buffer for 1 h), the rate in the case of A chain was considerably lower:

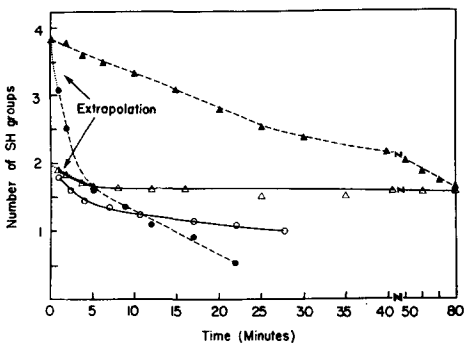

Fig. 2. Disappearance of sulfhydryl groups of separated A and B chains of insulin as a function of time. A solution of each chain in Tris buffer was allowed to oxidize in air at room temperature and aliquots were withdrawn at various times for the determination of SH groups. A chain: ●- - - -●, pH 8.8, 0.2 mM; ▲- - - -▲, pH 8. 0.1 mM in deoxygenated medium. B chain: ○—○, pH 8.8, 0.2 mM; △—△, pH 8.1 mM in deoxygenated medium.

Two of the 4 SH groups disappeared in 40 min, in contrast to 4 min at pH 8.8, and thereafter further change took place very slowly. In the case of B chain, however, initial loss of 0.3 SH group occurred in the same interval of 4 min. Experiments at pH values lower than 8 or at concentrations higher than 0.1 mM (at pH 8) could not be carried out because of the aggregation of B chain. Inclusion of EDTA or neocuprein (1 mM) in the solution of B chain did not abolish the initial loss of SH groups.

In view of MARKUS's[15] recent observation that a preferential reduction of inter-chain disulfide bonds to intra-chain disulfide occurs in the electrolytic reduction of insulin, it is possible that in the oxidation of the reduced A chain, the reverse might be true: The 2 SH groups which are involved in the formation of intra-chain disulfide bond could undergo oxidation in preference to those involved in the formation of inter-chain disulfide bonds. The disappearance of 0.3–0.6 SH groups of B chain is not understood. It apparently does not occur because of contamination with heavy metals since the inclusion of EDTA or neocuprein did not prevent this rapid loss. Conditions (deoxygenation and lower pH) which decreased the rates in the case of A chain did not affect the rate of disappearance of SH of B chain. Sulfhydryl groups have been shown to be involved in the aggregation reaction[16]; there is a possibility this decrease in sulfhydryl group of B chain is due to a reaction of this nature.

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