Activation of chicken liver fructose-1,6-bisphosphatase by oxidized glutathione

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Treatment of chicken liver fructose-1,6-bisphosphatase with oxidized glutathione (GSSG) leads to an increase in activity. This activation is markedly enhanced if treatment is performed in the presence of AMP or Mn^{2+}. The effects of AMP and Mn^{2+} appear to be synergistic. The maximal activation is over 13-fold and is accompanied by the disappearance of 4 sulfhydryl groups per molecule of enzyme. Both fructose 1,6-bisphosphate and fructose 2,6-bisphosphate can largely prevent this activation. Activation can be reversed by dithiothreitol or cysteine. It appears that GSSG activates this enzyme by thiol/disulfide exchanges with the enzyme's specific sulfhydryl groups.

Glutathione *Enzyme activation* *Fructose-1,6-bisphosphatase* *(Chicken liver)*

1. INTRODUCTION

Fructose-1,6-bisphosphatase (FBPase) (EC 3.1.3.11) is an enzyme which catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose-6-phosphate and P_i. The enzyme requires a bivalent cation, either Mn^{2+} or Mg^{2+}, for activity and is strongly inhibited by AMP [1,2] and fructose 2,6-bisphosphate [3,4]. The enzyme is believed to play a key role in gluconeogenesis.

The native 'neutral pH optimum' FBPase isolated from different sources is a tetramer composed of identical subunits of molecular masses ranging from 36 to 40 kDa [5–7]. A common feature of FBPase from various sources appears to be the presence of 4 highly reactive sulfhydryl (SH) groups per molecule of enzyme [8–12]. In contrast to a common phenomenon that most enzymes are inactivated by oxidation or modification of free SH groups [13,14], the catalytic activity of 'alkaline' rabbit liver FBPase was reported to be enhanced to varying extent by a number of SH reagents and disulfides (see [15] for review). It has been proposed that FBPase may be under metabolic control by virtue of a specific mechanism involving modification of one or more SH groups by some biological disulfides, resulting in the enhancement of catalytic activity [16]. The biological disulfides that were found to activate alkaline rabbit liver FBPase include cystamine, oxidized CoA, CoA glutathione disulfide, acyl carrier proteins, and homocystine. With these physiological compounds, the increase in activity ranged from 3- to 8-fold [15–18]. Pontremoli and co-workers [15–18] specifically pointed out that oxidized glutathione (GSSG) was without effect. It is noteworthy that the FBPase used in those studies [15–18] had been modified by proteolytic enzymes during isolation [19], resulting in the shift of optimum pH from neutral to alkaline range.

Gilbert [13,14] recently reported that phosphofructokinase (PFK) could be inactivated by GSSG through thiol/disulfide exchange and suggested the possible role of this biological disulfide in the regulation of PFK activity. Since PFK and FBPase are involved in a possible futile cycle central to the regulation of glycolysis and gluconeogenesis [20], we have reinvestigated the effect of GSSG on FBPase using the neutral pH optimum enzyme isolated from chicken liver.

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2. MATERIALS AND METHODS

Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase (564 U/mg) was obtained from Toyobo Shinko American Inc., New York, NY. GSSG, NAD, NADP, AMP, L-cysteine, dithiothreitol, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, and yeast phosphoglucose isomerase were all purchased from Sigma (St. Louis, MO). FBPase with optimum pH at neutral range was purified from chicken livers according to the procedure described [21]. Before use, the enzyme was dialyzed against 2000 vols of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA at 4°C for 8 h.

The activity of FBPase was assayed by the most frequently used coupling method [22] except that NADP and yeast glucose-6-phosphate dehydrogenase were replaced by NAD and *L. mesenteroides* glucose-6-phosphate dehydrogenase (which can use both NAD and NADP as its coenzyme), respectively. Unless otherwise stated, the assay mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 7.5), 0.1 mM EDTA, 0.5 mM $MnCl_2$, 0.25 mM NAD, 0.1 mM fructose 1,6-bisphosphate, 1 unit each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and an appropriate amount of FBPase. The assay mixture without substrate was preincubated in a cuvette at 25°C for 3 min. The reaction was initiated by the addition of substrate. The concentration of purified FBPase was determined by its extinction coefficient at 280 nm and the molecular mass of the enzyme was taken to be 144 kDa [21].

Modification of FBPase with GSSG was carried out in 0.1 M Tris-HCl buffer (pH 7.5) as described in detail in the legends to the table and figures. SH groups were measured spectrophotometrically at 412 nm by titration with 50 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [23] in 60 mM Tris-HCl buffer (pH 7.5) with or without 8 M urea. A molar extinction coefficient of 13600 for the liberated thionitrobenzoate anion was used for all calculations.

3. RESULTS

The concentration of GSSG in liver of fed rat was recently reported to be approx. 0.16 mM, but it can be as high as 0.4 mM [14]. The level of GSSG is expected to be higher under fasting conditions since fasting has been associated with an increase in the overall disulfide/thiol ratio in liver [24,25]. Attempts to obtain information from the literature on the level of GSSG in chicken liver have been unsuccessful to date. In this study, we have found that treatment of chicken liver FBPase with 0.1–0.45 mM GSSG at pH 7.5 resulted in a gradual increase in catalytic activity. As shown in fig.1, the catalytic activity increased about 1.8-, 2.5- or 3.7-fold after incubation for 12 h with 0.1, 0.2 or 0.45 mM GSSG, respectively. The activity of the enzyme incubated in the absence of GSSG (control) remained essentially unchanged throughout the 12-h incubation period.

As mentioned previously, a common feature of FBPase is the presence of 4 highly reactive SH groups per molecule of enzyme [8,12]. The reac-

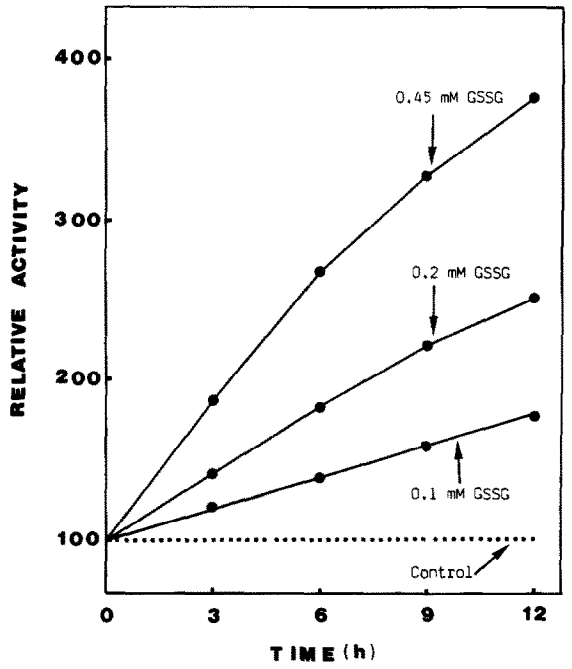

Fig.1. Activation of chicken liver FBPase by GSSG. A solution of FBPase containing 0.2 mg per ml (1.4 μM) in 0.1 M Tris-HCl buffer (pH 7.5) was incubated at 25°C with varying concentrations of GSSG as indicated. At the times indicated, aliquots were removed, diluted and assayed for enzyme activity under the conditions as described in section 2. The relative activity of 100 is equivalent to a specific activity (μmol/min per mg) of 9.2 under the present assay conditions.

tivity of these SH groups has been reported to be further enhanced by AMP [8,11,12] and Mn^{2+} [12]. We have found that the activation of FBPase by GSSG was markedly enhanced if treatment with GSSG was carried out in the presence of AMP or Mn^{2+}. Based on the initial rate of activation, the enhancing effects of AMP and Mn^{2+} appear to be synergistic. As shown in fig.2, the catalytic activity increased about 1.7-fold after treatment of the enzyme with 0.2 mM GSSG at pH 7.5 for 6 h. If this treatment was carried out in the presence of 0.2 mM AMP, 0.5 mM Mn^{2+} or 0.2 mM AMP plus 0.5 mM Mn^{2+}, the increase in activity was about 3.5-, 4- or 9-fold, respectively. Fig.2 also shows that the rate of increase in activity of the enzyme treated with GSSG in the presence of AMP and Mn^{2+} decreased sharply after 6 h or 9-fold increase in activity. The increase in activity reached about 11-fold after 12 h of incubation. It was found that the activity of this treated enzyme still continued to increase, although slowly, with further incubation (not shown). After 24 h of incubation, the activity of the treated enzyme increased over 13-fold, while the activity of the control decreased about 12%. It should be pointed out that if 0.5 mM Mn^{2+} was replaced by 5 mM Mg^{2+} in the assay system, the maximal activation observed with GSSG treatment was about 3.5-fold. However, if 0.5 mM Mn^{2+} and 5 mM Mg^{2+} were both present in the assay system, the activity of the GSSG-treated enzyme was equivalent to that assayed with 0.5 mM Mn^{2+} alone.

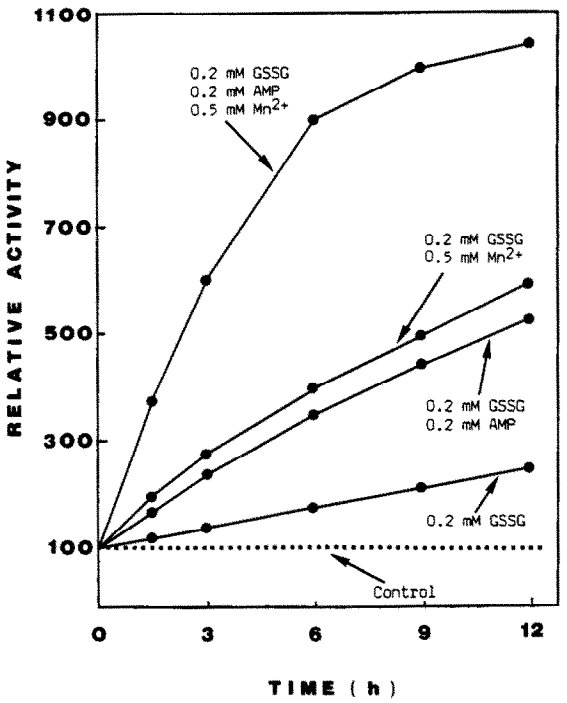

Fig.2. Effects of AMP and Mn^{2+} on the activation of chicken liver FBPase by GSSG. FBPase (0.2 mg/ml) was incubated at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) with 0.2 mM GSSG in the presence or absence of 0.2 mM AMP and/or 0.5 mM $MnCl_2$ as indicated. At the times indicated, aliquots were removed, diluted and assayed for enzyme activity under the conditions as described in section 2.

Activation of FBPase by GSSG was largely prevented if treatment was carried out in the presence of fructose 1,6-bisphosphate or fructose 2,6-bisphosphate, the latter being more effective (table 1). It is worthy of note that both fructose 1,6-bisphosphate and fructose 2,6-bisphosphate have been reported to decrease the reactivity of the SH groups of mammalian FBPase [12,18,26,27].

Activation by GSSG was readily reversed by reduction with dithiothreitol or cysteine, the

Table 1

Effects of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate on the activation of chicken liver FBPase by GSSG

Additions	Relative activity
None	100
0.5 mM GSSG	392
0.5 mM GSSG + 0.2 mM Fru-1,6-P_2	102
0.5 mM GSSG + 0.05 mM Fru-1,6-P_2	182
0.5 mM GSSG + 0.05 mM Fru-2,6-P_2	103
0.1 mM GSSG + 0.2 mM AMP + 0.5 mM Mn^{2+}	905
0.1 mM GSSG + 0.2 mM AMP + 0.5 mM Mn^{2+} + 0.2 mM Fru-1,6-P_2	104
0.1 mM GSSG + 0.2 mM AMP + 0.5 mM Mn^{2+} + 0.05 mM Fru-2,6-P_2	106

FBPase (0.2 mg/ml) was incubated at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) with GSSG and/or other ligands as indicated. After 12 h, aliquots were removed, diluted, and assayed for enzyme activity under the conditions as described in section 2. The relative activity of 100 is equivalent to a specific activity (μmol/min per mg) of 9.2

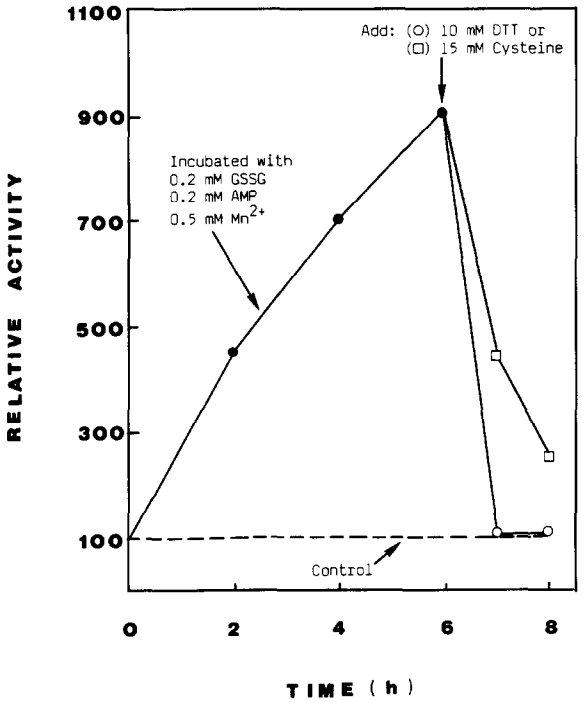

Fig.3. Reversal of the activation of chicken liver FBPase by GSSG. FBPase (0.2 mg/ml) was incubated with 0.2 mM GSSG, 0.2 mM AMP and 0.5 mM $MnCl_2$ at 25°C in 0.1 M Tris-HCl buffer (pH 7.5). After 6 h of incubation, cysteine or dithiothreitol (DTT) was added to the final concentration of 15 mM or 10 mM, respectively. The enzyme activity was assayed at the indicated times under the conditions as described in section 2.

former being more effective (fig.3). This indicates that the enzyme and the activator are linked by disulfide bonds.

Incubation of native enzyme (1.4–5.6 μM) with 50 mM DTNB in 60 mM Tris-HCl buffer (pH 7.5) for 30 min showed that only 4 SH groups per molecule of enzyme were titratable. These 4 reactive SH groups disappeared or were no longer detected after the enzyme had been treated with 0.2 mM GSSG, 0.2 mM AMP and 0.5 mM Mn^{2+} at pH 7.5 for 24 h. The total number of SH groups per molecule of enzyme titrated with DTNB in 8 M urea was found to be 24.3 and 20.6 for the native and the GSSG-treated enzymes, respectively.

4. DISCUSSION

Although many biological disulfides have been reported to activate alkaline rabbit liver FBPase, GSSG was specifically pointed out to be without effect [15–18]. It was recently suggested that GSSG may be one of the important biological disulfides serving as the 'third messenger' in the regulation of many enzymes, including PFK [13,14]. This prompted us to reinvestigate the effect of GSSG treatment on FBPase activity using the neutral pH optimum enzyme isolated from chicken liver. The results show that at the concentration which may occur in liver (0.2 mM [14]), GSSG alone is merely a weak activator. However, if treatment with GSSG is carried out in the presence of AMP or Mn^{2+} or especially AMP plus Mn^{2+}, the catalytic activity can be enormously enhanced. The maximal activation was found to be over 13-fold. It should be pointed out that the maximal activation induced by various disulfides reported in [15–18] ranged from 3- to 8-fold. Thus, the results of this study suggest that GSSG can potentially be an important biological disulfide activator of liver FBPase.

Like FBPase from other sources [8–12], the enzyme from chicken liver also contains 4 reactive SH groups per molecule of enzyme. Based on the results of titration studies with DTNB, it is considered that GSSG activates this enzyme by thiol/disulfide exchanges with these reactive SH groups. It has been previously reported that the reactivity of the SH groups of mammalian FBPase increased in the presence of AMP or Mn^{2+} [8,11,12] but decreased in the presence of fructose 1,6-bisphosphate or fructose 2,6-bisphosphate [12,18,26,27]. These appear to be in agreement with the results of this study that the activation of chicken liver FBPase by GSSG is enhanced by AMP or Mn^{2+} but inhibited by fructose 1,6-bisphosphate or fructose 2,6-bisphosphate.

Although activation by modification of specific SH groups has been suggested as a possible mechanism for the regulation of FBPase activity in vivo [15–18], there is still no direct evidence to support this hypothesis. Moser et al. [28] recently reported the isolation of the reduced and the oxidized forms of FBPase from rat liver. As compared with the reduced form, the oxidized form is less sensitive to AMP inhibition and bound much less tightly to phosphocellulose. These researchers did not report that the activity of the oxidized form was elevated. Research is currently underway in

this laboratory to determine if, under gluconeogenic conditions, chicken liver FBPase may exist in the oxidized form with elevated activity.

In assaying the activity of purified FBPase with the most frequently used coupling assay method [22], we obtained identical results when NADP was replaced by NAD, if glucose-6-phosphate dehydrogenase from *L. mesenteroides* rather than yeast was employed. This modified procedure can significantly reduce the cost of assaying the activity of purified FBPase.

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