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# Glutathione reductase directly mediates the stimulation of yeast glucose-6-phosphate dehydrogenase by GSSG

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Yeast glucose-6-phosphate dehydrogenase was inhibited by low NADPH concentrations in cell-free extracts, and de-inhibited by GSSG; extensive dialysis of the crude extract did not diminish the GSSG effect. Immunoprecipitation of glutathione reductase abolished the de-inhibition of glucose-6-phosphate dehydrogenase by GSSG. Purified glucose-6-phosphate dehydrogenase was inhibited by NADPH but not de-inhibited by GSSG, and upon addition of pure glutathione reductase GSSG completely de-inhibited the glucose-6-phosphate dehydrogenase.

## INTRODUCTION

Regulation of the pentose phosphate pathway is a central metabolic issue. The first two enzymes of this pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are almost completely inhibited at the physiological free [NADPH]/[$NADP^+$] ratio, in the order of 100:1 in rat liver [1]. Although the NADPH and $NADP^+$ concentrations have not been well established in yeast, the total [NADPH]/[$NADP^+$] ratio is, at the least, higher than 2:1 [2]. The strong inhibition of rat liver glucose-6-phosphate dehydrogenase by NADPH is relieved by GSSG, in a process attributed by Eggleston & Krebs to a protein cofactor of $M_r$ about 15000 [3]. Direct participation of glutathione reductase in this activation was ruled out in view of its very low activity in the liver extracts and the fact that its complete inhibition by $Zn^{2+}$ did not abolish the GSSG effect, which was not observed, however, with crystalline yeast glucose-6-phosphate dehydrogenase or crude yeast extracts [3]. The earlier findings by Eggleston & Krebs [3] were later confirmed and extended to mussel hepatopancreas extracts [4,5], although the $M_r$ of the putative cofactor has been raised to 100000 [5].

A critical appraisal of the effect of GSSG on hepatic glucose-6-phosphate dehydrogenase was carried out by Levy & Christoff, concluding that the effects previously described were largely the result of several artifacts, in the absence of evidence for the existence of a cofactor or for a direct effect of GSSG on glucose-6-phosphate dehydrogenase [6,7]. They suggested instead that GSSG could act indirectly via glutathione reductase oxidizing NADPH to $NADP^+$, both changes simultaneously enhancing the glucose-6-phosphate dehydrogenase activity [6]. It has been recently suggested that the event activating glucose-6-phosphate dehydrogenase could be mixed-disulphide formation with GSSG catalysed by thioltransferase [8], a low-$M_r$ enzyme fitting well with the $M_r$ initially predicted for the putative cofactor [3,4]. The present paper reports that, with both purified enzyme and crude yeast extracts, glucose-6-phosphate dehydrogenase is inhibited by NADPH and de-inhibited by GSSG. The activating effect of GSSG seems to be due to the direct action of glutathione reductase, since in crude extracts it was abolished by antibodies monospecific for that enzyme, and de-inhibition of purified glucose-6-phosphate dehydrogenase by GSSG was only observed in the presence of pure glutathione reductase.

## EXPERIMENTAL

*Saccharomyces cerevisiae* S288c $\alpha$ was grown on a medium containing 1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose. Growth was monitored by turbidimetry at 660 nm. Exponentially growing cells were collected by centrifugation, washed and disrupted during 3 min at 2°C in a Bühler homogenizer with 1 g of glass beads (0.3 mm) and 5 ml of buffer (1 mM-EDTA/100 mM-potassium phosphate buffer, pH 7.0) per g wet wt. of cells. The mixture was centrifuged at 3000 *g* for 5 min, and the supernatant was centrifuged again at 40000 *g* for 25 min to obtain the cell-free extract.

*S. cerevisiae* glutathione reductase was purified to homogeneity from Sigma type III enzyme as previously described [9]. Sigma type IX glucose-6-phosphate dehydrogenase was extensively dialysed against extraction buffer and used as yeast purified enzyme. Both glutathione reductase and glucose-6-phosphate dehydrogenase preparations were homogeneous, as shown by electrophoresis both in non-denaturing conditions and in the presence of SDS. Glutathione reductase and glucose-6-phosphate dehydrogenase activities were measured by the procedures described in [9] and [5] respectively. Protein was measured by the Lowry procedure [10], with bovine serum albumin as standard. The effect of GSSG on glucose-6-phosphate dehydrogenase activity was assayed as described by Levy & Christoff [6].

Antibodies against glutathione reductase were raised in New Zealand rabbits by inoculating gel slices from polyacrylamide-gel electrophoresis of glutathione reductase [11], after localization of its activity by monitoring under u.v. light the GSSG-dependent quenching of NADPH fluorescence. Whole blood was obtained 2 weeks after the first injection; after complement inactiva-

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tion, the plasma was precipitated with $(NH_4)_2SO_4$ and further purified by DEAE-cellulose chromatography and affinity chromatography on Sepharose linked to pure yeast glutathione reductase, by using the procedure previously described [11]. Immunodiffusion was performed in accordance with Ouchterlony & Nilsson [12]. Crossed immunoelectrophoresis [11] showed only one precipitated band after Coomassie Blue staining, corresponding to the glutathione reductase activity. Antibodies against *Anacystis nidulans* glutamine synthetase were raised and purified in the same way as described for yeast glutathione reductase antibodies.

## RESULTS AND DISCUSSION

Table 1 shows that yeast glucose-6-phosphate dehydrogenase was inhibited by low NADPH concentrations, the inhibition being efficiently reverted by GSSG. These results agree with those previously reported with rat or human liver extracts [3–7], although the GSSG effect had not been previously observed with yeast extracts [3]. Table 1 also shows that, in agreement with the results obtained by Levy & Christoff [6], the activating effect of GSSG on yeast glucose-6-phosphate dehydrogenase inhibited by NADPH did not diminish after 18 h dialysis. Similar results were obtained when cell-free extracts were chromatographed on Sephadex G-50 to exclude low-$M_r$ proteins (results not shown). Our results seem to rule out the participation of the low-$M_r$ cofactor proposed by Eggleston & Krebs [3] and originally supported by Rodriguez-Segade *et al.* [4], since their alleged cofactor was inactivated after dialysis and separated by ultrafiltration or Sephadex G-50 chromatography [3,4].

In the present paper the activation by GSSG of glucose-6-phosphate dehydrogenase has been expressed in two different ways: (*a*) as a percentage of the respective controls without NADPH (as was done in all previous papers [3–7]), and (*b*) as a percentage of the control assay carried out without added NADPH and GSSG. In our opinion this second procedure shows more accurately the activating effect of GSSG, since in the presence of this disulphide the NADPH produced by glucose-6-phosphate dehydrogenase is re-oxidized by glutathione reductase, whose activity (0.74 unit/ml) was very similar in our conditions to that of glucose-6-phosphate dehydrogenase (0.78 unit/ml), leading to an underestimation of this second enzyme. The alleged inhibition of glucose-6-phosphate dehydrogenase by GSSG previously reported [3] should be considered an artifact, at least in part due to the joint activity of glucose-6-phosphate dehydrogenase and glutathione reductase.

The influence of glutathione reductase on glucose-6-phosphate dehydrogenase de-inhibition by GSSG was studied in the past by inhibiting the first enzyme by various means. The maintenance of the GSSG effect after complete inhibition of glutathione reductase by $Zn^{2+}$ ions ruled out initially a possible role of this enzyme in the effect [3,4]. Nevertheless the discovery that $Zn^{2+}$ produced a spurious absorbance increase at 340 nm and that glutathione reductase inhibition by $Hg^{2+}$ ions blocked de-inhibition by GSSG [7] suggested a possible involvement of glutathione reductase. It should be noticed that $Hg^{2+}$ ions could additionally inhibit the activity of thioltransferase, a low-$M_r$ enzyme that could also be involved in glucose-6-phosphate dehydrogenase regulation [8].

To avoid the lack of a specific glutathione reductase inhibitor and the side effects of metal ions, we removed yeast glutathione reductase from crude extracts by means of purified monospecific antibodies, using as a control antibodies specific for *Anacystis nidulans* glutamine synthetase. The glutathione reductase activity of a dialysed crude extract decreased to 0.008 unit/ml after treatment with specific antibodies, while remaining at 0.32 unit/ml with control serum; the glucose-6-phosphate dehydrogenase activity remained at 0.44 unit/ml in either case. Table 2 shows that de-inhibition by GSSG of glucose-6-phosphate dehydrogenase was completely abolished upon immunoprecipitation of glutathione reductase. Such a result clearly indicates that the GSSG effect was directly mediated by glutathione reductase through NADPH oxidation, as previously suggested [6,7]. The homogeneity of the glutathione reductase used as antigen, the subsequent purification of the antibodies by affinity chromatography and the single immuno-

**Table 1. Inhibition by NADPH of *S. cerevisiae* glucose-6-phosphate dehydrogenase and its reversal by GSSG**

The activity of 10 $\mu$l of yeast cell-free extract (containing 50 $\mu$g of protein), either freshly prepared or after 18 h dialysis against extraction buffer, was determined in reaction mixtures containing in 1 ml final volume 50 mM-Tris/HCl buffer, pH 7.4, 3 mM-$MgCl_2$, 20 $\mu$M-$NADP^+$ and 1.5 mM-glucose 6-phosphate (G6P) and/or 70 $\mu$M-NADPH as indicated. The changes in absorbance at 340 nm were monitored during the period 1–11 min after enzyme addition. The results are expressed either as the percentage of the respective controls without NADPH, or as the percentage of the control without NADPH and GSSG added (shown by the asterisk *).

| Enzyme preparation | [GSSG] ($\mu$M) | $10^3 \times \Delta A_{340}$ −NADPH −G6P | −NADPH +G6P | −NADPH Net | +NADPH −G6P | +NADPH +G6P | +NADPH Net | Activity with NADPH (%) | |
|---|---|---|---|---|---|---|---|---|---|
| Crude extract | 0 | 1 | 81 | 80* | −3 | 51 | 54 | 67 | 67* |
| | 50 | 1 | 52 | 51 | −80 | 18 | 98 | 192 | 122* |
| | 100 | 2 | 45 | 43 | −170 | −31 | 139 | 323 | 173* |
| Dialysed crude extract (DCE) | 0 | 3 | 74 | 71* | −3 | 37 | 40 | 56 | 56* |
| | 100 | 2 | 57 | 55 | −236 | −71 | 165 | 300 | 232* |

**Table 2. Effect of anti-(*S. cerevisiae* glutathione reductase) antibodies on the de-inhibition by GSSG of glucose-6-phosphate dehydrogenase**

Samples (150 $\mu$l) of dialysed yeast cell-free extracts (DCE) (containing 0.75 mg of protein) were treated with 30 $\mu$l of purified antibodies solution containing 29 $\mu$g of anti-(*Anacystis nidulans* glutamine synthetase) IgG or 28 $\mu$g of anti-(yeast glutathione reductase) IgG (anti-GSSGrase IgG). The mixtures were incubated for 15 min at room temperature and an additional 12 h at 4°C, and subsequently centrifuged at 7500 *g* for 15 min. The glucose-6-phosphate dehydrogenase activities of both samples (0.012 ml) were then assayed as shown in the lower part of the Table in accordance with the conditions described in Table 1.

| System | [GSSG] ($\mu$M) | $10^3 \times \Delta A_{340}$ −NADPH −G6P | −NADPH +G6P | −NADPH Net | +NADPH −G6P | +NADPH +G6P | +NADPH Net | Activity with NADPH (%) | |
|---|---|---|---|---|---|---|---|---|---|
| DCE plus control IgG | 0 | 5 | 93 | 88* | 0 | 35 | 35 | 40 | 40* |
| | 100 | 5 | 51 | 46 | −187 | −59 | 128 | 278 | 145* |
| DCE plus anti-GSSGrase IgG | 0 | 6 | 98 | 92* | 1 | 41 | 40 | 43 | 43* |
| | 100 | 5 | 91 | 86 | −3 | 30 | 33 | 38 | 36* |

**Table 3. Effect of pure glutathione reductase on the de-inhibition of purified *S. cerevisiae* glucose-6-phosphate dehydrogenase by GSSG**

Purified yeast glucose-6-phosphate dehydrogenase (G6PDH) (2.1 $\mu$g) was dissolved in 100 mM-potassium phosphate buffer, pH 7, containing 1 mM-EDTA in 1 ml final volume (final activity 0.45 unit/ml) either in the absence or in the presence of 1.6 $\mu$g of pure yeast glutathione reductase (GSSGrase) (final activity 0.35 unit/ml). The glucose-6-phosphate dehydrogenase activities of both samples (10 $\mu$l) were then assayed as indicated in the lower part of the Table in accordance with the conditions described in Table 1.

| System | [GSSG] ($\mu$M) | $10^3 \times \Delta A_{340}$ −NADPH −G6P | −NADPH +G6P | −NADPH Net | +NADPH −G6P | +NADPH +G6P | +NADPH Net | Activity with NADPH (%) | |
|---|---|---|---|---|---|---|---|---|---|
| Purified G6PDH | 0 | −1 | 77 | 78* | −2 | 31 | 33 | 42 | 42* |
| | 100 | 0 | 80 | 80 | −2 | 33 | 35 | 44 | 45* |
| Purified G6PDH +GSSGrase | 0 | 0 | 84 | 84* | 1 | 32 | 31 | 37 | 37* |
| | 100 | 0 | 55 | 55 | −160 | −46 | 114 | 207 | 136* |

precipitation band obtained after crossed immunoelectrophoresis rule out the participation in the de-inhibitory effect of GSSG of any other protein different from glutathione reductase.

The direct influence of yeast glutathione reductase on de-inhibition of yeast glucose-6-phosphate dehydrogenase by GSSG was finally demonstrated by the reconstruction experiment carried out with both pure enzymes summarized in Table 3. Purified yeast glucose-6-phosphate dehydrogenase was inhibited by NADPH, but not de-inhibited by GSSG when assayed alone. However, when pure yeast glutathione reductase was also present, GSSG addition produced a complete de-inhibition of glucose-6-phosphate dehydrogenase, similar to that observed in cell-free extracts, fully corroborating the results given in Table 2. Our results contradict those of Eggleston & Krebs, who were unable to show any GSSG effect either with crystalline yeast glucose-6-phosphate dehydrogenase or with the enzyme in crude yeast extracts [3].

The regulation *in vitro* by GSSG of glucose-6-phosphate dehydrogenase activity [4,5,13] is thus directly mediated, at least in yeast, by re-oxidation of NADPH catalysed by glutathione reductase, as previously proposed by Levy & Christoff [6,7], in a process that should be considered a de-inhibition [13] rather than an activation. The existence of any other low-$M_r$ or high-$M_r$ protein cofactor different from glutathione reductase is not required, either in yeast crude extracts (Tables 1 and 2) or with homogeneous yeast enzymes (Table 3). The possibility that GSSG could, in addition, modulate glucose-6-phosphate dehydrogenase activity directly, as has been suggested [8], should not be lightly dismissed, although evidence for such a mechanism has yet to be established. The direct effect of glutathione reductase in the regulation of the hexose monophosphate shunt could, perhaps, be related with the previously described regulation of glutathione reductase by redox interconversion [9,14], a process of possible physiological significance [15].

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