BIOCHEMICAL JOURNAL LETTERS

Reversal effect of oxidized glutathione on the inhibition of glucose-6-phosphate dehydrogenase by NADPH

It was reported by Eggleston & Krebs (1974) that a cofactor co-operates with oxidized glutathione (GSSG) in reversing the inhibition of rat liver glucose-6-phosphate dehydrogenase by NADPH. Rodriguez-Segade *et al.* (1979) have found that the cofactor required for GSSG to be effective was a polypeptide of M_r 10000 or 15000 in rat liver and mussel hepatopancreas respectively. Other properties of the rat liver and mussel hepatopancreas cofactor have also been studied (Rodriguez-Segade *et al.*, 1978, 1980; Rodriguez-Segade & Freire, 1982).

In assays to determine the effect of GSSG in reversing the inhibition of glucose-6-phosphate dehydrogenase by NADPH, 0.033 mM-ZnCl₂ has been used to inhibit glutathione reductase in order to prevent any interference of this enzyme in the reversal assay (Eggleston & Krebs, 1974; Rodriguez-Segade *et al.*, 1978, 1979, 1980; Mize & Langdon, 1962). Recently, Levy & Christoff (1983) reported that this Zn²⁺ concentration induces changes in the A_{340} of rat liver extract, even in the absence of NADPH, NADP⁺ and glucose 6-phosphate. They claim that this effect of Zn²⁺ results from the interaction of this cation with some macromolecular component present in the rat liver extract, and could be mistaken for glucose-6-phosphate dehydrogenase activity.

In the conditions of Levy & Christoff's (1983) experiments we found that the absorbance change induced by Zn²⁺, in the absence of glucose 6-phosphate, is similar in both rat liver and mussel hepatopancreas extracts. However this absorbance change represents only 7% and 23% of that induced by glucose 6-phosphate in mussel hepatopancreas and rat liver extracts respectively. In our reversal assay the net absorbance changes were determined automatically by monitoring of pairs of cuvettes in which only one contained substrate. Similar results (approx. 5% difference) are obtained by using the procedure of Levy & Christoff (1983) in which absorbance changes produced in the presence or absence of glucose 6-phosphate are separately read against air. Therefore, according to our data, the presence of Zn²⁺ cannot account for the reversal by GSSG of the inhibition by NADPH of glucose-6-phosphate dehydrogenase, especially in mussel hepatopancreas.

Eggleston & Krebs (1974) have reported the inhibition of rat liver glucose-6-phosphate dehydrogenase by GSSG; however, Levy & Christoff (1983) argued that there is no interaction between GSSG and glucose-6-phosphate dehydrogenase, and that the apparent inhibi-

tion observed by Eggleston & Krebs (1974) is due to the glutathione reductase activity. This is based on the fact that, when Hg²⁺ is added to the rat liver extracts to inhibit the glutathione reductase, GSSG does not affect the glucose-6-phosphate dehydrogenase activity. Likewise, they claim that the reversal by GSSG of the inhibition by NADPH in rat liver should be explained as a result of the NADPH consumed by the glutathione reductase reaction during the reversal assay.

Concerning the inhibition of glucose-6-phosphate dehydrogenase by GSSG, we are unable to understand Levy & Christoff's (1983) reasoning, since they themselves admit to observing what they call the apparent inhibition of glucose-6-phosphate dehydrogenase by GSSG in experiments in which NADPH, one of the substrates of glutathione reductase, is not added to the medium. Nevertheless, to settle the point we determined the effects of Hg²⁺, GSSG and Hg²⁺ plus GSSG on the activity of glucose-6-phosphate dehydrogenase in both rat liver and mussel hepatopancreas extracts. In these experiments NADPH was not added, so little or no interference from glutathione reductase was possible. We found that GSSG and Hg²⁺, at the same concentrations as those used by Levy & Christoff (1983), separately inhibited the enzyme from both sources, whose activity was restored in whole (mussel hepatopancreas) or in part (rat liver) when Hg²⁺ was added in combination with GSSG.

Those results seem to confirm the observation of Eggleston & Krebs (1974) and Rodriguez-Segade *et al.* (1978), and show that Hg²⁺ interferes with the GSSG-glucose-6-phosphate dehydrogenase interaction. This being so, the reversal of the inhibition produced by GSSG would be expected to be suppressed by this cation.

In order to determine the extent to which glutathione reductase could be involved in the effect of the GSSG, we have evaluated the amount of NADPH that the glutathione reductase is able to consume during the assay, in the absence of Zn²⁺ or Hg²⁺. In the assay with rat liver extracts, 9.6 units of glutathione reductase are present, which means that this enzyme consumes, during the 10 min of the assay, 1.4 nmol of the 80 nmol of NADPH present. However, for the removal of NADPH to relieve its inhibition of glucose-6-phosphate dehydrogenase to the same extent as is actually observed in the presence of 0.1 mM-GSSG in the reaction mixture (20.2–37.4 units), the amount of NADPH removed should be at least 30 times higher to have significant influence on the reversal effect *in vitro*. In mussel hepatopancreas extracts the participation of the glutathione reductase in the reversal effect is even less likely to be significant, since the activity of this enzyme in the assay reaction is only 5.2 units.

In the light of these results, and others presented previously (Rodriguez-Segade *et al.*, 1979, 1980) where

the effect of GSSG was studied with purified enzyme and cofactor, the reversal of the inhibition of glucose-6-phosphate dehydrogenase by NADPH cannot be attributed to the glutathione reductase mechanism.

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Eggleston, L. V. & Krebs, H. A. (1974) *Biochem. J.* **138**, 425–435

Levy, H. R. & Christoff, M. (1983) *Biochem. J.* **214**, 959–965

Mize, C. E. & Langdon, R. G. (1962) *J. Biol. Chem.* **237**, 1589–1595

Rodriguez-Segade, S. & Freire, M. (1982) *Comp. Biochem. Physiol.* **72B**, 117–121

Rodriguez-Segade, S., Freire, M. & Carrion, A. (1978) *Biochem. J.* **170**, 577–582

Rodriguez-Segade, S., Carrion, A. & Freire, M. (1979) *Biochem. Biophys. Res. Commun.* **89**, 148–154

Rodriguez-Segade, S., Eguiraun, A. & Freire, M. (1980) *Comp. Biochem. Physiol.* **65B**, 579–581

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The effect of oxidized glutathione on NADPH inhibition of glucose-6-phosphate dehydrogenase is indirect

Eggleston & Krebs (1974) provided evidence that oxidized glutathione (GSSG) and an unidentified cofactor could overcome the potent inhibition, by physiological concentrations of NADPH, of rat liver glucose-6-phosphate dehydrogenase. This appeared to provide an attractive mechanism for the physiological control of this enzyme and, hence, of the hexose-monophosphate shunt. Experiments reported by Rodriguez-Segade *et al.* (1978, 1979, 1980) appeared to support the findings of Eggleston & Krebs (1974) in rat liver extracts, to extend them to extracts from mussel hepatopancreas, and to identify and partially characterize the cofactor. In our own attempts to extend these findings (Levy & Christoff, 1983) we were unable to show any direct effect of GSSG on rat liver glucose-6-phosphate dehydrogenase or any evidence for the existence of a cofactor that mediates such an effect. Instead, we presented evidence that the earlier findings were partly the result of two artifacts: (a) the use of ZnCl_2 to inhibit hepatic glutathione reductase, which causes artifactual absorbance changes easily mistaken for glucose-6-phosphate dehydrogenase activity, and (b) the incorrect use of controls lacking glucose 6-phosphate. We presented evidence that in experiments *in vitro* GSSG appears to relieve NADPH inhibition of glucose-6-phosphate dehydrogenase by oxidizing NADPH to NADP^+ , catalysed by glutathione reductase (Levy & Christoff, 1983).

Rodriguez-Segade *et al.* (1985) now report anomalous absorbance changes, of about the same magnitude as those we described, when ZnCl_2 is added to rat liver

extracts. They also state that, in their reversal assays, net absorbance changes (in which one of a pair of cuvettes contains no substrate) differ by only approx. 5% from results in which absorbances in the presence or absence of substrate are read separately against air. These two methods for subtracting the absorbance change in the absence of added substrate must, of course, give the same result. The important point we made (Levy & Christoff, 1983), and which Rodriguez-Segade *et al.* (1985) appear to disregard, is that it is incorrect to subtract the absorbance change in the cuvette lacking substrate at all. This absorbance change is caused by glucose 6-phosphate in the extract, but because the concentration of glucose 6-phosphate used in the assay saturates the enzyme, any additional substrate added with the extract cannot alter the reaction velocity (Levy & Christoff, 1983). The interpretation of the effect of GSSG and NADPH inhibition of glucose-6-phosphate dehydrogenase in untreated rat liver extracts is completely erroneous if this 'correction' is made, as can be seen by examining our data for the high-speed supernatant in Table 1 of our paper (Levy & Christoff, 1983).

Rodriguez-Segade *et al.* (1985) state that they were unable to understand our reasoning that inhibition of hepatic glucose-6-phosphate dehydrogenase by GSSG is only apparent, since we admit to observing apparent inhibition in experiments in which no NADPH is added. Attempting to settle this point, they show that Hg^{2+} (which they add, as we did, to inhibit glutathione reductase without introducing the artifactual absorbance changes caused by Zn^{2+}) and GSSG, added separately, each inhibit glucose-6-phosphate dehydrogenase, whereas when they are added together they do not. This is precisely what one would expect and, in fact, what we also reported (see our Table 2 and our statement that $2 \mu\text{M-Hg}^{2+}$ inhibits the enzyme by approx. 15%; Levy & Christoff, 1983). Contrary to what Rodriguez-Segade *et al.* (1985) state, the failure to add NADPH to their assay mixtures does not eliminate interference from glutathione reductase activity. The NADPH generated from NADP^+ by the action of glucose-6-phosphate dehydrogenase is rapidly reoxidized by GSSG and glutathione reductase, diminishing the net absorbance increase at 340 nm, which can be erroneously interpreted as inhibition of glucose-6-phosphate dehydrogenase.

The role played by glutathione reductase in these experiments is central to the correct interpretation of the effect of GSSG. In this regard it is not clear, as Rodriguez-Segade *et al.* (1985) claim, why 9.6 units of glutathione reductase present in their rat liver extracts can only consume 1.4 nmol of NADPH in 10 min. In every rat liver extract we have examined, glutathione reductase activity is very high. To assess its role in experiments on glucose-6-phosphate dehydrogenase, one must assay glutathione reductase under the same conditions as those used in those experiments. We noted, for example, that low concentrations of EDTA in the glucose-6-phosphate dehydrogenase assay mixture profoundly stimulate glutathione reductase (Levy & Christoff, 1983). The large effect of glutathione reductase on absorbance changes in glucose-6-phosphate dehydrogenase assays in rat liver extracts is clearly illustrated in Table 1 of our paper (Levy & Christoff, 1983), by comparing the effects of increasing GSSG concentrations in cuvettes lacking glucose 6-phosphate but containing NADPH, with those in cuvettes containing glucose 6-phosphate but lacking