Volume 336, number 2, 208–210 FEBS 13408 December 1993

SH-containing compounds as allosteric effectors of glyceraldehyde-3-phosphate dehydrogenase

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Received 22 September 1993

The rate of hydrolysis of 3-phosphoglyceroyl-holoenzyme, a covalent intermediate of glyceraldehyde-3-phosphate dehydrogenase catalyzed reaction, is considerably decreased in the presence of micromolar concentrations of reduced glutathione, cysteine or dithiothreitol with K_i values of 0.78 μM, 0.6 μM and 10 μM, respectively. The maximal effect is achieved at a molar ratio [effector]/[tetrameric enzyme] close to unity, which points to subunit cooperativity involved in the stabilization of the covalent intermediate against hydrolysis. The effect is specific for acylholoenzyme conformation and insignificant in the case of hydrolysis of acylated apoenzyme species. The ability of the effectors to stabilize the reaction intermediate against spontaneous hydrolysis, in which water replaces inorganic phosphate as the acyl group acceptor, may be a factor contributing to the specificity and effectiveness of the enzyme catalysis.

Glyceraldehyde-3-phosphate dehydrogenase; Acyl-enzyme hydrolysis; Allosteric effector; SH-compound

1. INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GPDH) catalyses oxidation of glyceraldehyde-3-phosphate to form the E-3-phosphoglyceroyl·NADH complex. Reduced coenzyme is then replaced by NAD^+, and the resulting E-3-phosophoglyceroyl·NAD^+ complex is subject to phosphorolysis producing 1,3-bisphosphoglycerate [1,2]. Each active center of the tetrameric GPDH molecule contains an essential highly reactive cysteine residue. To maintain the enzyme in a native state, SH-protective reagents (such as dithiothreitol or β-merceptoethanol) are often used in purification and assay procedures. In our recent studies on rabbit muscle GPDH we have unexpectedly observed an effect of several SH-containing compounds which could not be attributed to the protection of the essential cysteine residue. The results rather pointed to a specific enzyme–effector interaction leading to stabilization of a particular conformational state of the protein. Such an assumption was supported by the results of a more detailed study summarized in the present communication.

2. MATERIALS AND METHODS

Glyceraldehyde-3-phosphate was prepared by the method of Szewzuk [3]. 1,3-Bisphosphoglycerate was prepared and purified as described in [4]. GPDH was isolated from rabbit skeletal muscle by the method of Hill et al. [5]. Hydrolysis of 3-phosphoglyceroyl-holoenzyme was followed as described in the figure legends. Experiments on the hydrolysis of 3-phosphoglyceroyl-apoenzyme were carried out in 50 mM HEPES, pH 7.5, 5 mM EDTTA, 64 μM apo-GPDH. The reaction was initiated by the addition of 256 μM 1,3-bisphosphoglycerate. At fixed time intervals, aliquots containing 6.4 μM GPDH were taken from the mixture and supplemented with 0.1 mM NAD^+. The increase in absorbance at 360 nm due to appearance of Racker band was measured.

Hydrolysis of *p*-nitrophenylacetate was followed at 400 nm, using $\varepsilon = 16500\ M^{-1} \cdot cm^{-1}$ for *p*-nitrophenolate ion [6].

All experiments throughout the work were carried out at 20°C. The data were analyzed with a personal computer using a program Graph PAD in Plot/Version 3.0 N by means of non-linear regression analysis techniques [7].

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3. RESULTS

3.1. *Hydrolysis of holoenzyme-3-phosphoglyceroyl produced in the reaction of glyceraldehyde-3-phosphate oxidation*

Fig. 1 illustrates typical results obtained in experiments carried out with high enzyme concentrations in the presence of excess concentrations of NAD^+ and glyceraldehyde-3-phosphate. Under these conditions, the progress curve consists of a 'burst' of NADH corresponding to formation of an equilibrium concentration of the acyl-enzyme·NADH complex. The linear part of the plot shows the rate of NADH production due to hydrolysis of the acyl-enzyme.

As seen in Fig. 1, curve 2, reduced glutathione (GSH) inhibits the hydrolysis of the acyl-holoenzyme; under the conditions employed, the inhibition was nearly complete. A similar effect was observed with other SH-containing compounds, such as cysteine, β-mercaptoethanol, dithiothreitol. To characterize the effect in quantitative terms, the inhibition constants (K_i) were determined from dependencies of hydrolysis rate con-

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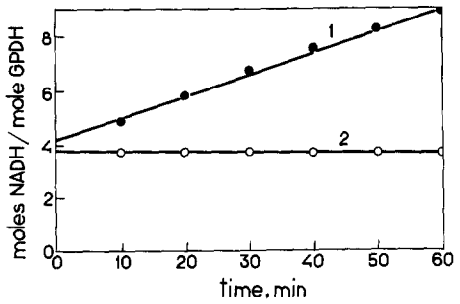

Fig. 1. Effect of reduced glutathione on the rate of hydrolysis of 3-phosphoglyceroyl-holoenzyme produced in the reaction of glyceraldehyde-3-phosphate oxidation. The reaction was carried out in 50 mM HEPES pH 7.5, 5 mM EDTA, 0.2 mM NAD^+, 0.5 mM glyceraldehyde-3-phosphate, 6 μM GPDH and was initiated by the addition of glyceraldehyde-3-phosphate. The increase in absorbance at 340 nm was followed. 1, no additions; 2, in presence of 0.025 mM GSH. Hydrolysis rate constants were 1.3 min^{-1} and 0.055 min^{-1} in the absence and in presence of GSH, respectively.

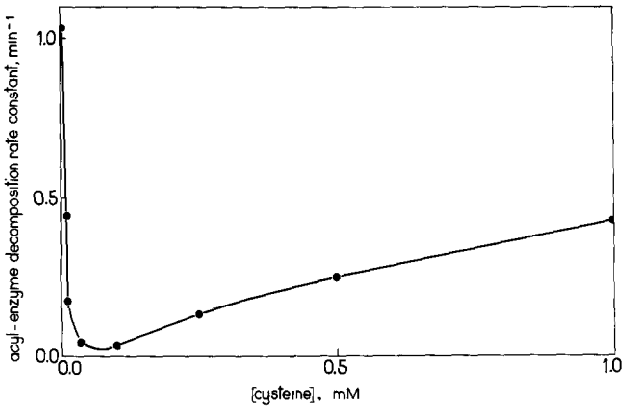

Fig. 2. Different effects of low and high cysteine concentrations on the rate of acyl-enzyme hydrolysis. The reaction mixture contained 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.2 mM NAD^+, 0.5 mM glyceraldehyde-3-phosphate and 5 μM GPDH. Cysteine concentrations are shown in the figure.

stants on concentrations of the corresponding effectors. The K_i values were found to be 0.78 μM (glutathione), 0.6 μM (cysteine), 10 μM (dithiothreitol). Increasing the concentration of the reagent up to millimolar values resulted in the acceleration of acyl-enzyme decomposition (Fig. 2). This result was expected, since under such conditions the SH-containing reagent can serve as a nucleophilic acceptor of the acyl group.

The high potency of the tested compounds to inhibit hydrolysis of acylated GPDH is noteworthy. As shown in Fig. 3, 1 mol of GSH added per mol of the tetramer provides complete protection. This may indicate that the binding of the effector to a single subunit of the tetramer is sufficient to stabilize each active center in a conformation wherein the covalent acyl-holoenzyme intermediate is resistant to hydrolysis. The figure also shows that the effect is specific for the reduced form of glutathione.

3.2. *Hydrolysis of 3-phosphoglyceroyl-GPDH formed by the acylation of the enzyme with 1,3-bisphosphoglycerate*

In this series of experiments, a direct method of following hydrolysis has been employed, starting with the acyl-GPDH intermediate synthesized by the acylation of holo- or apoenzyme by 1,3-bisphosphoglycerate. In the case of holoenzyme, acylation is accompanied by the disappearance of absorbance at 360 nm (Racker band) due to covalent modification of the essential SH group involved in the charge transfer complex formation [8]. Hydrolytic decomposition of the acyl-enzyme intermediate can then be followed by the reappearance of the Racker band. Fig. 4 shows the results of an experiment of this series. It is seen that the rate of hydrolysis is markedly reduced in the presence of low concentrations of dithiothreitol. Similar results were obtained with GSH as an effector. Together with the results described in the previous section, these data suggested that in the presence of SH-containing compounds, the 3-phosphoglyceroyl-enzyme·NAD^+ complex becomes stabilized against hydrolysis. To get some insight into the mechanism of the effect, we have performed experiments with another form of the acylated enzyme, namely, with 3-phosphoglyceroyl-apo GPDH. The results obtained revealed no significant effect of dithiothreitol (hydrolysis rate constants were 0.11 min^{-1} and 0.058 min^{-1} in the absence and presence of 0.1 mM DTT, respectively). It therefore appears that the action of the effectors is specific for the holo-conformation of the covalent intermediate. To obtain additional support to this conclusion, we have studied the effect of SH-containing compounds on the rate of hydrolysis of *p*-nitrophenylacetate, the reaction that is catalysed by the apo-form of GPDH and proceeds via the formation of an acetyl-apoenzyme covalent intermediate. The effect of low concentrations of dithiothreitol was negligible, whereas millimolar concentrations produced acceleration probably due to acyl group transfer to the nucleophilic acceptor.

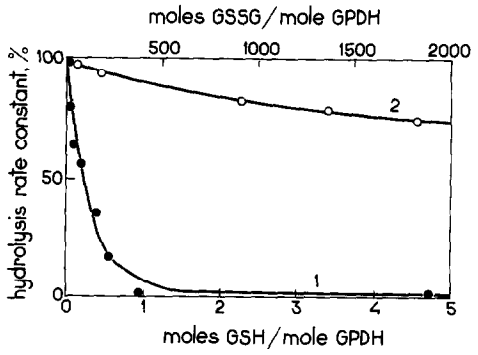

Fig. 3. The effect of glutathione on the rate of hydrolysis of acyl-enzyme. 1, reduced glutathione (lower scale); 2. oxidized glutathione (upper scale).

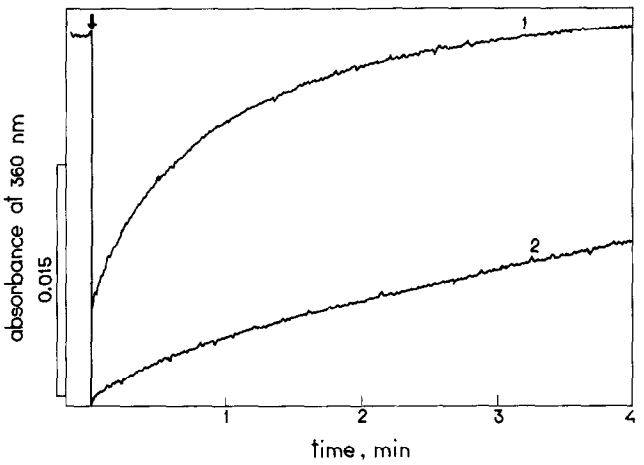

Fig. 4. Formation of 3-phosphoglyceroyl-enzyme · NAD^+ complex by acylation of the holoenzyme with 1,3-bisphospholycerate and hydrolysis of the complex. The reaction was initiated by the addition of 32.4 μM 1,3-bisphosphoglycerate (final concentration) to the mixture containing 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.5 mM NAD^+, 8.1 μM GPDH (indicated by the arrow). The rapid drop in absorbance is due to enzyme acylation; this is followed by the slow reappearance of the Racker band as a consequence of acyl-enzyme hydrolysis. 1, no additions; 2, in the presence of 0.05 mM dithiothreitol. Hydrolysis rate constants were 1.15 min^{-1} and 0.12 min^{-1} in the absence and in presence of dithiothreitol, respectively.

The effect of SH-containing compounds was only observed when the hydrolytic conversion of the reaction intermediate took place, but was absent when the overall rate of glyceraldehyde-3-phosphate oxidative phosphorylation was followed under each of the three sets of conditions employed (50 mM glycine, pH 9.0, 50 mM HEPES, pH 7.5, 50 mM potassium phosphate, pH 7.0).

4. CONCLUSIONS

Information on the mechanism of the GPDH-catalysed reaction indicates that the reaction proceeds via the conversion of the 3-phosphoglyceroyl-enzyme·NADH complex, which is resistant to phosphorolysis, into the 3-phosphoglyceroyl-enzyme·NAD^+ complex. The exchange of NADH for NAD^+ greatly accelerates the rate of acyl transfer to an acceptor-nucleophil [9–11]. Thus, the covalent 3-phosphoglyceroyl-holoenzyme intermediate can adopt two alternative conformational states differing in the accessibility of the acyl-thioester bond to phosphorolysis or hydrolysis. The results of this study present evidence for the existence of a third conformation, that of 3-phosphoglyceroyl-enzyme·NAD^+ complex resistant to hydrolysis. Our data suggest that this conformation can be stabilized by SH-compounds such as GSH, cysteine, dithiothreitol. No effect is, however, seen when the overall reaction of the glyceraldehyde-3-phosphate oxidative phosphorylation takes place, and this indicates that the main function of the effectors is to ensure protection of the 3-phosphoglyceroyl-holoenzyme intermediate against wasteful hydrolysis. Taking into account sufficiently high (millimolar) cellular concentrations of GSH [12], it seems likely that the conformation of the acyl-enzyme·NAD^+ complex stabilized by an SH-containing ligand would prevail under physiological conditions.

The mechanism of the allosteric effect exhibited by SH-containing compounds remains obscure. Of considerable interest is the low stoichiometry of an effector-to-GPDH molar ratio sufficient to achieve maximal effect. This suggests that the effector binding site may be involved in a system of interactions responsible for subunit cooperativity in the tetrameric GPDH molecule. We have also observed that 'effector-stabilized' and an 'effector-free' acyl-enzyme·NAD^+ conformations differ in character of functional interactions between GPDH active centers. This subject is under study in our laboratory.

Acknowledgements: This work was supported by the Russian Foundation of Fundamental Investigations (Grant N 93-04-20215) and by a grant from State research programme 08.05 'Newest methods of bioengineering, subprogram Engineering in Enzymology'.

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