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Interaction of glyceraldehyde-3-phosphate dehydrogenase with SH-containing compounds: evidence for the binding of L-cysteine and for the dependence of the binding on the functional state of the enzyme

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Abstract **Incorporation of L-[^{35}S]cysteine into rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was detected following incubation of the enzyme in a mixture containing glyceraldehyde-3-phosphate, NAD^+ and the labeled cysteine. Insignificant binding occurred in the absence of either the substrate or NAD^+, suggesting that formation of an acylated enzyme form was a prerequisite for the binding. Stoichiometry of the binding depended on the number of functioning active centers; up to 4 moles of L-[^{35}S]cysteine bound per mole tetramer with fresh enzyme preparations. The L-[^{35}S]cysteine incorporation depended on pH and was maximal when a group having pK_a of 8.5 is protonated. To clarify the relevance of this finding to the effect of SH-containing compounds previously shown to decrease the rate of 3-phosphoglyceroyl-enzyme hydrolysis [Kuzminskaya et al., FEBS Lett. 336 (1993) 208–210], the pH-dependence of the effect of glutathione on the hydrolysis rate was determined and found to be close to the pH-dependence of L-[^{35}S]cysteine binding.**

Key words: SH-containing compounds; Acyl-enzyme hydrolysis; Glyceraldehyde-3-phosphate dehydrogenase

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes a two-step reaction. First, glyceraldehyde-3-phosphate is oxidized to form a covalent intermediate at the essential Cys-149, complexed with NADH. Following NADH dissociation from 3-phosphoglyceroyl-enzyme·NADH complex, NAD^+ binds to acyl-enzyme intermediate. The second step, phosphorolysis then occurs, yielding 1,3-biphosphoglycerate [2,3]. The replacement of NADH by NAD^+ greatly accelerates the rate of acyl transfer to an acceptor-nucleophile [4,5], and this implies that specific, functionally important differences exist between the 3-phosphoglyceroyl-enzyme·NADH and 3-phosphoglyceroyl-enzyme·NAD^+ conformations. The structural basis of these differences remains, however, unknown since no X-ray studies on the ternary complexes formed by GAPDH have been as yet performed. At the same time, information on the factors influencing conformational states of the acylated enzyme forms is of importance for better understanding the molecular mechanisms of enzyme functioning and regulation. Such considerations warrant our interest as to the effect of low molecular weight SH-containing compounds on the stability of 3-phosphoglyceroyl-GAPDH intermediate reported in our previous communication [1]. Significant deceleration of the acyl-enzyme hydrolysis observed in the presence of micromolar concentrations of cysteine, reduced glutathione or dithiothreitol suggested that there is a specific interaction between the acylated enzyme and an effector. The purpose of this study was to experimentally test the ability of GAPDH to bind a low molecular weight SH-containing compound and to examine the correlation between the binding of the ligand and its effect.

2. Materials and methods

L-[^{35}S]Cysteine as a hydrochloride (20–150 μCi/mmol) was obtained from Amersham. GAPDH was purified from rabbit skeletal muscle [6]. Immobilization of the enzyme was carried out as described [7] using CNBr-activated Sepharose 4B prepared before each experiment (30 mg CNBr per 1 ml of the packed gel). Holoenzyme was dissolved in 0.1 M sodium phosphate, 5 mM EDTA, pH 8.3, protein concentration, 1.0–1.5 mg/ml. Under these conditions, 200–500 μg protein per ml of the packed gel became immobilized (determined as described [8]). The binding of L-[^{35}S]cysteine to the immobilized GAPDH was measured as follows. Immobilized GAPDH was incubated with 2 mM DTT for 1 h (or left overnight), washed with 100 vol. of a buffer with desired pH and suspended in the same buffer (1 ml of the buffer per 1 ml of the packed gel). The reaction mixture contained 0.2–0.3 ml of the suspension, 0.5 mM NAD^+, 20 μM L-[^{35}S]cysteine and 0.5 mM GAP. After the addition of the latter component and mixing, the gel was separated by centrifugation (10,000 × *g*) for 1 min, and the radioactivity of the supernatants was determined. Controls lacking substrate were run in parallel. The amount of L-[^{35}S]cysteine incorporated into the immobilized enzyme was estimated as the difference between the standard and control samples.

3. Results

The possibility that SH-containing compounds (e.g. cysteine and reduced glutathione) can specifically interact with GAPDH, was tested by measuring the binding of L-[^{35}S]cysteine under the conditions resembling those employed in the studies on the effect of SH-containing compounds on the 3-phosphoglyceroyl-enzyme hydrolysis [1]. GAPDH immobilized on CNBr-activated Sepharose was used in these experiments. After incubation of the matrix-bound enzyme in an appropriate reaction mixture containing the label, the gel was quickly removed by centrifugation, and the supernatant was analyzed for the remaining L-[^{35}S]cysteine. In this way, specific incorporation of the label into the enzyme could be followed owing to the fact that control samples which lacked but one component of the reaction mixture were run in parallel. As shown in Fig. 1, up to 4 moles of L-[^{35}S]cysteine can be incorporated per tetrameric GAPDH in the presence of both GAP and NAD^+. Curve 2

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAP, glyceraldehyde-3-phosphate; DTT, dithiothreitol.

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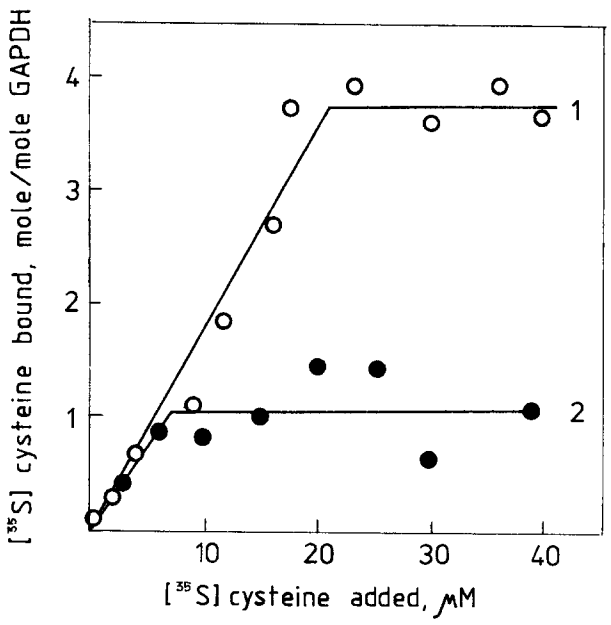

Fig. 1. The binding of [^{35}S]cysteine to GAPDH immobilized on Sepharose 4B. Line 1 = 0.2 ml of the reaction mixture contained 30 mM HEPES-NaOH, pH 7.0, 5 mM EDTA, 0.5 mM NAD^+, 0.5 mM GAP, 1.0–40 μM L-[^{35}S]cysteine (0.015–0.6 μCi) and 1 μM immobilized GAPDH tetramer. Line 2 = the same as line 1, but the immobilized enzyme was washed with 100 vols. of sodium phosphate buffer (30 mM), pH 7.0, to remove bound NAD^+. NAD^+ was also omitted from the reaction mixture. Control samples contained all the components excepting GAP. Time of incubation (including centrifugation) did not exceed 5 min. The amount of the labeled cysteine incorporated in the enzyme was estimated from the decrease of L-[^{35}S]cysteine content in the supernatant (the data obtained with the sample lacking GAP were subtracted from the data obtained with the sample containing GAP).

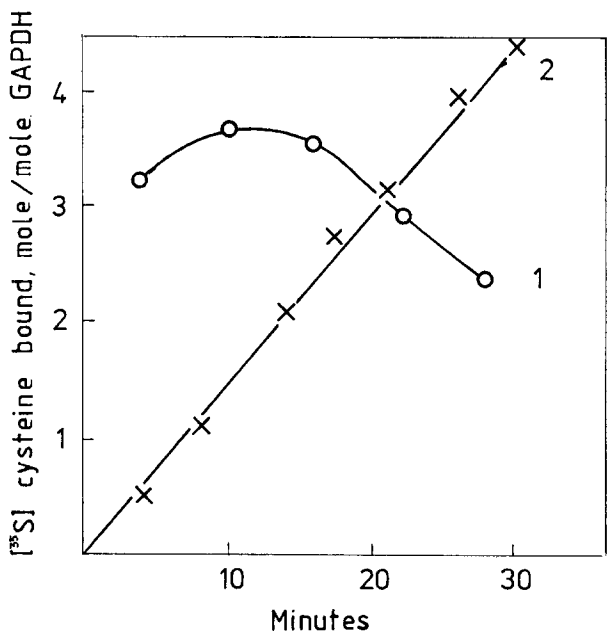

Fig. 2. Time dependence of the binding of L-[^{35}S]cysteine to immobilized GAPDH. The experimental conditions were the same as in Fig. 1, L-[^{35}S]cysteine concentration, 20 μM. At a fixed time interval, the immobilized enzyme suspension was separated by centrifugation, and an aliquot (10 μl) of the supernatant was taken to determine the concentration of L-[^{35}S]cysteine. The remaining suspension was then mixed and the above procedure repeated. Line 1 = the data obtained in the presence of GAP minus the data obtained in the absence of GAP. Line 2 = the data obtained in the absence of GAP.

refers to the sample where NAD^+ was omitted from the reaction mixture. It is seen that the incorporation of the label is significantly reduced. Some binding was however detected, possibly due to the presence of the firmly bound NAD^+ in the protein. In another experiment performed with an aged preparation of the immobilized GAPDH, complete removal of NAD^+ has been achieved; in this case no binding of L-[^{35}S]cysteine was detected in the absence of added NAD^+. We have also observed that enzyme preparations not pretreated with DTT (see section 2) incorporated less L-[^{35}S]cysteine (2.4 ± 0.2 moles per mole tetramer).

Taken together, the results obtained support the idea that the binding of L-[^{35}S]cysteine depends on the integrity of essential Cys-149 and on the catalytic conversion of GAP into 3-phosphoglyceroyl-enzyme. The L-[^{35}S]cysteine incorporation into the protein might be a fast process; as shown in Fig. 2, the maximal effect was observed at the earliest time of measurement possible in our experiments. As noted above, some binding of L-[^{35}S]cysteine occurred in the absence of GAP. This 'non-specific' binding was normally subtracted from the measurements made using the complete reaction mixture. One can see from Fig. 2, curve 2, that the 'non-specific' binding progressively increased in time; incorporation of the cysteine into the matrix gel supporting the immobilized enzyme could partially account for the effect. Taking into consideration this time dependence of the 'non-specific' binding, we used the shortest time of incubation possible under the conditions of our experiments.

The binding of L-[^{35}S]cysteine to the enzyme exhibited distinctive pH dependence. As shown in Fig. 3, it is controlled by

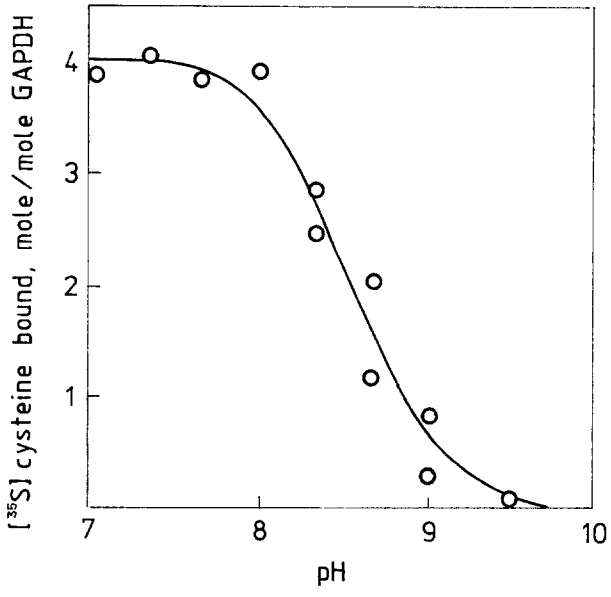

Fig. 3. The binding of L-[^{35}S]cysteine to immobilized GAPDH as a function of pH. 0.2 ml of the reaction mixture contained 50 mM HEPES-NaOH (pH 7.0–8.0), 50 mM sodium veronal-HCl buffer (pH 8.0–8.5), or 50 mM glycine-NaOH buffer (pH 9.0–9.5), 5 mM EDTA, 0.5 mM NAD^+, 0.5 mM GAP, 20 μM L-[^{35}S]cysteine (0.3 μCi), 1 μM tetramer GAPDH. See Fig. 1 for further details.

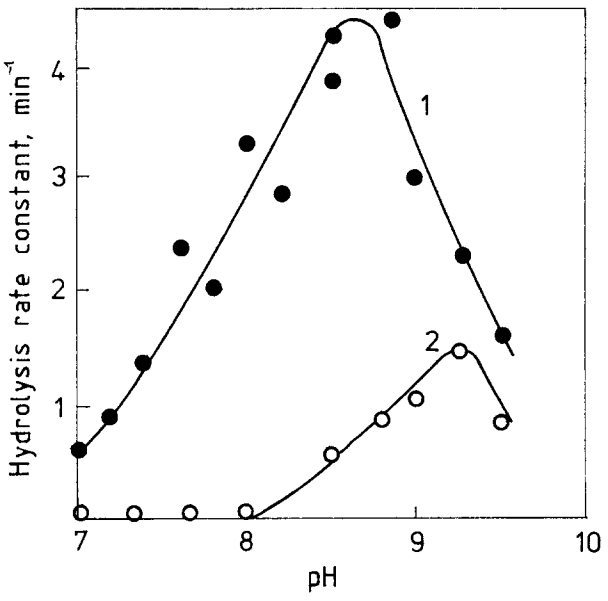

Fig. 4. pH-dependencies of the rate constants of 3-phosphoglyceroyl-enzyme hydrolysis performed in the absence (1) or in the presence (2) of 10 μM reduced glutathione. The reaction was carried out in 50 mM HEPES-NaOH (pH 7.0–8.5) or in 50 mM CHES-NaOH (pH 8.5–9.5), 5 mM EDTA, 0.2 mM NAD^+, 0.2 mM GAP, 8.5 μM soluble GAPDH and initiated by the addition of GAP.

an acidic group with pK_a of 8.5. In view of these results, we were interested to investigate the pH dependence of the effect of SH-containing compounds on the hydrolysis of 3-phosphoglyceroyl-enzyme. As shown in our previous study [1], cysteine, reduced glutathione and DTT significantly decreased the rate of acyl-enzyme hydrolysis; in these experiments the reaction was carried out at pH 7.5. We thought that if a correlation exists between the binding of a SH-containing compound and its effect on hydrolysis, the pH dependence of the effect must be close to the pH dependence of the binding. The results shown in Fig. 4 argue in favour of this proposal. The inhibitory effect of reduced glutathione is maximal at pH 8 and disappears at pH above 9, i.e. displays a pH-dependence closely resembling the pH-dependence of the binding of L-[^{35}S]cysteine.

4. Discussion

The evidence we have presented supports the suggestion that GAPDH is capable of catalyzing the binding of a low molecular weight thiol. In fact, the effective binding of L-[^{35}S]cysteine was only observed under the conditions where catalytic conversion of GAP into 3-phosphoglyceroyl-enzyme occurred. Similar results were obtained in the absence or in presence of inorganic phosphate in the reaction medium, which implies that a single catalytic turnover yielding the acylated enzyme form was sufficient to ensure the binding. The extent of binding depended on the integrity of the essential Cys-149. With the fully active enzyme tetramer one mole of radiolabeled cysteine incorporated per mole enzyme subunit; at present, we have no definite information on the mechanism of the process involved, and can only propose a hypothetical explanation for the findings. We speculate that upon acylation of the enzyme by its substrate, conditions are created for the activation of a low molecular weight thiol, necessary for its binding to the protein. The activation might proceed through a nucleophilic attack of the cysteine thiol group on the transition state acyl cation (which is suggested to appear after the binding of NAD^+ to 3-phosphoglyceroyl-enzyme [9]. A thioacetal formed in this way might serve as a source of a thiolate anion form of the cysteine transferred to an appropriate acceptor, most likely a cysteine residue of the protein. Such an intramolecular transfer resulting in the formation of a mixed disulfide would strongly depend on the conformational state of the enzyme and might only be possible in the acylated enzyme form. Protonation of a group with pK_a near 8.5 is also a prerequisite for the binding. A likely candidate for such a group is the SH-group of the cysteine in solution.

Irrespective of the mechanism of cysteine incorporation, the modification alters functional properties of the enzyme. This is manifested in the decreased susceptibility of 3-phosphoglyceroyl-enzyme to hydrolysis.

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