Mildly oxidized GAPDH: the coupling of the dehydrogenase and acyl phosphatase activities

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Received 2 April 1999; received in revised form 21 April 1999

Abstract The hydrogen peroxide-induced 'non-phosphorylating' activity of D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown to be a result of the successive action of two forms of the enzyme subunits: one catalyzing production of 1,3-bisphosphoglycerate, and the other performing its hydrolytic decomposition. The latter form is produced by mild oxidation of GAPDH in the presence of a low hydrogen peroxide concentration when essential Cys-149 is oxidized to the sulfenate derivative. The results obtained with a C153S mutant of *Bacillus stearothermophilus* GAPDH rule out the possibility that intrasubunit acyl transfer between Cys-149 and a sulfenic form of Cys-153 is required for the 'non-phosphorylating' activity of the enzyme.

Key words: D-Glyceraldehyde 3-phosphate dehydrogenase; Acyl phosphatase; Hydrogen peroxide

1. Introduction

As was recently shown in our group, incubation of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the presence of low hydrogen peroxide concentrations results in oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate in the absence of inorganic phosphate. The observed activity could be immediately blocked by the addition of low molecular weight thiols. Since the products of the process were identified as 3-phosphoglycerate and NADH, the reaction was specified as 'non-phosphorylating dehydrogenase activity' [1]. A possible role of this activity in the uncoupling of oxidation and phosphorylation in glycolysis and its significance in metabolic regulation has been discussed [2]. A more detailed study of the enzyme treated with low hydrogen peroxide concentrations showed that sulfenic acid derivatives of cysteine residues were involved in the catalytic process. Sulfenic acids are known to be present among the products of hydrogen peroxide-induced oxidation of GAPDH, together with sulfinic and sulfonic acids [3]. In the presence of mild oxidizing agents (iodosobenzoate, trinitroglycerin) all functionally important cysteine residues of GAPDH can be oxidized to give a stabilized form of sulfenic acid. Such a modification results in a complete disappearance of the dehydrogenase activity and converts the enzyme into an acyl phosphatase [4–6].

Participation of a sulfenic acid derivative of a cysteine residue in the 'non-phosphorylating' GAPDH activity detected under our experimental conditions led us to suggest acyl transfer from the thioester intermediate to a sulfenic acid yielding a sulfenyl carboxylate derivative which is easily hydrolyzed. Given that the appearance of the 'non-phosphorylating' activity was accompanied by only a minor change in the essential SH group content (no more than 0.9 SH groups per tetramer), we hypothesized that some other cysteine residue could be involved. An intrasubunit acyl transfer between active site Cys-149 and the closely spaced Cys-153 (oxidized to a sulfenic acid derivative) has been suggested [1,7]. In this paper, results are presented which rule out such a mechanism and we give an alternative explanation of the experimental facts.

2. Materials and methods

NAD^+, HEPES, glycine, iodoacetamide, and EDTA were from Sigma, β-mercaptoethanol (ME) was obtained from Ferak, hydrogen peroxide was purchased from Merck. Glyceraldehyde 3-phosphate was prepared by the method of Szewczuk et al. [8].

GAPDH was isolated from rabbit muscles by the method of Scopes and Stoter [9] complemented by gel filtration on a Sephadex G-100 column to remove traces of myoglobin.

Site-directed mutagenesis on *Bacillus stearothermophilus* GAPDH was performed using the method of Kunkel et al. [10]. The *Escherichia coli* strain used for C153S and C149A mutant enzyme production was DH5α transformed with a pBluescript II SK containing the *gap* gene under the *lac* protomer (pSKBstII) [11]. Purification of C153S and C149A mutant enzyme forms was carried out as previously described by Talfournier et al. [12]. Purity of the enzymes was checked by electrophoresis on a 10% SDS polyacrylamide gel and by mass spectrometry. Protein concentration was determined at 280 nm using $A_{0.1\%} = 1.0$. The hydrogen peroxide concentration was determined at 230 nm using $\varepsilon = 72.7\ M^{-1}\ cm^{-1}$.

Phosphorylating GAPDH activity was followed at 340 nm. The reaction was carried out at pH 8.9 and was initiated by the addition of 5 μg of the enzyme to the mixture containing 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, 0.5 mM NAD^+ and 0.5 mM glyceraldehyde 3-phosphate.

'Non-phosphorylating' GAPDH activity was followed at 340 nm by a steady increase in NADH concentration due to the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate at pH 7.6 in the mixture containing 50 mM HEPES, 5 mM EDTA, 0.5 mM NAD^+, 0.5 mM glyceraldehyde 3-phosphate, 0.7 μM GAPDH, and was initiated by the addition of glyceraldehyde 3-phosphate.

The mild oxidation of GAPDH (rabbit muscle, *B. stearothermophilus*) was performed by incubation of the enzymes with hydrogen peroxide at 20°C, pH 7.6 for 30 min. The reaction was initiated by the addition of hydrogen peroxide to a final concentration of 7–14 μM to the mixture containing 50 mM HEPES, 5 mM EDTA, 0.5 mM NAD^+, and 7 μM GAPDH. To achieve complete oxidation of all

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); ME, β-mercaptoethanol

0014-5793/99/$20.00
PII: S0014-5793(99)00627-4

essential cysteine residues, the enzymes were incubated with 60 μM hydrogen peroxide for 30 min. Before starting the experiments on oxidation, enzyme preparations were pretreated with 2 mM ME. Excess ME was then removed by passing the enzyme solution through a Sephadex G-50 column.

3. Results and discussion

To obtain more direct information on the role of Cys-153 in the 'non-phosphorylating' GAPDH activity, we turned to the investigation of a mutant form of the enzyme, in which Cys-153 was replaced by serine. The C153S mutant and the wild type *B. stearothermophilus* GAPDH were both observed to catalyze the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate under the conditions previously employed in experiments performed with the rabbit muscle enzyme. The activities were found to be 3.0 U/mg and 3.6 U/mg for the C153 mutant and the wild type, respectively. This indicated that the ability to catalyze the 'non-phosphorylating' reaction is a common property of GAPDHs of different origin. The second conclusion which follows from these studies is that a sulfenic acid derivative which participates in catalysis must originate from Cys-149 (the only cysteine residue existing in the C153S mutant of *B. stearothermophilus* GAPDH), and any role of Cys-153 is to be ruled out.

Therefore, the mildly oxidized GAPDH preparation contains a certain amount of Cys-149 in the form of a sulfenic acid derivative. We made an attempt to determine the proportion of this form among other products of Cys-149 oxidation in the preparations of GAPDH subjected to different extents of oxidative modification. The experimental approach was based on the liability of sulfenic acid to reduction by arsenite; incubation of an oxidized enzyme with arsenite results in the disappearance of the 'non-phosphorylating' activity [1]. As shown in Fig. 1, curve 1, the amount of sulfenic acid estimated in GAPDH preparations preincubated with different concentrations of hydrogen peroxide never exceeded 22% of the total amount of Cys-149 residues, which corresponds to about 0.9 essential cysteines per enzyme tetramer. In the presence of low hydrogen peroxide concentrations, cysteine residues are predominantly oxidized to sulfenic acid derivatives. Upon increasing the hydrogen peroxide concentration, the portion of sulfenic acid among the products of oxidation diminishes at the expense of increasing amounts of the other products of oxidation (sulfinic and sulfonic acids, Fig. 1, curve 2) which are no longer liable to reduction by arsenite. Completely oxidized enzyme contains practically no SOH groups. Similar results were obtained on the muscle enzyme and the *B. stearothermophilus* C153S mutant.

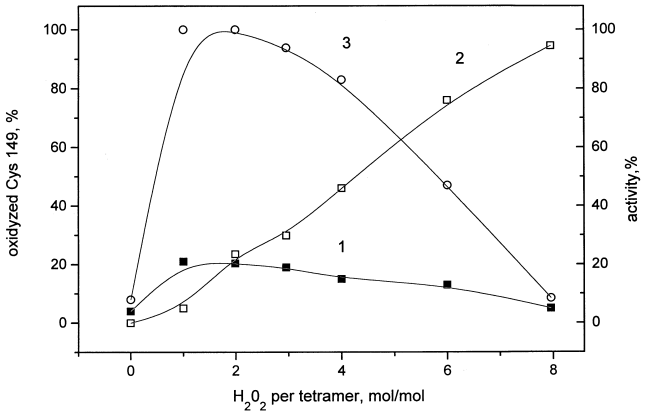
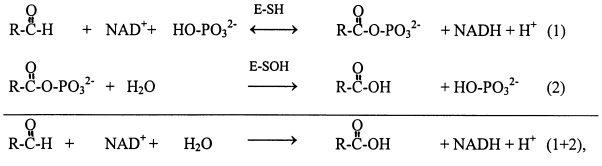


Fig. 1. Content of the sulfenic acid derivatives of Cys-149 among other products of its oxidation by hydrogen peroxide. Correlation with the 'non-phosphorylating' activity. Curve 1, sulfenic acid derivatives of Cys-149 (left ordinate); 2, other products of oxidation of Cys 149 (left ordinate); 3, 'non-phosphorylating' GAPDH activity (right ordinate). The reaction mixture contained 50 mM HEPES, pH 7.6, 0.5 mM NAD^+, 7 μM rabbit muscle GAPDH and hydrogen peroxide as indicated. Following 30 min incubation at 20°C the samples were supplemented with arsenite to a final concentration of 50 mM, and again incubated for 1 h. The percentage of SOH groups in the preparations of oxidized GAPDH was estimated as $(A_{red} - A_{ox})/A \times 100\%$, where A_{ox} stands for the phosphorylating dehydrogenase activity of the enzyme after the oxidation with hydrogen peroxide, A_{red} for the phosphorylating dehydrogenase activity of the oxidized GAPDH after the treatment with sodium arsenite, and A for the activity of the native enzyme. The percentage of other products of oxidation was determined as $(A - A_{red})/A \times 100\%$.

The results presented in Fig. 1 indicate that the 'non-phosphorylating' dehydrogenase activity (curve 3) is maximal under the conditions where about one Cys-149 per tetramer is oxidized to a sulfenic acid derivative, while the other Cys-149 are practically not affected. Thus, reduced (SH) and mildly oxidized (SOH) forms of the essential Cys-149 are located on different subunits of the enzyme tetramer. This excludes a possibility of a direct acyl transfer between a subunit producing 3-phosphoglyceroyl and a subunit able to hydrolyze it. The only possibility is a coupling of the dehydrogenase reaction (1) and the acyl phosphatase one (2), in case the reaction medium is admixed with inorganic phosphate:



where R stands for -CHOH-CH_2-O-PO_3^{2-}.

To obtain more experimental support for the above scheme, it seemed reasonable to demonstrate the coupling of the dehydrogenase and acyl phosphatase activities performed by different tetrameric enzyme species. To this end, a mixture of tetrameric GAPDH species (one containing all Cys-149 residues in the SH form, and the other containing a sulfenic acid derivative of the essential cysteine) was used. To prepare the latter form, GAPDH was oxidized under the mild conditions (see Table 1, B). The SH groups which remained unmodified were treated with iodoacetamide to completely block the free cysteines (iodoacetamide does not affect SOH groups). The enzyme prepared in this way (Table 1, C) contained only cysteines in the SOH form and was devoid of both the phosphorylating and 'non-phosphorylating' activities, since the latter reaction is only operative when the enzyme contains free cysteines as well as sulfenic acid derivatives. At the same time, the reduced enzyme (Table 1, A) contained only reduced cysteines and showed only the phosphorylating activity. A mixture of the two preparations (A+C) in a ratio of one subunit containing Cys-149 in the sulfenic acid form to three subunits containing intact Cys-149 possessed the 'non-phosphorylating' activity (see Table 1). The results evidence the coupling of the dehydrogenase and acyl phosphatase reactions carried out by different tetrameric enzyme species. Table 1 also shows that the completely oxidized enzyme

Table 1
Properties of different GAPDH preparations

Enzyme preparation	Phosphorylating dehydrogenase activity (U/mg)	'Non-phosphorylating' activity (U/mg)
A. Reduced	80	<0.04
B. Mildly oxidized	65	1.4
C. Preparation B modified by iodoacetamide	0	0
D. Completely oxidized	0	0
E. Mildly oxidized C149A mutant	0	0
A+D[a]		<0.04
A+C[a]		0.9
A+E[a]		<0.04

A–D, rabbit muscle GAPDH; E, *B. stearothermophilus* GAPDH.
A. An enzyme preparation was incubated with 2 mM ME for 10 min, and then passed through a Sephadex column.
B. 7 μM of the reduced GAPDH (A) was incubated in the presence of 10 μM H_2O_2 for 30 min.
C. Preparation B was titrated with 2 mM iodoacetamide solution to complete disappearance of phosphorylating dehydrogenase activity.
D. 7 μM of the reduced GAPDH (A) was incubated in the presence of 60 μM H_2O_2 to complete disappearance of phosphorylating dehydrogenase activity.
E. The enzyme preparation was oxidized under the conditions described in B.
[a]'Non-phosphorylating' activity was determined in a sample (3 ml) containing 200 μg oxidized and 150 μg reduced enzyme preparations.

form (D) mixed with the reduced enzyme is ineffective in the coupling reaction due to the absence of SOH groups. No activity was observed when the C149A mutant form of the *B. stearothermophilus* enzyme lacking the essential cysteine residue but having Cys-153 was subjected to the mild oxidation and mixed with the reduced enzyme (Table 1). This definitively excluded a possibility of participation of some other oxidizable groups in catalysis of the reaction.

All these data support the conclusion that the 'non-phosphorylating' dehydrogenase activity is a result of the coupling of two reactions, the phosphorylating dehydrogenase reaction on the one hand, and the acyl phosphatase reaction on the other, which are carried out by different subunits of GAPDH (possessing the SH or the SOH form of Cys-149, respectively); the subunits may be either located within a single tetrameric enzyme molecule or belong to different tetramers. The coupling of the dehydrogenase (1) and acyl phosphatase (2) reactions proceeds under a stationary inorganic phosphate concentration since the second reaction is irreversible. The process can therefore take place at very low phosphate concentrations, even at the expense of impurities present in the components of the reaction mixture. Under the conditions of our experiments, addition of 1–10 mM inorganic phosphate to the reaction mixture did not influence the rate of the 'non-phosphorylating' dehydrogenase activity, which suggests that the acyl phosphatase reaction was a rate-limiting one. Increasing inorganic phosphate concentration (up to 10 mM) which accelerates the phosphorylating dehydrogenase activity hindered the measurements. For this reason, the coupled reaction was usually followed in the presence of 1 mM inorganic phosphate.

It should be pointed out that the possibility to couple the dehydrogenase and acyl phosphatase activities of GAPDH was previously demonstrated by Ehring and Colowick [4]. Native GAPDH was incubated in the reaction mixture containing all components of the dehydrogenase reaction until equilibrium had been established. A preparation of GAPDH oxidized by iodosobenzoate was then added, and a steady-state increase of NADH was observed in the system due to the irreversible hydrolysis of 1,3-bisphosphoglycerate. The preparation of oxidized GAPDH was completely devoid of dehydrogenase activity but was active as an acyl phosphatase. A peculiarity of the mildly oxidized GAPDH used in our experiments is that one and the same enzyme preparation can possess both the dehydrogenase and acyl phosphatase activities (exhibited by different active centers) and thus is able to catalyze the coupled reaction.

So far the acyl phosphatase activity of GAPDH was considered an alternative to the dehydrogenase activity: oxidation of Cys-149 residues by iodosobenzoate resulted in the appearance of the acyl phosphatase activity and the complete loss of the dehydrogenase activity. The main interest has been focused on the mechanism of the transformation of the enzyme function, whereas the physiological significance of the phenomenon was not discussed. Little and O'Brien demonstrated that a GAPDH preparation oxidized by H_2O_2 under mild conditions was a mixture of different components: along with intact reduced cysteine residues it contained various products of their oxidation, including sulfenic acid derivatives [3]. The authors have not, however, studied the catalytic properties of the preparation, centering their interest on the characteristics of the oxidation products. In the present work we have managed to show that the dehydrogenase and acyl phosphatase activities can be performed by different subunits of a mildly oxidized enzyme located either within a single tetramer or within different tetrameric species, and their successive action eventually leads to the uncoupling of oxidation and phosphorylation.

In this way, the product of the dehydrogenase reaction, 1,3-bisphosphoglycerate, can undergo further transformation either in the reaction catalyzed by 3-phosphoglycerate kinase producing ATP, or in the acyl phosphatase reaction catalyzed by the mildly oxidized GAPDH form. Although the said acyl phosphatase reaction is much slower than the 3-phosphoglycerate kinase reaction, it is irreversible and does not require the presence of ADP.

The mild oxidation of GAPDH occurs in the presence of rather low hydrogen peroxide concentrations, which can be observed in the cell under some conditions, such as oxidative stress. Bearing in mind the high intracellular concentrations of the enzyme, one may suggest that in such cases detectable amounts of GAPDH capable of catalyzing the dehydrogenase reaction not coupled with phosphorylation could be formed. The physiological significance of this transformation, which is readily reversible in the presence of reducing reagents, deserves further study.

Acknowledgements: This work was supported by the Russian Foundation of Basic Research (Grant 99-04-48076), by the Program for the Support of Scientific Schools in Russia (Grant 96-15-98083), by the Centre National de la Recherche Scientifique and the University Henri Poincareé, Nancy 1.

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