Rabbit muscle GAPDH: non-phosphorylating dehydrogenase activity induced by hydrogen peroxide

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Abstract Incubation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with micromolar hydrogen peroxide concentrations does not alter the catalytic properties of GAPDH in the reaction of oxidative phosphorylation of glyceraldehyde-3-phosphate, but endows the enzyme with the ability to catalyze the reaction in the absence of inorganic phosphate, producing NADH and 3-phosphoglycerate. The reaction is supposed to occur as a result of intramolecular acyl transfer from Cys-149 to a sulfenic acid form of Cys-153, followed by hydrolysis of the intermediate. The 'mildly oxidized' form of the enzyme can be easily converted back to the form unable to catalyze glyceraldehyde-3-phosphate oxidation in the absence of phosphate, by the addition of thiols.

Key words: Glyceraldehyde-3-phosphate dehydrogenase; Hydrogen peroxide; Thiol; Oxidation

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes oxidative phosphorylation of glyceraldehyde-3-phosphate in a two step reaction [1]. As a result of the first, oxidative step, 3-phosphoglyceroyl enzyme·NADH complex is formed at each of the four active centers of the tetramer. The covalent intermediate wherein 3-phosphoglyceroyl moiety is bound to Cys-149 of the active center is resistant to acyl transfer reactions. The second step of the reaction, i.e. deacylation, can only occur following NADH dissociation and binding of NAD^+ to the acyl enzyme. The structural basis of NAD^+-induced conformational changes which make the thioester carbonyl of the covalent intermediate liable to nucleophilic attack by inorganic phosphate is obscure, since no X-ray studies of the ternary complex have been performed on GAPDH.

Little is also known about the mechanism of deacylation which can take place in the absence of inorganic phosphate. It has long been known that the 3-phosphoglyceroyl enzyme is subject to slow hydrolytic decomposition [2,3], but the structural basis of the acyl transfer reaction remained unclear. In the course of our studies devoted to this problem it was observed that the rate of hydrolytic decomposition of 3-phosphoglyceroyl enzyme progressively increased upon storage of the crystalline enzyme preparations at pH 7.5–8.0. Since the reaction was shown to result in the accumulation of 3-phosphoglycerate, it was attributed to non-phosporylating dehydrogenase activity. Low molecular weight thiols (cysteine, reduced glutathione, etc.) effectively inhibited the activity without influencing the rate of oxidative phosphorylation reaction catalyzed by the enzyme [4,5]. This led us to the assumption that the hydrolytic activity (the non-phosphorylating dehydrogenase activity) could depend on the spontaneous oxidation of some functional groups of the enzyme molecule, other than the essential Cys-149 residues. A hypothesis was suggested, according to which accumulation of the 'mildly oxidized' form of GAPDH could occur under aerobic conditions (especially in the presence of hydrogen peroxide); this creates a possibility of uncoupling glyceraldehyde-3-phosphate oxidation and phosphorylation [6].

In the present work the mechanism of conversion of GAPDH into the form possessing the non-phosphorylating dehydrogenase activity was studied and the accumulation of this form on incubation with micromolar hydrogen peroxide concentrations was demonstrated.

2. Materials and methods

NAD^+, HEPES, glycine, 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 Szewzuk [7]. 1,3-Bisphosphoglycerate was prepared and purified as described in [8]. GAPDH was isolated from rabbit muscles by the method of Scopes [9] with following gel filtration on a Sephadex G-100 column to remove traces of myoglobin. The protein concentration was determined at 280 nm, using $A_{0.1\%} = 1.0$ for the holoenzyme and $A_{0.1\%} = 0.8$ for the apoenzyme. The hydrogen peroxide concentration was determined at 230 nm using $\varepsilon = 2.7\ M^{-1}\ cm^{-1}$.

The 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 a mixture containing 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, 0.5 mM NAD^+ and 0.5 mM glyceraldehyde-3-phosphate.

The non-phosphorylating GAPDH activity was followed at 340 nm by the accumulation of NADH due to the oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate in the absence of inorganic phosphate. The reaction was carried out at pH 7.6 in a mixture containing 50 mM HEPES, 5 mM EDTA, 0.5 mM NAD^+, 0.5 mM glyceraldehyde-3-phosphate and 7 μM GAPDH and was initiated by the addition of glyceraldehyde-3-phosphate. Two stages were observed in the reaction: a fast initial burst of NADH accumulation corresponding to the formation of the 3-phosphoglyceroyl enzyme·NADH complex (the pre-steady state) was followed by a slower accumulation of NADH due to acyl enzyme hydrolysis (the steady state). The steady state rate of NADH formation was used to calculate the activity. The NADH burst value corresponding to the number of acylated active centers was calculated by subtraction of the steady state rate from the total rate of NADH accumulation.

The acylation of the enzyme by 1,3-bisphosphoglycerate and the hydrolytic decomposition of 3-phosphoglyceroyl enzyme were followed at 360 nm by the drop in absorbance in the Racker band region [10] and its reappearance. The extinction coefficient for the charge transfer complex was determined by titration of excess GAPDH

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Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12; ME, β-mercaptoethanol

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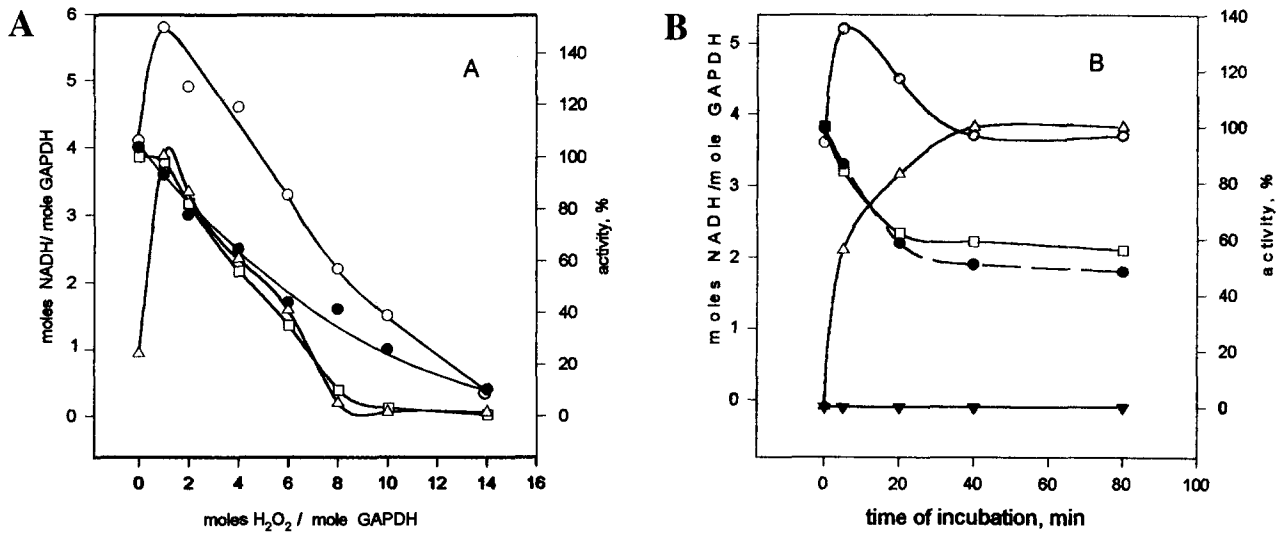

Fig. 1. The influence of hydrogen peroxide on the parameters of the two dehydrogenase reactions. A: 6.7 μM GAPDH was incubated for 1 h at 20°C and pH 7.6 in 50 mM HEPES, containing 5 mM EDTA, 0.5 mM NAD^+ and hydrogen peroxide in the concentrations indicated, then both activities were measured. The non-phosphorylating activity at zero point (no hydrogen peroxide) corresponds to spontaneous oxidation of the enzyme under the indicated conditions. Right ordinate: □, the phosphorylating dehydrogenase activity (100% corresponds to 100 U/mg); the non-phosphorylating dehydrogenase activity (100% corresponds to 0.25 U/mg): △, measured without additions; ▼, in the presence of 0.1 mM ME. Left ordinate: the number of acylated sites (○) measured without additions; ●, in the presence of 0.1 mM ME. B: GAPDH (11 μM) was incubated at 20°C and pH 7.6 in 50 mM HEPES, 5 mM EDTA and 0.5 mM NAD^+ in the presence of 50 μM H_2O_2. At indicated time intervals samples were taken to measure the activities together with the number of sites acylated in the reaction (see Section 2 for details).

with certain amounts of NAD^+ and was found to be 1000 M^{-1} cm^{-1} at pH 7.6.

To obtain the'reduced' GAPDH form (i.e. to reduce to a minimum a small non-phosphorylating activity usually present in GAPDH preparations) the enzyme was incubated with 2 mM ME for 30 min. Excess ME was then removed by passing the enzyme solution through a Sephadex G-50 column.

3. Results and discussion

Fig. 1A shows the effect of hydrogen peroxide on the two activities. It is seen that in the presence of 1–2 equivalents of H_2O_2 per tetramer GAPDH the non-phosphorylating activity is highly activated, while the phosphorylating dehydrogenase activity is little changed. At higher hydrogen peroxide concentrations both the non-phosphorylating and phosphorylating dehydrogenase activities are proportionally reduced, which can be explained by the oxidation, under these conditions, of the essential Cys-149, which is needed for both reactions. These results show that experimental conditions can be found under which the non-phosphorylating activity is selectively induced with minimal alteration of the phosphorylating one.

The oxidation of Cys-149 was also checked by the disappearance of absorbance at 360 nm (Racker band) due to decomposition of the charge transfer complex [10]. As shown in Table 1, the number of Cys-149 residues oxidized upon 1 h incubation of the enzyme with 1 μM H_2O_2 does not exceed 0.1 (sample B) (2.5% of the total amount), which is in good agreement with the degree of alteration of the dehydrogenase activity under these conditions (see Fig. 1A). Incubation with higher hydrogen peroxide concentrations leads to the more pronounced oxidation of Cys-149 (sample D). Table 1 also shows that addition of low (100 μM) concentrations of ME immediately blocks the non-phosphorylating activity (sample E), but does not restore the absorbance at 360 nm (i.e. does not reduce the modified Cys-149 residues to Cys-SH, the form involved in charge transfer complex formation).

Taken together, the results presented in Fig. 1A and in Table 1 clearly demonstrate that the rise of hydrolytic activity exhibited by the enzyme after treatment with micromolar hydrogen peroxide concentrations had no relation to the oxidation of the essential Cys-149. Pronounced oxidation of this residue can only be detected at higher concentrations of H_2O_2; it is accompanied by a decrease in absorbance at 360 nm and by the lowering of the dehydrogenase activity meas-

Table 1
The influence of hydrogen peroxide on GAPDH properties

Enzyme preparation	SH groups of Cys-149	Non-phosphorylating GAPDH activity (U/mg)
A: 'Reduced' GAPDH form	3.9	0.004
B: The same as A but after 60 min incubation with 1 μM H_2O_2	3.8	0.17
C: The same as B but after addition of 100 μM mercaptoethanol (without any subsequent incubation)	3.8	0.001
D: The same as A but after 30 min incubation with 10 μM H_2O_2	3.0	0.24
E: The same as D but after addition of 100 μM ME (without any subsequent incubation)	3.14	0.001

7 μM GAPDH was incubated with hydrogen peroxide at 20°C and pH 7.6 in 50 mM HEPES, containing 5 mM EDTA and 0.5 mM NAD^+. The stoichiometry of Cys-149 oxidation was followed by the decrease in the absorption at 360 nm.

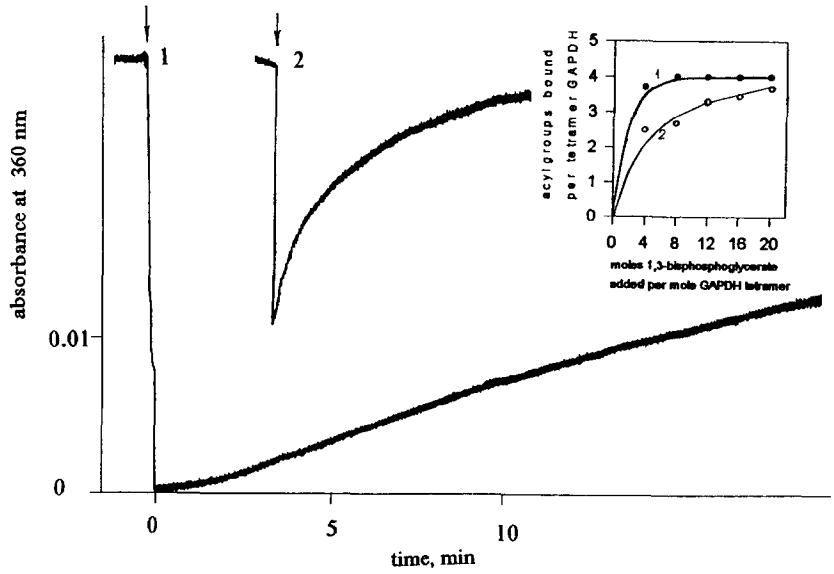

Fig. 2. Acylation of GAPDH by 1,3-bisphosphoglycerate and hydrolysis of the acyl enzyme. The reaction mixture contained 50 mM HEPES pH 7.6, 5 mM EDTA, 0.5 mM NAD^+, 7 μM GAPDH. At the moment indicated by the arrow, 1,3-bisphosphoglycerate was added to achieve a final concentration of 28 μM. The drop in absorbance is due to the enzyme acylation; the slow reappearance of the Racker band is related to acyl-enzyme hydrolysis. 1, 'reduced' enzyme; 2, the same as 1, but after 30 min incubation with 1 μM H_2O_2. Insert: Dependence of the stoichiometry of enzyme acylation on the concentration of 1,3-bisphosphoglycerate.

ured in the presence of inorganic phosphate. At the same time, the integrity of Cys-149 proved to be obviously necessary for both enzyme activities, phosphorylating and non-phosphorylating (see Fig. 1A).

To reconcile these facts, we suggest the following mechanism of hydrogen peroxide-induced alterations of GAPDH functional properties. Very low H_2O_2 concentrations oxidize some functional group of the enzyme molecule (other than Cys-149, but located near the active center), producing a new group which readily accepts the acyl transferred from Cys-149. The next step of the reaction is hydrolysis of the intermediate and release of 3-phosphoglycerate. A good candidate for the role of the acyl acceptor can be a hydroxyl of sulfenic acid, the product of mild oxidation of cysteine residues in proteins. A sulfenic acid derivative of Cys-149 in GAPDH has been shown to exhibit high acyl phosphatase activity, especially with 1,3-bisphosphoglycerate as a substrate [11–13]. Taking into account this information, we suppose that the first target of GAPDH oxidation in the presence of very low H_2O_2 concentrations might be a cysteine residue, most likely Cys-153, which is four residues removed from the essential Cys-149. Cys-153 possesses much greater reactivity than the remaining cysteine residues in the GAPDH molecule [14]. Several lines of evidence indicate that a close interrelationship exists between the conformational state of the catalytic center and the microenvironment of Cys-153. Following modification of the enzyme with 5,5′-dithiobis-(2-nitrobenzoate), an intramolecular disulfide bond is formed between Cys-149 and Cys-153 [15] which indicates that the two residues can be brought into proximity.

Evidence in favor of the above hypothesis was obtained in the following two series of experiments. First, the proposal that sulfenic acid is formed as a product of mild oxidation of GAPDH by hydrogen peroxide was supported by the re-

Table 2
Reversibility of the effect of low H_2O_2 concentrations on the non-phosphorylating GAPDH activity

Enzyme preparation	Non-phosphorylating GAPDH activity (U/mg)
A: 'Reduced' GAPDH form	0.002
B: 'Oxidized' GAPDH form	0.25
B.1: The same as B, but after incubation with	
5 mM sodium arsenite	0.19
50 mM sodium arsenite	0.001
B.2: The same as B, but in the presence of 100 μM ME	0.001
B.3: The same as B, but after incubation with 0.5 mM hydroxylamine	0.002
B.4: The same as B, but in the presence of 50 mM glycyl-glycine	0.001

A: GADPH solution, 70 μM, was treated with 2 mM ME as described in Section 2.
B: Solution A was diluted with 50 mM HEPES, 5 mM EDTA, pH 7.6 to achieve a protein concentration of 21 μM. NAD^+ was then added to a final concentration of 1.5 mM, and incubation with 10 μM H_2O_2 was performed for 10 min.
B.1: Solution B was supplemented with sodium arsenite to achieve a final concentration of 5 mM or 50 mM. After 60 min incubation at 20°C, the solution was freed of arsenite on a Sephadex G-50 column equilibrated with the above HEPES buffer.
B.2: Solution B was supplemented with ME to achieve a final concentration of 100 μM. The activity was measured without any preincubation.
B.3: Solution B was supplemented with NH_2OH to achieve a final concentration of 0.5 mM. After 30 min incubation the solution was freed from hydroxylamine on a Sephadex G-50 column equilibrated with the above HEPES buffer.
B.4: Solution B was diluted with glycyl-glycine buffer pH 7.6 (the final concentration of the buffer, 50 mM; the GAPDH concentration, 7 μM); the activity was measured without any preincubation.

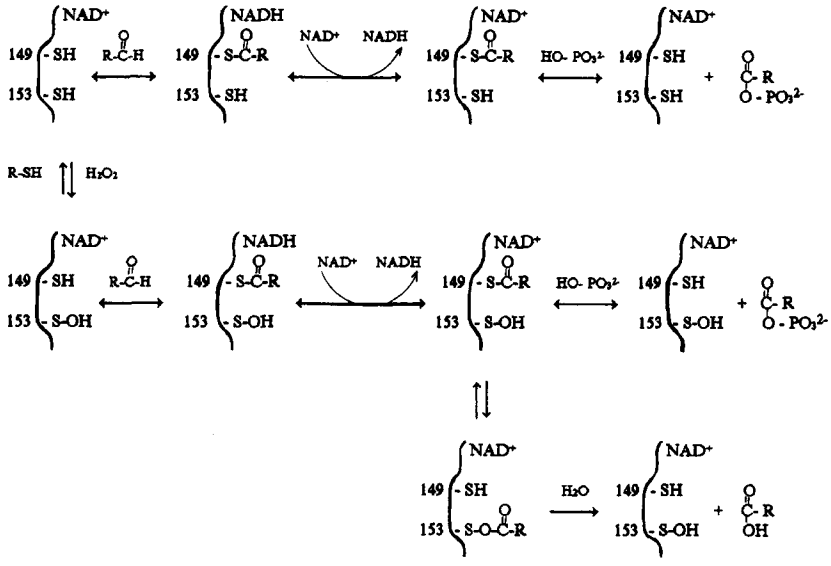

Fig. 3. Hypothetical mechanism of the arising of the non-phosphorylating activity upon oxidation of GAPDH by H_2O_2.

R–C–H is glyceraldehyde-3-phosphate, C–R is 1,3-bisphosphoglycerate, $O-PO_3^{2-}$ C–R is 3-phosphoglycerate. OH

sults of studies on the reversibility of the oxidation (Table 2). The characteristic property of sulfenic acids is their susceptibility to reduction even by very mild reductants, such as sodium arsenite [16]. The effect of this reducing agent on the properties of the oxidized GAPDH was then studied. As seen in Table 2, the hydrolytic activity induced by hydrogen peroxide completely disappears upon incubation of the enzyme with 50 mM sodium arsenite. This points toward the possibility of participation of sulfenic acid in this reaction. Sulfenic acids also readily react with a number of amino compounds with the formation of corresponding sulfenamides [17]. The effect of incubation of the oxidized GAPDH in the presence of hydroxylamine is shown in Table 2 (B.3). The disappearance of the hydrolytic activity observed in this experiment can be explained by the reaction of sulfenic acid, which is essential for this activity, with hydroxylamine. Table 2 also shows that complete inhibition of the hydrolytic activity was also observed in the presence of 50 mM glycylglycine. Some α-amino acids (glycine, alanine, phenylalanine) were also shown to inhibit the reaction, whereas β-alanine exhibited no effect. We speculate that a reaction between sulfenic acid and an α-amino acid may occur with formation of sulfenamide. The effect of α-amino acids appeared to be reversible (the hydrolytic activity was restored after removal of the reagents by gel filtration).

The second piece of evidence supporting our suggestion about the transfer of a 3-phosphoglyceroyl group to a sulfenic acid produced under mild GAPDH oxidation was gained from the experiments documented in Fig. 1A,B. It was found that incubation of the enzyme in the presence of low H_2O_2 concentrations resulted in an increase of the NADH burst corresponding to the number of sites acylated in the reaction. As shown in Fig. 1, before the addition of hydrogen peroxide about four active sites are determined to be acylated, without additions as well as in presence of ME. After 1 h incubation with 1 μM hydrogen peroxide (Fig. 1A) or after a short period of incubation with 50 μM hydrogen peroxide (Fig. 1B), Cys-149 residues are little affected, as can be seen from the phosphorylating dehydrogenase activity, but the total number of acylated sites increases, exceeding four, and non-phosphorylating activity appears. The 'superfluous' sites as well as the non-phosphorylating activity immediately disappear on the addition of reducing agent, and the number of acylated sites is brought into correspondence with the phosphorylating dehydrogenase activity. At longer incubation times or at higher hydrogen peroxide concentrations, the number of Cys-149 residues progressively diminishes, as witnessed by the decrease in phosphorylating dehydrogenase activity. At the same time, the number of acylated sites, determined from the initial burst of NADH in the absence of ME, remains considerably higher,

Table 3
The effect of hydrogen peroxide and thiols on the acylation of GAPDH by 1,3-bisphosphoglycerate and on the deacylation of the acyl enzyme

Enzyme preparation	Acylated active sites	Deacylation rate constant (min^{-1})
A: 'Reduced' GAPDH form	3.7	0.06
B: H_2O_2-treated form	2.5	1.8
C: The same as B, but after addition of ME to a final concentration of 100 μM	3.9	0.04

See legend to Fig. 2 for details.

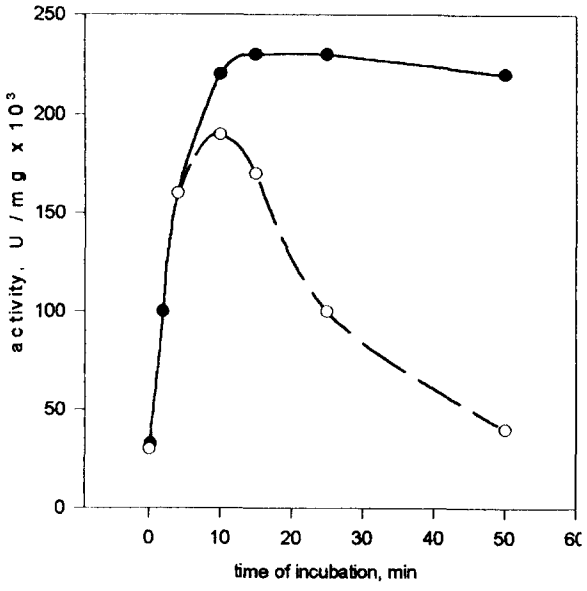

Fig. 4. Effect of NAD^+ on the non-phosphorylating dehydrogenase activity. 7 μM GAPDH was incubated at pH 7.6 in a mixture containing 50 mM HEPES, 5 mM EDTA and 10 μM H_2O_2: ●, in the presence of 0.5 mM NAD^+; ○, in the absence of NAD^+ (the incubation was performed on the enzyme treated with Norit to remove the enzyme-bound NAD^+). At indicated time intervals, aliquots were taken to measure the activities. The phosphorylating dehydrogenase activity (not shown) was not altered in either case.

as expected on the basis of phosphorylating activity value. In the presence of ME the number of acylated sites corresponds to the extent of the enzyme inactivation.

The most likely explanation of these data is the assumption that in the oxidized enzyme a rapid transfer of acyl groups from the active site Cys-149 to another group (probably the sulfenic acid form of Cys-153) takes place, followed by the instant acylation of the vacant Cys-149. As a result, 'additional' acylated sites are detected.

It is noteworthy that these excessive acylation sites disappear virtually instantly under the conditions in which the active site cysteine residues (Cys-149) probably still remain oxidized, since their number in the presence of 0.1 mM ME corresponds to the extent of the phosphorylating dehydrogenase activity. The instantaneous inhibition of the non-phosphorylating activity in the presence of low thiol concentrations may indicate that upon interaction of the oxidized form with thiol, an intermediate is formed (possibly a disulfide), which is unable to accept the acyl group and perform hydrolysis.

Another approach to verify the proposal on the transfer of the 3-phosphoglyceroyl group from the active center Cys-149 to some other acceptor was based on the investigation of enzyme acylation with 1,3-bisphosphoglycerate. As shown in Fig. 2, upon addition of 4 mol of 1,3-bisphosphoglycerate per mol of enzyme tetramer, the almost complete disappearance of the Racker band was observed with the reduced enzyme form; this corresponded to acylation of 3.7 active sites per tetramer (Table 3A). In the case of the enzyme treated with H_2O_2, the number of acylated sites was considerably lowered (see Fig. 2 and Table 3B), and increased again in the presence of 100 μM ME (Table 3C). Thus, oxidation of some groups was probably responsible for the difference in the stoichiometry of acylation. The possibility of the oxidation of Cys-149 SH groups in these conditions is to be excluded on the basis of the results presented in Fig. 2 (insert), which demonstrate that acylation of all active sites can be achieved in the presence of an excess of 1,3-bisphosphoglycerate, and also in Table 1B. Another possibility is the oxidation of some other groups resulting in the appearance of additional acyl acceptor sites. In such a case, initial acylation of all four active site Cys-149 residues can be followed by the transfer of a part of the 3-phosphoglyceroyl groups to the additional sites. Assuming that the process is sufficiently rapid, one can expect that the number of acylated Cys-149 residues measured under the employed experimental conditions will be lowered. This is in agreement with the results obtained (Table 3). The acceleration of the rate of deacylation observed upon incubation of the enzyme with H_2O_2 is probably due to the appearance of the sites which accept 3-phosphoglyceroyl groups from Cys-149 residues and are able to transfer them to water. Summarizing, the whole body of data presented in this communication is consistent with the following scheme (Fig. 3).

According to our hypothesis, GAPDH can exist in two easily interconvertible forms, which differ in acyl transfer properties. The first, 'reduced' form is practically devoid of the ability to transfer the 3-phosphoglyceroyl group to water, whereas the 'oxidized' form can catalyze the acyl transfer to both inorganic phosphate and water.

The mechanism of acyl transfer to inorganic phosphate must be identical with the two enzyme forms, since they catalyze the phosphorylating dehydrogenase reaction at similar rates. The mechanism of acyl transfer to water is suggested to occur via a stage of intramolecular transfer of a 3-phosphoglyceroyl group to the sulfenic form of Cys-153, with subsequent hydrolysis.

Although consistent with the experimental data, this mechanism remains hypothetical, since identification of the sulfenic form of Cys-153 has not yet been achieved. The enzyme oxidized by H_2O_2 was rather unstable and could only exist under particular conditions. Thus, as shown in Fig. 4, the presence of NAD^+ was necessary to maintain it in a conformation active in the non-phosphorylating reaction; the effect of NAD^+ is believed to be due to the stabilization, via specific conformational changes, of the sulfenic form of Cys-153. The instability of the sulfenic acid derivatives of cysteine residues hampers their direct identification. According to our results, the 'oxidized' enzyme form could not even be distinguished from the 'reduced' one on the basis of SH group titration performed with 5,5′-dithio(bis)-nitrobenzoate or with *p*-chloromercuribenzoate.

In conclusion, we have demonstrated the possibility of transformation of GAPDH which catalyzes the oxidation of substrate coupled with phosphorylation in glycolysis, into a form capable of performing the oxidation in the absence of phosphate. The transformation is induced by low concentrations of H_2O_2 and is instantaneously reversed in the presence of low concentrations of reducing agents. We suppose that the interconversion between the two enzyme forms can occur under physiological conditions and play a role in the regulation of glycolysis. The data obtained lend support to the hypothesis [6] that under aerobic conditions, especially in the presence of reactive oxygen species, triosephosphate oxidation and NADH production are no longer tightly coupled to phosphorylation and can occur in the absence of ADP. Thus, under aerobic conditions, when the concentration of ADP is

very low, glycolysis is not completely blocked, supplying mitochondria with substrates. This process might be connected with the 'mild' uncoupling of respiratory phosphorylation in mitochondria which, as was recently suggested by Skulachev [18,19], represents one of the earliest lines of antioxygen defence.

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References

[1] Branden, C.I. and Eklund, H. (1980) in: J. Jeffery (Ed.), Dehydrogenases Requiring Nicotinamide Coenzymes. Birkhauser Verlag, Basel, pp. 41–84.
[2] Byers, L.D. and Koshland Jr., D.E. (1975) Biochemistry 14, 3661–3669.
[3] Kanchunger, M.S., Leong, P.M. and Byer, L.D. (1979) Biochemistry 18, 4373–4379.
[4] Kuzminskaya, E.V., Asryants, R.A. and Nagradova, N.K. (1993) FEBS Lett. 336, 208–210.
[5] Schmalhausen, E.V., Muronetz, V.I. and Nagradova, N.K. (1995) FEBS Lett. 375, 18–20.
[6] Schmalhausen, E.V. and Muronetz, V.I. (1997) Biosci. Rep. (in press).
[7] Szewzuk, K., Wolny, H. and Baranowsky, T. (1961) Acta Biochem. Polon. 8, 201–207.
[8] Furfine, C.S. and Velick, S.F. (1965) J. Biol. Chem. 240, 844–855.
[9] Scopes, R.K. and Stoter, A. (1982) Methods Enzymol. 90, 479–491.
[10] Racker, E. and Krimsky, I. (1952) J. Biol. Chem. 198, 731–743.
[11] Little, C. and O'Brien, P.J. (1969) Eur. J. Biochem. 10, 533–538.
[12] Allison, W.S. and Connor, M.J. (1970) Arch. Biochem. Biophys. 136, 383–391.
[13] Benitez, L.V. and Allison, W.S. (1974) J. Biol. Chem. 249, 6234–6243.
[14] Vas, M. and Boross, L. (1974) Eur. J. Biochem. 13, 237–244.
[15] Wassarman, P.M. and Major, J.P. (1969) Biochemistry 8, 1076–1082.
[16] Parker, J.D. and Allison, W.S. (1969) J. Biol. Chem. 244, 180–189.
[17] Allison, W.S., Benitez, L.V. and Johnson, C.L. (1973) Biochem. Biophys. Res. Commun. 52, 1403–1409.
[18] Skulachev, V.P. (1994) Biochemistry (Moscow) 59, 1910–1912.
[19] Skulachev, V.P. (1996) Q. Rev. Biophys. 29, 169–202.