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The activation of glycolysis performed by the nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase in the model system☆

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Abstract

Influence of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) on glycolysis was investigated. The addition of GAPN—which oxidizes glyceraldehyde-3-phosphate directly to the 3-phosphoglyceric acid—led to the strong increase in the rate of lactate accumulation in the rat muscle extract with low ADP content. The lactate accumulation was also observed in the presence of GAPN in rat muscle extract, which contained only ATP and no ADP. This can be the evidence of the "futile cycle" stimulated by GAPN. Here ADP can be regenerated from ATP by the phosphoglycerate kinase reaction. The high resistance of GAPN from *Streptococcus mutans* towards inactivation by natural oxidant—H_2O_2 was showed. This feature distinguishes GAPN from phosphorylating glyceraldehyde-3-phosphate dehydrogenase, which is very sensitive to modification by hydrogen peroxide. A possible role of the oxidants and non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in the regulation of glycolysis is discussed.

Keywords: Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase; Regulation of glycolysis; Uncoupling of oxidation and phosphorylation; Futile cycle; Hydrogen peroxide

Yeast extracts prepared by autolysis have been known to exhibit a low rate of glycolysis because they are limited by a low Pi and ADP concentration. Meyerhof [1] demonstrated that the addition of ATPase to these extracts raised the glycolytic rate to that achieved by the addition of arsenate. Later, Harary [2] showed that the addition of muscle acylphosphatase to the same glycolytic system also raised the glycolytic rate, like ATPase or arsenate. The author assumed that this enzyme could play a regulatory role in glycolysis: in the lack of ADP or phosphate, the muscle acylphosphatase capable of hydrolyzing 1,3-DPG could allow glycolysis to occur at a more rapid rate at the sacrifice of ATP synthesis. In 1996, Raugei et al. [3] showed that the expression of human muscle acylphosphatase in *Saccharomyces cerevisiae* increased strongly the rate of ethanol production. A mild oxidation of phosphorylating glyceraldehyde-3-phosphate dehydrogenase in the presence of air oxygen or hydrogen peroxide leads to oxidation of a part of the active site cystein residues to sulfenic acid derivatives, resulting in the induction of the acylphosphatase activity with a simultaneous decrease in the dehydrogenase activity [4,5]. The reduced active sites of the enzyme retain the ability to oxidize glyceraldehyde-3-phosphate yielding 1,3-diphosphoglycerate, while the oxidized active sites catalyze irreversible cleaving of 1,3-diphosphoglycerate. This combinative activity of GAPDH could also lead to the acceleration of glycolysis at low Pi and ADP concentrations. Such an effect was recently shown in our laboratory [6].

☆ *Abbreviations:* 1,3-DPG, 1,3-diphosphoglycerate; DTNB, 5,5′-dithiobis(nitrobenzoic) acid; GAPDH, phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9); G-3-P, glyceraldehyde-3-phosphate; LDH, lactate dehydrogenase (EC 1.1.1.27); ME, β-mercaptoethanol; PEP, phosphoenolpyruvate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 3-PG kinase, 3-phosphoglycerate kinase.

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Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase catalyzes the irreversible oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate with concomitant reduction of NAD(P) [G-3-P + NAD(P) + $H_2O \rightarrow$ 3-PG + NAD(P)H + $2H^+$] [7]. It was shown that the *Escherichia coli* mutant strain, which expressed GAPN but lacked GAPDH, could live at the aerobic conditions (using glucose but not pyruvate) and could not grow at all without oxygen. In this case the first point of substrate phosphorylation was probably skipped and the sum ATP yield in that type of glycolysis was zero [8].

We postulated that the presence of GAPN in the full glycolytic system with GAPDH must prevent the blockage of glycolysis in the lack of ADP. To test such a possibility, we studied the influence of the addition of GAPN on the glycolytic pathway. Animals do not have GAPN that is why the experiments were performed using a rat muscle extract.

Materials and methods

Materials. ATP, ADP, DTNB, and glyceraldehyde-3-phosphate were purchased from Sigma; NAD, hydrazine hydrochloride, and lactate dehydrogenase (EC 1.1.1.27) were from ICN; hydrogen peroxide was from Merck; β-mercaptoethanol was from Ferak, activated charcoal "Norite A" was from "Serva." Phosphorylating glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was isolated from rabbit muscles by the Scopes' method [9]. Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) was purified from the *E. coli* DH5α and transformed with the plasmid pskBstII with the gene of NADP-dependent GAPN from *Streptococcus mutans*. The purification was performed according to the method developed by Crow and Wittenberger [10] and modified by Branlant et al. [11]. The authors are grateful to Prof. Branlant (University of Nancy, France) for a gift of pskBstII plasmid with the gene of GAPN.

Both GAPDH and GAPN were stored as the suspension in the saturated ammonium sulfate solution at −20 °C. The concentrations of the rabbit muscle GAPDH and GAPN were determined spectrophotometrically. $A^{0.1\%}$ at 280 nm is 0.83 and 1.0 for GAPDH (apo- and holo-enzyme, respectively) and 1.0 for GAPN (apo-enzyme).

GAPN was isolated in the apo-form, while rabbit muscle GAPDH was isolated in the holo-form. The apo form of the rabbit muscle GAPDH was obtained by the incubation of the enzyme with the activated charcoal ("Norite A"). Five milligram charcoal was added per mg of protein and the suspension was incubated for 10 min at room temperature. Then the charcoal was separated from the protein solution by centrifugation. The content of NAD was estimated spectrophotometrically by the absorbance ratio A_{280}/A_{260}. The ratio for the apo-form was no less than 1.8.

Muscle extract preparing. White laboratory rat was decapitated. Rat muscles were minced into small pieces by scissors and two volumes of cold buffer containing 20 mM KH_2PO_4, 0.8 mM $MgCl_2$, 3.4 mM KCl, and 68.4 mM NaCl (pH 7.6) were added. After 15 min of incubation, debris was removed by centrifugation (10 min, 12,000*g*). To remove fine particles, the extract was centrifuged for 10 min at 30,000*g*. To remove glycolytic substrates, the supernatant was applied on a Sephadex G-25 column ("Pharmacia") and a protein fraction was eluted by 10 mM KH_2PO_4, 0.8 mM $MgCl_2$, pH 7.6. The entire protein concentration in the muscle extract was determined by the biuret method.

Lactate measurement. Aliquots were taken from the reaction mixture and $HClO_4$ (final concentration of 1 M) was added to the samples to stop glycolysis. The samples were neutralized by the addition of saturated Na_2CO_3 solution. To measure lactate content, 0.1 ml of a sample was added to 0.9 ml of the assay system containing 50 mM glycine, 50 mM hydrazine, 1 mM NAD, and 10 U LDH (pH 9.0). The mixture was incubated at 25 °C for 1 h, together with a blank sample containing 1 ml of the assay system; then NADH concentration was determined.

The modification of sulfhydryl groups of GAPDH and GAPN by hydrogen peroxide or DTNB. The solution of DTNB (10 mM stock in 50 mM KH_2PO_4, pH 8.0) was added to the solution of the enzyme (GAPDH or GAPN, 20 μM of the protein) in 50 mM KH_2PO_4 (pH 8.0) the end concentration of DTNB at 300 μM. The solution of hydrogen peroxide (88 mM stock in water) was added to the solution of the enzyme (GAPDH or GAPN, 20 μM of the protein) in 50 mM KH_2PO_4 (pH 8.0) the end concentration of H_2O_2 at 1 mM.

In both cases the mixture was stirred and the aliquots were sampled during the incubation at 25 °C. The reaction was "stopped" by the rapid dilution (201 times) of the modified enzyme solution in the activity buffer.

A molar extinction coefficient (ε_{412} nm) of 13,600 $M^{-1}\,cm^{-1}$ for the anion of thionitrobenzoic acid was used to determine the number of moles of SH-groups reacted with DTNB.

Enzyme activity assays. The activities of GAPDH or GAPN were measured spectrophotometrically by the detection of the increase in the absorbance at the 340 nm during the reaction of NAD or NADP reduction at 25 °C. The activity buffer for GAPDH was 1 mM NAD, 1 mM G-3-P, 50 mM KH_2PO_4, 50 mM glycine, 5 mM EDTA, and 5 mM ME (pH 9.0), for GAPN was 1 mM NADP, 0.5 mM G-3-P, 50 mM KH_2PO_4, and 5 mM ME (pH 8.2). The homogeneous enzyme preparations have the activities close to the maximal known: 105 U/mg for GAPDH and 65 U/mg for GAPN.

Results

To study the effect of GAPN addition on glycolysis, a muscle extract, liberated from glycolytic substrates, was used. The samples were pre-incubated with ME (or without GAPN and ME, but with H_2O_2) for 30 min at 20 °C in the reaction mixture containing NAD and NADP. After the incubation, glycolysis was started by the addition of ADP and G-3-P, and then the samples were incubated for 45 min at 37 °C. After that NADH was added to 3 mM in the sample and the incubation was continued for 15 min at 37 °C to convert the pyruvate to lactate (GAPN is NADP-dependent enzyme and we need to supply the mixture by the additional NADH for LDH reaction). Then the reaction was stopped by $HClO_4$ and lactate content was measured (see Materials and methods). The experiments performed demonstrate the effect of the addition of GAPN on lactate accumulation during incubation with G-3-P and ADP. It can be seen in Fig. 1 (bar 1) that the minimal rate of lactate accumulation was observed under optimal conditions for GAPDH functioning—in the presence of ME (without addition of GAPN). The addition of GAPN strongly promotes the lactate accumulation (Fig. 1, bars 3 and 4). The concentration of GAPN needed for stimulation of glycolysis to the level close to the maximal possible lactate uptake (in the system used) was 3.5 μM (Fig. 1, bar 4), while the addition of 1 μM GAPN

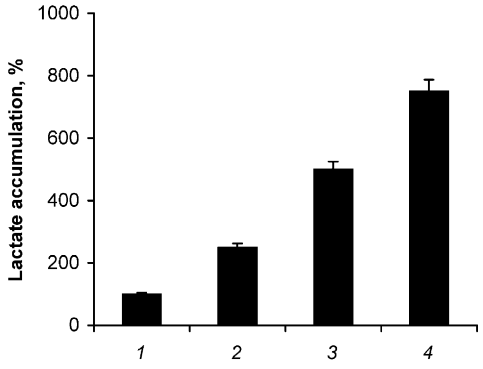

Fig. 1. Lactate accumulation in the course of incubation of muscle extract in the presence of GAPN or 100 μM H_2O_2. The reaction mixture contained muscle extract, prepared as described in Materials and methods (1 mg/ml of total protein), 10 mM KH_2PO_4, 0.8 mM $MgSO_4$ (pH 7.6), 2 mM NADP, 1 mM NAD, 1 mM ADP, and 3 mM G-3-P, plus: 100 μM H_2O_2 (bar 2) or 1 μM GAPN and 2 mM ME (bar 3) or 3.5 μM GAPN and 2 mM ME (bar 4). Control sample (bar 1) contained 2 mM ME instead of H_2O_2 and no GAPN. Lactate content in the presence of 2 mM ME only was accepted for 100%. The data are given as means of three separate experiments ± SD.

was insufficient for the full expression of the effect (Fig. 1, bar 3). These results suggest that increased lactate accumulation observed in the extract in the presence of GAPN and mercaptoethanol could be attributed to the non-phosphorylating dehydrogenase activity of the exogenous protein. In the absence of GAPN and ME but with H_2O_2 an increase in lactate production is also observed presumably due to acylphosphatase activity of oxidized GAPDH (Fig. 1, bar 2).

Moreover, GAPN together with PG kinase and reduced GAPDH appeared to act as an ATPase in the presence of ATP. Accumulation of lactate was observed in the course of incubation of samples with GAPN in the presence of ME, G-3-P, and ATP, omitting ADP (Fig. 2, bar 3). Only traces of lactate were detected in the samples containing mercaptoethanol only (Fig. 2, bar 1). Evidently, the combination of GAPN and reduced GAPDH with PG kinase in the presence of G-3-P results in ATP hydrolysis yielding ADP, which is used in pyruvate kinase reaction (Fig. 3). The non-phosphorylating dehydrogenase reaction (reaction 5) results in 3-PG formation, which is phosphorylated in the presence of ATP by PG kinase (reaction 3) yielding ADP for pyruvate kinase reaction (reaction 4). 1,3-DPG obtained in this reaction can be recycled to G-3-P by the reduced GAPDH and NADH (reaction 1). Thus, in this case, ATP hydrolysis allows glycolysis to proceed. The ATPase cycle was already performed with pure GAPDH and GAPN [12], here we showed the possibility of such a process in the glycolytic system. The same effect was obtained for the lactate accumulation in the presence of 100 μM H_2O_2 (without ME) (Fig. 2, bar 2). In this case another design of the above-mentioned "futile cycle" can be postulated (Fig. 3, reactions 1–3).

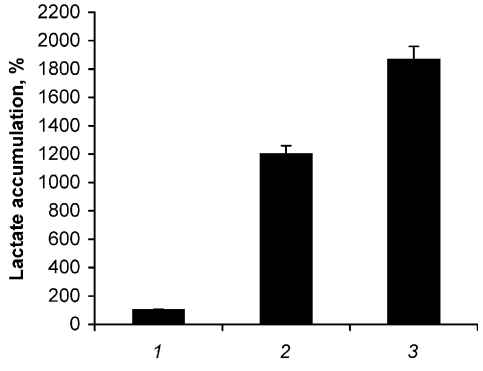

Fig. 2. The influence of GAPN and hydrogen peroxide on lactate accumulation during the incubation of muscle extract in the presence of 1 mM ATP, instead of ADP. The experiment conditions are given in the legend to Fig. 1; 1 mM ATP was added instead of ADP. bar 1—Control sample with 2 mM ME, bar 2—sample with no ME but H_2O_2 at concentration of 100 μM was added, and bar 3—sample with 2 mM ME and 3.5 μM GAPN. The data are given as means of three experiments ± SD.

There is some evidence to suggest that GAPDH is very sensitive to modification by the thiol reagents comparing to other glycolytic enzymes. In accordance with the data of Hyslop et al. [13], no detectable effects of up to 5 mM H_2O_2 were observed on the kinetic parameters of any glycolytic enzymes except for a small effect on the maximal velocity of hexokinase (25% decrease) and almost complete inhibition of GAPDH (99%). The basic principles of the catalytic mechanism of GAPDH and GAPN are the same [11]. That is why we performed a comparison of the accessibility of the essential sulfhydryl groups of *S. mutans* GAPN and muscle GAPDH to modification.

We used a well-known natural oxidant—H_2O_2. The 1 mM hydrogen peroxide did not inactivate GAPN at all during the time used. NADP did not affect the ability of GAPN to react with H_2O_2 (Fig. 4, curves 1 and 2). At the same time GAPDH was rapidly inactivated by hy-

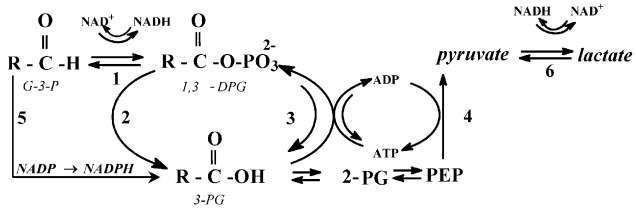

Fig. 3. Possible scheme of the flux design at the level of glycolytic oxidoreduction. The coupling of dehydrogenase and acylphosphatase reactions (the reactions 1, 2) results in 3-PG formation; the GAPN gives 3-PG directly (5). 3-PG is phosphorylated in the presence of ATP by PG kinase (3) yielding ADP for pyruvate kinase reaction (4). The 1,3-DPG formed can either be converted to 3-PG by oxidized GAPDH again or transformed to G-3-P by reduced GAPDH with subsequent oxidation to 3-PG by GAPN. The end product of all processes is lactate, which can be obtained in lactate dehydrogenase reaction (6). R=$(OPO_3^{2-})CH_2CH(OH)$–.

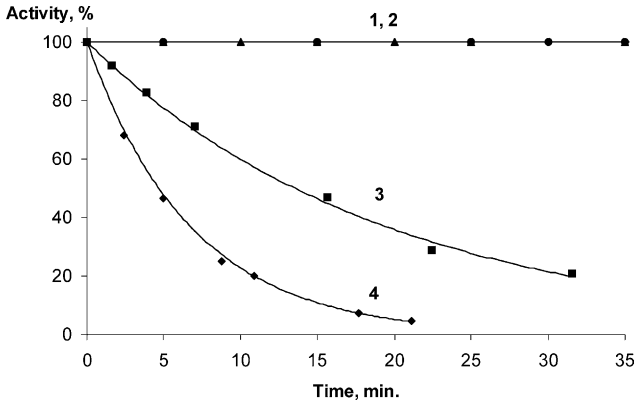

Fig. 4. The decrease in activity of the enzyme in the course of incubation of GAPN or rabbit muscle GAPDH with H_2O_2. (1) The residual activity of apo GAPN, (2) the residual activity of GAPN in the presence of 3 mM NADP, (3) the residual activity of apo-rabbit muscle GAPDH, and (4) the residual activity of rabbit muscle GAPDH in the presence of 3 mM NAD. The solution of hydrogen peroxide was added to the solution of the enzyme (GAPDH or GAPN, 20 μM proteins) in 50 mM KH_2PO_4 (pH 8.0) until the end concentration of H_2O_2 at 1 mM.

drogen peroxide and NAD promoted inactivation (Fig. 4, curves 3 and 4).

The discrepancy between these enzymes was also proved when we used dithiobis(nitrobenzoic) acid and measured the residual enzyme activity after the incubation with DTNB. The results are shown in Fig. 5. One can see that the inhibition of the enzyme activity is much more rapid for the muscle GAPDH (curves 1 and 2) than for GAPN (curves 3 and 4). It should also be noted that the cofactor "protects" GAPN.

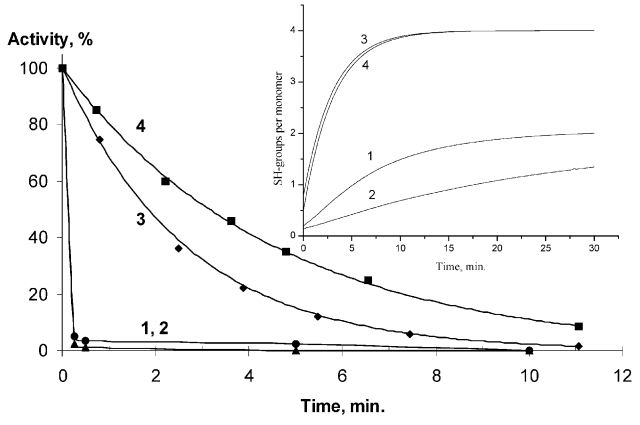

Fig. 5. The decrease in activity of the enzyme in the course of incubation of GAPN or rabbit muscle GAPDH with DTNB. (1) The residual activity of apo-rabbit muscle GAPDH, (2) the residual activity of rabbit muscle GAPDH in the presence of 3 mM NAD, (3) the residual activity of apo GAPN, and (4) the residual activity of GAPN in the presence of 3 mM NADP. The inset: number of sulfhydryl groups of rabbit muscle GAPDH or GAPN reacting with DTNB vs. time. (1), (2) GAPN, apo and with 3 mM NADP, respectively; (3, 4) rabbit muscle GAPDH, apo and with 3 mM NAD, respectively. The experiments were performed as described in Materials and methods. The solution of DTNB was added to the solution of the enzyme (GAPDH or GAPN, 20 μM proteins) in 50 mM KH_2PO_4, (pH 8.0) until the end concentration of DTNB at 300 μM.

All cysteins were modified in both enzymes (four per rabbit muscle GAPDH monomer and two per GAPN monomer). It is noteworthy that the "half-level" of modification (two cysteins per monomer for GAPDH and 1 cystein per monomer for GAPN) can be reached much more rapidly in the case of GAPDH. (See Fig. 5, the inset. The extinction of the enzyme solution at 412 nm was detected.) At this level GAPDH is fully inactivated while GAPN retains about 20% of its activity.

Thus, the active center of muscle GAPDH is much more sensitive to the exogenous modifications than that of GAPN from *S. mutans*.

Discussion

The data of Fig. 1 show the same effects of mildly oxidized GAPDH and the addition of GAPN on lactate accumulation. Hence, GAPN can work as the couple "reduced GAPDH/mildly oxidized GAPDH" performing a "shunted" metabolic flux. In other words the introduction of the glyceraldehyde-3-phosphate dehydrogenase with the hydrolytic cleavage of the acyl-enzyme intermediate leads to the same effect as the presence of the acylphosphatase system. This influence of GAPN depends on the amount of GAPN introduced in the extract (Fig. 1), while the effect of acylphosphatase activity depends on the level of the oxidized GAPDH in the mixture [6]. During such pathways ATP should be produced mainly in pyruvate kinase reaction, bypassing 3-PG kinase reaction, and the total ATP yield per mole of G-3-P should be close to zero. One can imagine the uncoupling of oxidation and phosphorylation in glycolysis due to the injection of the "bypassing" enzyme in the system or the mild oxidation of glyceraldehyde-3-phosphate dehydrogenase [6].

Under anaerobic conditions, substrate oxidation with zero ATP yield would be energetically useless. But under aerobic conditions, the glycolytic pathway is not the only source of energy; moreover, it provides NADH and pyruvate for oxidative phosphorylation and substrates for biosynthesis. That is why the glycolysis must not be stopped even during intensive respiration [14]. The data from Fig. 1 suggest two possible mechanisms to overcome ADP and Pi deficiency and to support the glycolytic flux.

The experiment depicted in Fig. 2 claimed the possibility of the presence of two types of futile cycles in glycolysis. First type—the oxidized GAPDH/phosphoglycerate kinase recirculation of phosphoglycerate (see Fig. 3) and the second type—GAPN/ reduced GAPDH/ phosphoglycerate kinase recirculation of phosphoglyc-

erate (also see Fig. 3 for the scheme). Such futile cycles can drive the glycolytic flux even in the absence of ADP.

Figs. 4 and 5 outline the sensitivity of muscle GAPDH cysteines to modification. Moreover, one can see that in the case of modification by the natural oxidant—H_2O_2—GAPN stays fully active while GAPDH activity is strongly decreased. It is noteworthy that the concentration of hydrogen peroxide was very high in the inactivation experiment comparing with the physiological rates [15]. We hypothesize that the very structure of the active center of muscle GAPDH is specifically designed to perform the rapid modification with subsequent functioning as the acylphosphatase. These data on peroxide inactivation are in good agreement with the evidence of high sensitivity of GAPDH from *S. mutans* to inactivation by air O_2 and the high resistance of GAPN from the same organism towards inactivation by O_2 [16]. The increase in the velocity of inactivation of holo-GAPDH by hydrogen peroxide is also in good agreement with the fact of the pKa decrease of the essential Cys of the holo-GAPDH [17].

We assume that mild and reversible oxidation of GAPDH caused by low, physiological concentration of H_2O_2 or some other oxidants (like O_2) may play a regulatory role [6,14,18]. On the other hand one can imagine the significance of the special enzymes—the glycolytic uncouplers that perform this function in their native, non-modified state. The ferredoxin-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase and NAD-dependent GAPN were found in some hyperthermophilic anaerobic archaea [19–22]. It was shown that phosphorylating G-3-P dehydrogenases were specialized for 3-PG reduction and probably functioned only in gluconeogenesis in these organisms. According to the literary data NADP-dependent GAPN from *S. mutans* provides this organism with the reductive equivalents. This fact demonstrates that the presence of GAPN can also take place even in the organism where the glycolysis is the only energy source. Moreover, the *S. mutans* strain with the deleted gapN gene could not grow in the presence of air [23].

The presence of NADP-dependent GAPN in all green plants [7] could play a remarkable role for driving the G-3-P oxidation under the conditions of intensive photophosporylation, when the cytosol was shown to be depleted of ADP and Pi. G-3-P produced in the Calvin cycle is transported to the cytosol. GAPN converts it to the PGA producing NADPH. PGA is then transported back into the chloroplast. A triosophosphate driven NADPH shuttle mechanism was suggested [7]. Thus, one can observe the obvious necessity of NADP-dependent GAPN to make this process unidirectional. On the basis of the present study we postulate that such a fact is the evidence of the need of glycolytic uncoupling driven by the ADP- and Pi-independent irreversible oxidation of G-3-P. As for the model system of muscle extract, the experiments performed in this work by an independent "enzyme-probe" tool [24]—GAPN—elucidated the possibility of the important regulatory step at the level of glycolytic oxidoreduction in the animal cell.

Acknowledgments

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