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Autophosphorylation of Glyceraldehydephosphate Dehydrogenase and Phosphorylation of Protein from Skeletal Muscle Microsomes†

Richard M. Kawamoto*,‡ and Anthony H. Caswell
Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101
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ABSTRACT: Glyceraldehydephosphate dehydrogenase purified from rabbit skeletal muscle is autophosphorylated with MgATP. Half-maximal phosphorylation is achieved around 0.3 mM. The phosphorylation is Ca^{2+} independent. The phosphoenzyme complex is labile in alkaline conditions and stable in moderately acid media. The complex is readily hydrolyzed by 0.1 M neutral hydroxylamine, indicating the complex formed is a high-energy acyl phosphate. The phosphorylation is reduced by nicotinamide adenine dinucleotides, reduced form (NADH), glyceraldehyde 3-phosphate, and nicotinamide adenine dinucleotide (NAD^+). The enzyme is also dephosphorylated by these metabolites although to a lesser extent by NAD^+. Calsequestrin isolated from rabbit skeletal muscle inhibits the phosphorylation of the enzyme. The phosphoenzyme behaves as a kinase catalyzing the phosphorylation of proteins of M_r 80 000 and 72 000 found in the skeletal muscle terminal cisternae/triad preparation. This reaction is enhanced by NADH. The phosphate found in the protein substrate has been shown to be the same phosphate initially involved in the phosphorylation of glyceraldehydephosphate dehydrogenase.

Glyceraldehydephosphate dehydrogenase (glyceraldehyde-P dehydrogenase) has been exhaustively investigated as a key enzyme in glycolysis (Harris & Waters, 1976). The enzyme is responsible for the oxidative phosphorylation of glyceraldehyde 3-phosphate (GAP)[1] by NAD^+ and inorganic phosphate. It has been isolated from a variety of sources including yeast (Krebs et al., 1953), lobster (Allison & Kaplan, 1964), and rabbit (Caputto & Dixon, 1945). Amino acid sequencing has revealed that glyceraldehyde-P dehydrogenase is a highly conserved protein among different organisms.

In general, glycolytic enzymes have been described as soluble proteins found in the cytosolic milieu. However, glyceraldehyde-P dehydrogenase has been shown to exhibit specific binding to erythrocyte membranes (Kant & Steck, 1975). The interaction is of an ionic nature, and elution of glyceraldehyde-P dehydrogenase can be effected by physiological concentrations of salt. Moreover, ATP, NADH, and GAP can also cause specific dissociation of glyceraldehyde-P dehydrogenase from erythrocyte membranes. The site of interaction has been identified as the anion channel (band 3) of erythrocytes (Yu & Steck, 1975). As yet, the physiological significance of the interaction has not been determined.

Recently, glyceraldehyde-P dehydrogenase has been identified as a component involved in the formation of triad junctions of rabbit skeletal muscle. The purified protein has been demonstrated to promote re-formation of triad junctions previously disrupted mechanically by French press treatment (Corbett et al., 1985; Caswell & Corbett, 1985). The catalytic function is specific for T tubules and heavy terminal cisternae components as glyceraldehyde-P dehydrogenase fails to promote association of transverse tubules with nonjunctional light terminal cisternae SR or longitudinal SR. The mechanism by which glyceraldehyde-P dehydrogenase re-forms triad junctions is uncertain. However, it is possible that the enzyme is involved in organizing the membrane-bound "anchoring" protein to facilitate the interaction with the "spanning" protein to form the junctional complex.

The discovery of a glycolytic enzyme involved in an entirely unrelated catalytic function suggests the possibility that this protein participates in functions separate from glycolysis. We now report the autophosphorylation of glyceraldehyde-P dehydrogenase by MgATP which is apparently independent of glycolysis. Evidence is presented indicating that the phos-

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[1] Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GAP, glyceraldehyde 3-phosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NH_2OH, hydroxylamine; $(NH_4)_2SO_4$, ammonium sulfate; PCA, perchloric acid; SDS, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; TC, terminal cisternae; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

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phoprotein formed is an acyl phosphate and that the protein catalyzes the phosphorylation of membrane proteins of skeletal muscle microsomes.

Materials and Methods

Purification of Glyceraldehyde-P Dehydrogenase. The complete procedure for the purification of glyceraldehyde-P dehydrogenase has been described previously (Corbett et al., 1985), and only a brief protocol will be presented. Rabbit back muscle was homogenized in 1.0 mM Tris-HCl, pH 7.0, 100 mM KCl, and 0.5 mM Tris–EDTA. The homogenate was centrifuged at 16300*g* for 20 min, and the resulting supernatant was recentrifuged at 125000*g* for 1 h. The supernatant was treated with $(NH_4)_2SO_4$ at 75% saturation. The resulting protein pellet was discarded, and additional $(NH_4)_2SO_4$ was added to the supernatant to achieve 100% saturation. The precipitated protein was collected, applied to a hydroxyapatite column equilibrated with 5 mM histidine, pH 7.0, and eluted with a shallow sodium phosphate gradient. The large protein peak corresponding to glyceraldehyde-P dehydrogenase was pooled and precipitated with 100% saturating levels of $(NH_4)_2SO_4$. The protein was applied to a Sephacryl S-200 molecular sieve column and eluted with 5 mM histidine, pH 7.0. The glyceraldehyde-P dehydrogenase fraction was collected and dialyzed against 10 mM imidazole, pH 7.0, 10 mM sodium acetate, and 1 mM dithioerythritol. Aliquots were stored frozen.

The purity of GAPD was determined by SDS–polyacrylamide gel electrophoresis, and no other major proteins were detected.

Preparation of Terminal Cisternae/Triads and Isolation of Calsequestrin. Terminal cisternae/triads were isolated from rabbit skeletal muscle (sacrospinalis) according to the method of Caswell et al. (1976). Calsequestrin was extracted from terminal cisternae/triads isolated from rabbit skeletal muscle as previously described (Caswell & Corbett, 1985). The vesicles were initially treated with 2 mg of Triton X-100/mg of protein and centrifuged at 123000*g* for 1 h. The pellet was dissolved in 0.3 M NaCl and Zwittergent 3-14 (2 mg/mg of protein) and applied to a hydroxyapatite column equilibrated with 0.2 M NaCl, 0.2% Lubrol PX, and 10 mM Tris-HCl, pH 7.0. Calsequestrin was eluted with a sodium phosphate gradient (0–1.0 M) using a constant-volume gradient mixer. Calsequestrin was identified by SDS–PAGE.

Phosphorylation of Glyceraldehyde-P Dehydrogenase. Purified glyceraldehyde-P dehydrogenase was incubated with 1 mM [^{32}P]ATP in a buffer containing 10 mM imidazole, pH 7.0, 10 mM sodium acetate, 1 mM magnesium acetate, 1 mM dithioerythritol, and 0.1 mM EGTA (phosphorylation medium). Reactions were normally carried out at 30 °C for 10 min. Phosphorylation reactions were stopped with ice-cold 0.5 N HCl. Protein was precipitated with 0.5 N ice-cold perchloric acid with 500 μg of BSA as carrier. The precipitate was pelleted in an Eppendorf microfuge, and the pellets were resuspended in 1 mL of 0.5 N ice-cold Cl_3CCOOH. The protein was pelleted again and washed 3 times with 1 mL of 0.5 N Cl_3CCOOH. The final pellet was dissolved in 100 μL of 0.5 N NaOH and assayed for incorporated ^{32}P by liquid scintillation.

Phosphorylation was also conducted in the presence of various effectors. Reaction mixtures contained 10 mM NADH, NAD^+, or GAP. When calsequestrin was used, it was present in equimolar amounts to glyceraldehyde-P dehydrogenase tetramer.

In experiments with hydroxylamine, 0.1 M NH_2OH neutralized to pH 7.0 with Tris was added to an unquenched phosphorylation reaction mixture containing glyceraldehyde-P dehydrogenase The mixture was acid-quenched 5 min after the NH_2OH addition as described, and the ^{32}P retained in the protein pellet was determined.

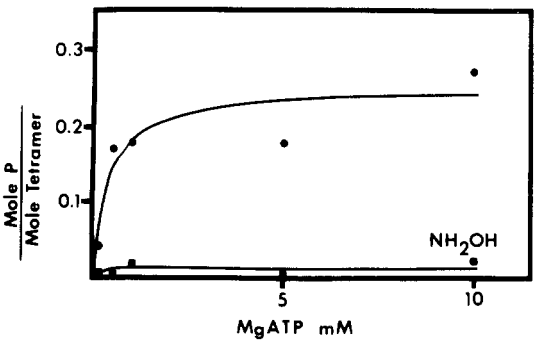

FIGURE 1: Phosphorylation of glyceraldehyde-P dehydrogenase as a function of MgATP concentration. (●) Glyceraldehyde-P dehydrogenase (200 μg/mL) was incubated with various Mg[^{32}P]ATP concentrations for 10 min at 30 °C. Reactions were quenched with 0.5 N HCl. (■) 0.1 M NH_2OH/Tris, pH 7.0, was added after 10 min as before.

In experiments estimating the pH stability of the phosphoprotein, glyceraldehyde-P dehydrogenase (1 mg/mL) was incubated in the above phosphorylation medium. After 10 min, the enzyme was separated from ATP by HPLC using a TSK G4000 SW molecular sieve column with 0.5 mL/min flow rate. The elution buffer contained 4 mM Tris, pH 7.0, and 0.1 M KCl. Aliquots of 150 μL were incubated in various pH solutions for 1 h at 23 °C. The mixtures were acid-precipitated as above, and ^{32}P retained was determined. Control samples were acid-quenched immediately following HPLC separation of the enzyme from ATP. The pH conditions were maintained by using a system of 50 mM phosphoric acid/50 mM glycine, and the pHs were adjusted with KOH.

Autoradiographic Analysis of TC/Triad Protein Phosphorylation. Phosphorylation reactions were conducted in the medium described earlier with the substitution of 10 mM MOPS, pH 7.0, in place of imidazole. Reactions were conducted in 80-μL volumes. When glyceraldehyde-P dehydrogenase and TC/triads were used in combination, 5 μg of glyceraldehyde-P dehydrogenase was added to 20-μg TC/triad proteins. Reactions were terminated with the addition of SDS solubilization buffer and boiling for 1 min. SDS–PAGE was performed by the system of Laemmli (1970). Gels were stained and destained within 5 h and dried overnight. Dried gels were developed on Kodak XRP-1 film with an enhancing screen.

Results

The phosphorylation of glyceraldehyde-P dehydrogenase as a function of MgATP concentration is shown in Figure 1. The reaction shows a MgATP concentration dependence with half-maximal phosphorylation achieved around 0.3 mM. Glyceraldehyde-P dehydrogenase is a tetrameric enzyme, and phosphorylation of multiple sites is a possibility. Generally, phosphorylation in the range of 0.3–0.4 mol of phosphate/mol of tetramer was observed. However, values of 1 mol of phosphate/mol of tetramer were also obtained. The variability observed in the amount of phosphorylation occurring may be a reflection of the enzyme stability (stored frozen) or the instability of the phosphoenzyme complex. Nucleotide specificity was also investigated (not shown). ADP and GTP, as well as creatine phosphate (1 mM), did not alter the GAPD phosphorylation in the presence of 0.5 mM [^{32}P]ATP. Figure 1 also shows the sensitivity of the phosphorylated glycer-

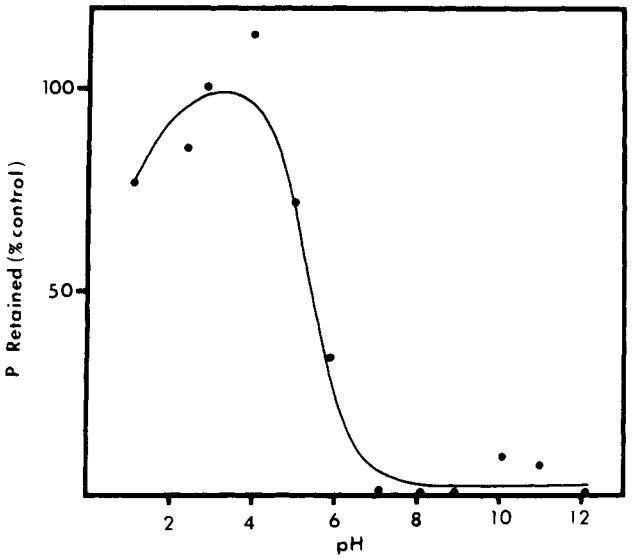

FIGURE 2: pH stability of phosphorylated glyceraldehyde-P dehydrogenase. Phosphorylated enzyme was separated from ATP by molecular sieve chromatography as described under Materials and Methods. Aliquots were incubated in various pH media for 1 h at 23 °C before acid quench and acid precipitation of the protein.

aldehyde-P dehydrogenase to neutral hydroxylamine. The phosphoenzyme complex was treated with hydroxylamine (0.1 M) prior to acid denaturation of the enzyme over a range of concentrations of MgATP. This concentration of hydroxylamine completely dephosphorylated the phosphoprotein over the entire concentration range of MgATP used in the initial phosphorylation reaction. In data not shown, parallel experiments were conducted in which phosphorylated glyceraldehyde-P dehydrogenase was treated with 0.1 M NH_2OH/Tris or with Tris alone at a similar concentration to NH_2OH. Rapid dephosphorylation occurred in the presence of NH_2OH but not in the Tris-treated sample, showing that dephosphorylation was not simply an ionic strength effect.

Figure 2 is a pH stability profile of the phosphorylated glyceraldehyde-P dehydrogenase complex. The phosphoenzyme complex is most stable at pH 4 while exhibiting a very dramatic lability in neutral and alkaline conditions and a slight drop in stability at highly acid pH. The phosphoenzyme complex was not tested in the extreme acid/base conditions (pH <1 or >12). The instability of the phosphoprotein in alkaline conditions argued against an ester phosphate and suggested that the enzyme forms an acyl phosphate.

The rate of phosphorylation is relatively slow with an initial rate of 0.16 mol of phosphate (mol of tetramer)$^{-1}$ min^{-1} (Figure 3), although faster rates have been observed. Maximum phosphorylation occurred by 15 min.

The effect of various metabolites of enzyme glycolytic function on the extent of phosphorylation was investigated (Figure 3). Addition of 10 mM NADH resulted in a 93% reduction in the phosphorylation compared to control. Both 10 mM NAD^+ and glyceraldehyde phosphate had a moderate effect by reducing the level of phosphorylation by 40% and 68% of control, respectively (data not shown). Inorganic phosphate (not shown) had no effect on this phosphorylation. In addition, calsequestrin, which has been shown to inhibit the oxidative phosphorylation of glyceraldehyde phosphate by the enzyme (Caswell & Corbett, 1985), was also found to prevent enzyme phosphorylation (figure 3). The reduction was by approximately 84% of control.

The action of the metabolites on phosphorylation could occur either by inhibiting phosphorylation or by catalyzing de-

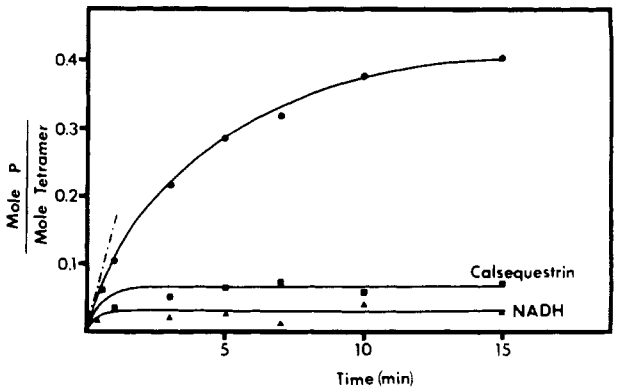

FIGURE 3: Effect of NADH and calsequestrin on the phosphorylation of glyceraldehyde-P dehydrogenase. Enzyme (250 μg/mL) was phosphorylated as described under Materials and Methods. (●) Control phosphorylation; (▲) phosphorylation in the presence of 10 mM NADH; (■) phosphorylation in the presence of 110 μg/mL calsequestrin in equimolar amounts to enzyme tetramer.

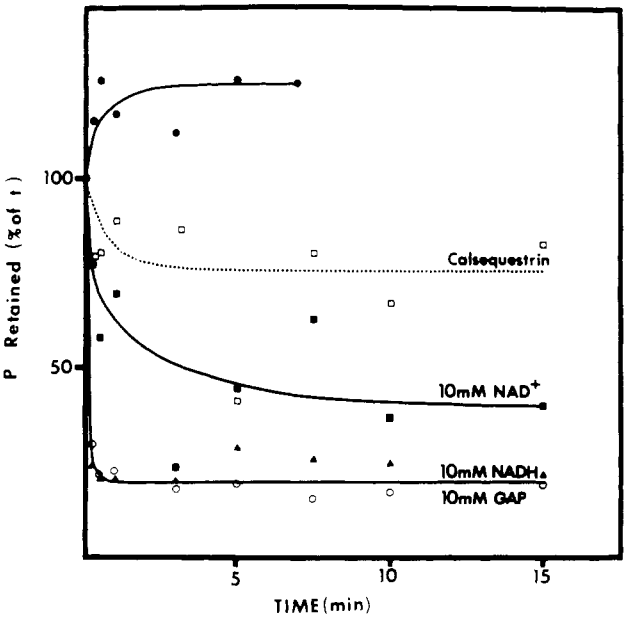

FIGURE 4: Effect of NAD^+, NADH, glyceraldehyde 3-phosphate, and calsequestrin on phosphorylated glyceraldehyde-P dehydrogenase. Enzyme (250 μg/mL) was phosphorylated as described under Materials and Methods. After 10 min of phosphorylation, various metabolites were added (t_0). (●) No addition; (■) 10 mM NAD^+; (▲) 10 mM NADH; (○) 10 mM GAP. Also, enzyme (125 μg/mL) was phosphorylated as described under Materials and Methods. At t_0, calsequestrin in an equimolar amount (55 μg/mL) to glyceraldehyde-P dehydrogenase was added (□). Reactions were acid-quenched at the times shown in the figure.

phosphorylation of the enzyme–phosphate complex. The preformed phosphoenzyme was incubated with these metabolites (Figure 4). Both GAP and NADH caused a rapid and nearly complete dephosphorylation, dropping the phosphoenzyme complex to 20% of the control value by 30 s. NAD^+ also produced a significant but incomplete dephosphorylation, lowering phosphorylation to approximately 45% of control. Calsequestrin was found to cause only a slight diminution of the phosphoenzyme.

During the same time period when dephosphorylation was catalyzed by the metabolites, the control conditions showed a small increase in phosphorylation toward a maximal value, indicating that a steady-state phosphorylation had not been achieved at the time of metabolite additions. Analogous experiments in which the phosphoenzyme was removed from

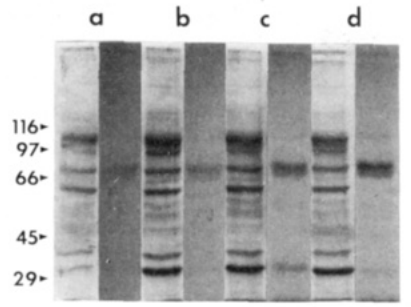

FIGURE 5: Effect of glyceraldehyde-P dehydrogenase and NADH on the phosphorylation of terminal cisternae/triad proteins. Specific reactions are described below. Reactions were performed in a volume of 60 μL. Each reaction was stopped by adding Laemmli solubilization buffer and boiling for 1 min. The proteins were separated by SDS–PAGE, and the gel was autoradiographed. In the figure, each lane is composed of the Coomassie-stained gel (left half) and the corresponding autoradiogram (right half) beside it. Molecular weights noted are $\times 10^{-3}$. Reaction conditions were as follows: (a) TC/triads (20 μg) were incubated with [^{32}P]ATP/Mg for 10 min at 30 °C in the phosphorylation media described. (b) Same as (a) with the addition of glyceraldehyde-P dehydrogenase (5 μg) to the reaction. (c) Glyceraldehyde-P dehydrogenase was phosphorylated with [^{32}P]ATP as described under Materials and Methods. The prephosphorylated enzyme (5 μg) was incubated with TC/triads (20 μg) for 10 min at 30 °C. (d) 5 μg of glyceraldehyde-P dehydrogenase prephosphorylated as in (c) was treated with 10 mM NADH for 5 min at 30 °C. The mixture was then added to TC/triads and incubated for 10 min at 30 °C.

unreacted ATP by molecular sieve chromatography using HPLC prior to incubation with the metabolites produced similar data.

The possibility that glyceraldehyde-P dehydrogenase had been dephosphorylated and the phosphate had been transferred to calsequestrin was investigated. In experiments similar to that of Figure 4, the acid protein precipitate containing both glyceraldehyde-P dehydrogenase and calsequestrin was treated with 0.5 N NaOH. The treatment was found to release all of the radiolabel, demonstrating that the ^{32}P was still in the form of acyl phosphate. This may be taken as evidence that the phosphate is still associated with glyceraldehyde-P dehydrogenase for it would be unlikely that this enzyme would transfer phosphate to calsequestrin still in the form of a high-energy acyl phosphate.

The formation and cleavage of the high-energy acyl phosphate bond of the enzyme suggested the possibility of an acceptor molecule(s) for the phosphate group. Figure 5 demonstrates the phosphorylation of TC/triad proteins as affected by glyceraldehyde-P dehydrogenase. When isolated TC/triads were incubated wiith [γ-^{32}P]ATP and Mg^{2+}, a small amount of phosphorylation was detected (lane a). Phosphorylation was most apparent on proteins with molecular weights of 80000 and 72000. When untreated purified glyceraldehyde-P dehydrogenase was added to the TC/triads, an increase in the phosphorylation of these two protein bands occurred (lane b). The protocol was reversed, and enzyme was first incubated with radiolabeled ATP and Mg^{2+}. After 10 min, the mixture was combined with TC/triads (lane c). A clear enhancement in the phosphorylation of the M_r 80000 and 72000 bands was observed. In addition, a small amount of phosphorylation was observed in proteins with molecular weights of 54000 and 100000. The phosphorylated band at 34000 daltons is presumably the residual label associated with glyceraldehyde-P dehydrogenase. The experiment is complicated by the presence of glyceraldehyde-P dehydrogenase as an extrinsic protein bound to the vesicle. Therefore, the control phosphorylation (lane a) could have occurred as a consequence of the endogenous enzyme. In a similar experiment, prephosphorylated enzyme was treated with NADH prior to incubation with TC/triad vesicles (lane d). A significant further enhancement in the phosphorylation of the 80000- and 72000-dalton proteins occurred. However, the phosphorylation of the 54000- and 100000-dalton bands did not appear affected whereas the phosphorylation of glyceraldehyde-P dehydrogenase was diminished. The phosphorylation of the 34000-dalton band was not always visible in these Laemmli gels as might be predicted considering the alkaline conditions employed which tend to hydrolyze the acyl phosphate. In other experiments, 0.1 M neutralized NH_2OH was added to the mixture following the 10-min phosphorylation reaction. The reaction was stopped 5 min later with solubilization buffer. The analysis by SDS–PAGE resulted in an identical pattern of phosphorylation of TC/triad proteins. These phosphoproteins were also found to be stable in 0.5 N NaOH, indicating that the phosphorylated TC/triad proteins were not acyl phosphates.

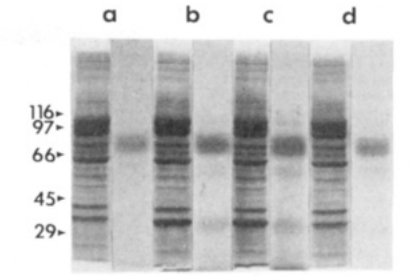

FIGURE 6: Effect of NADH on the phosphorylation of triad protein. Treatment of individual reactions for SDS–PAGE was as described in Figure 5. Reaction conditions were as follows: (a) TC/triads (20 μg) incubated with [^{32}P]ATP/Mg in the phosphorylation media for 10 min at 30 °C. (b) Glyceraldehyde-P dehydrogenase (5 μg) prephosphorylated with [^{32}P]ATP was incubated with TC/triads (29 μg) for 10 min at 30 °C. (c) Prephosphorylated enzyme (5 μg) was treated with 10 mM NADH for 5 min at 30 °C. The mixture was incubated with 20 μg of TC/triads for 10 min at 30 °C. (d) TC/triads were incubated with 10 mM NADH and [^{32}P]ATP/Mg in the phosphorylation media for 10 min at 30 °C.

The effect of NADH on the phosphorylation of TC/triad proteins is further evaluated in Figure 6. Lanes a, b, and c substantiate the results described in Figure 5 as lane c demonstrates the effectiveness of NADH in the phosphorylation of the 80000- and 72000-dalton proteins. Lane 6d shows the results of incubating TC-triads with NADH, [γ-^{32}P]ATP and Mg^{2+} in the absence of added enzyme. The phosphorylation detected in the 80000- and 72000-dalton bands is slightly greater than that in control without NADH (lane a). This observation argues that NADH behaves as a catalyst in the phosphorylation of TC/triad proteins. Yet the phosphorylation is less than that observed in conditions in which prephosphorylated enzyme was employed with or without NADH (lanes b and c). This would imply that the enzyme is involved in the phosphorylation of TC/triad proteins and the enhancement in phosphorylation detected in lane d is the consequence of NADH interaction with the endogenous enzyme associated with the TC/triads. The phosphorylation of the added enzyme is clearly discernible in Figures 5 and 6. The extent is low compared with that of the 80000-dalton band. However, the acyl phosphate of glyceraldehyde-P dehydrogenase is likely to be unstable as previously stated.

We next explored whether the acyl phosphate of the enzyme served as the phosphate donor for vesicle phosphorylation or whether the phosphoenzyme was the active catalyst of phosphorylation of the vesicle by ATP. In Figure 7, lanes a and b again demonstrate the enhancement in phosphorylation of the 80000- and 72000-dalton bands by NADH. In lane c,

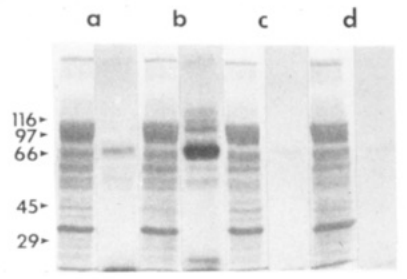

FIGURE 7: Effect of phosphorylating glyceraldehyde-P dehydrogenase with unlabeled ATP prior to incubation with TC/triads and [^{32}P]ATP. Treatment of individual reaction for SDS–PAGE was as described in Figure 5. Reaction conditions were as follows: (a) 5 μg of glyceraldehyde-P dehydrogenase prephosphorylated with [^{32}P]ATP. The mixture was added to TC/triads (20 μg) and incubated for 10 min at 30 °C. (b) 5 μg of enzyme prephosphorylated with [^{32}P]ATP was treated with 10 mM NADH for 5 min at 30 °C. The mixture was added to TC/triads (20 μg) and incubated for 10 min at 30 °C. (c) 5 μg of enzyme was prephosphorylated with unlabeled ATP. The mixture was added to TC/triads (20 μg) simultaneously with [^{32}P]ATP. The amount of radiolabel was equal to that utilized in (a) and (b). (d) 5 μg of enzyme prephosphorylated with unlabeled ATP was treated with 10 mM NADH for 5 min at 30 °C. The mixture was added to TC/triads coincident with the addition of [^{32}P]ATP. Amount of label equalled that used in lanes a–c.

the enzyme was prephosphorylated with nonradioactive ATP and Mg^{2+} before being incubated with TC/triads. Lane d was similar except NADH was added to prephosphorylated glyceraldehyde-P dehydrogenase prior to addition of TC/triads. Carrier-free [^{32}P]ATP was added to the vesicles at the same time that they were incubated with the enzyme. Thus, lanes a and c are identical except for the time of label addition; similarly, lanes b and d are identical except for the time of labeling. Clearly, the amount of phosphorylation detected in the 80 000- and 72 000-dalton bands is far less in lanes c and d than in either lane a or lane b. This observation indicates that the phosphate which is incorporated into the 80 000- and 72 000-dalton protein bands is the same phosphate as that which initially reacted with the enzyme.

The effect of Ca^{2+} concentration on the phosphorylations thus far described was investigated (data not shown). The autophosphorylation of GAPD was independent of Ca^{2+} concentration over a range from 10 nM to 100 μM. The phosphorylation of TC/triad proteins mediated by enzyme and NADH was also found to be Ca^{2+} insensitive. The phosphorylation of the vesicle proteins was not augmented by addition of Ca^{2+} (10 nM–100 μM). There was a small decrease in the phosphorylation of the vesicle proteins as [Ca^{2+}] was elevated. However, this response may have been due to the activation of an endogenous Ca^{2+}-activated phosphatase present in the TC/triad vesicles.

Discussion

We have presented evidence demonstrating the autophosphorylation of a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, with MgATP. Although the enzyme catalyzes the oxidative phosphorylation of GAP, the enzyme is not itself phosphorylated in this reaction, and ATP is not a substrate. The reaction mechanism as described by Segal & Boyer (1954) indicates that the enzyme–substrate complex consists of a mercaptal linkage with GAP which is first oxidized and then subjected to phosphorolysis by inorganic phosphate. Therefore, our observations indicate a wholly different phenomenon in which the phosphate of ATP is covalently incorporated to form an enzyme–phosphate complex.

Acyl phosphates exhibit properties which clearly distinguish them from ester phosphates, being susceptible to nucleophilic attack by hydroxylamine and being labile in alkaline conditions (Koshland, 1952; Walsh et al., 1970; Post & Kume, 1973; Black & Wright, 1955). Acyl phosphates such as acetyl phosphate, succinyl phosphate, and aspartyl phosphate have been shown to have a distinct U-shaped pH stability profile in which the phosphate bond is labile at the pH extremes while remaining relatively stable at the intermediate pHs. Similar profiles have been demonstrated in acyl phosphoenzyme intermediates in which the complex is stable in mildly acid conditions but becomes increasingly unstable in alkaline media. Variability exists in the reported results, and presumably, this can be attributed to different reaction conditions as well as the differences in the molecular environment surrounding each type of phosphoenzyme complex. However, in each case the enzyme–phosphate bond was shown to be susceptible to hydroxylamine hydrolysis. We have now demonstrated that the phosphorylated glyceraldehyde-P dehydrogenase exhibits qualitatively similar pH sensitivity and lability in neutral hydroxylamine. It is unlikely that the phosphorylation is occurring at sites other than at a carboxyl residue. Histidyl phosphates are stable in base (Boyer et al., 1962), cysteinyl phosphates are stable at the pH extremes (Akerfeldt, 1960), and tyrosyl phosphates are stable in either acid or base conditions (Plimmer, 1941).

Alternative possibilities for the observed phosphorylation of GAPD might be (1) contaminating kinase or (2) contaminating phosphorylated proteins. The latter possibility seems unlikely as SDS gels have indicated no contaminating bands. Furthermore, it would seem improbable that a contaminant would respond to the metabolites of GAPD utilized in the experiments. The possibility of a contaminating protein kinase also seems unlikely as known kinases catalyze the formation of ester phosphate on proteins while the phosphoenzyme formed by GAPD is an acyl phosphate.

An increasing literature has accumulated describing the regulatory significance of ester phosphates (seryl and threonyl) in proteins, particularly in controlling protein kinases. On the other hand, protein acyl phosphates have been described as high-energy intermediates in transport enzymes (Na/K- and Ca-ATPases) and in carboxylate kinases involved in substrate level phosphorylation (ATP citrate-lyase, 3-phosphoglycerate kinase, and acetate kinase). Therefore, precedent would suggest that the phosphoenzyme formed here is an intermediate in further reactions rather than a regulator of glycolytic activity.

Four agents tested diminished the phosphorylation of the enzyme, but calsequestrin clearly acts by a different mechanism than NADH, NAD^+, and GAP. We previously demonstrated that calsequestrin binds to and inhibits the glycolytic enzyme activity of glyceraldehyde-P dehydrogenase. By analogy with the interaction of the anion channel of erythrocytes, it may be concluded that acid groups on calsequestrin bind in the alkaline NAD^+ binding site of the enzyme. Calsequestrin also inhibits enzyme phosphorylation but does not enhance dephosphorylation of the phosphoenzyme.

NADH, GAP, and to a smaller extent NAD^+ activate dephosphorylation of the enzyme. It is possible that GAP exerts its action by causing reduction of NAD^+ present in the holoenzyme and that the primary activator of dephosphorylation is NADH. Dephosphorylation of acyl phosphate enzymes is normally associated with energy transduction either in active transport or in transfer of phosphate to a substrate. The rapid dephosphorylation of glyceraldehyde-P dehydrogenase by NADH is therefore puzzling. We are currently investigating whether a phosphorylated product occurs. The rapid de-

struction of the phosphoenzyme by the glycolytic substrates further argues against a role of enzyme phosphorylation as a regulator of glycolysis.

We have demonstrated that the enzyme enhances the phosphorylation of proteins of TC/triads. Its principal substrates are proteins of M_r 80000 and 72000 found in the TC/triad vesicle preparations. Some caution is necessary in assigning radiolabel to specific Coomassie-stained proteins since a minor protein having similar molecular weight can be heavily labeled. With this proviso, the 80000-dalton protein is a major protein present in both T tubules and terminal cisternae but is almost absent from nonjunctional terminal cisternae and is low in longitudinal reticulum. This protein has been identified as the "anchor" which recognizes the spanning protein of the triad and holds it to the membrane. A protein of M_r 72000 is the major protein of T tubules and is not detectable in sarcoplasmic reticulum. Thus, the phosphorylated proteins may be specific triadic entities.

The kinase activity is markedly enhanced by the addition of NADH. While NADH causes dephosphorylation of the enzyme, it enhances the phosphorylation of TC/triad protein. It is therefore possible that the enzyme is responsible for forming a phosphorylated intermediate from ATP which is the proximal substrate for the vesicle phosphorylation. Moreover, these data demonstrate that phosphate is transferred directly or indirectly from the phosphoenzyme to the vesicle substrates. In data not presented, the phosphorylation of TC/triad proteins was found to be reduced when incubated with type C-1 protein phosphatase. The sensitivity to protein phosphatase indicates that the vesicle phosphoprotein is an ester phosphate.

In summary, we have demonstrated the autophosphorylation of glyceraldehydephosphate dehydrogenase. The phosphoenzyme behaves as a kinase giving rise to phosphorylated proteins, and the catalytic step is enhanced by NADH. Furthermore, it may be a unique and previously undescribed class of kinase representing a new mechanism of protein regulation. A possible role of protein phosphorylation on ion flux in T tubules and sarcoplasmic reticulum is currently being investigated.

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Registry No. NAD, 53-84-9; NADH, 58-68-4; GAP, 142-10-9; GAPD, 37250-87-6; MgATP, 1476-84-2; protein kinase, 9026-43-1.

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