Regulation of Glycogen Synthetase

SPECIFICITY AND STOICHIOMETRY OF PHOSPHORYLATION OF THE SKELETAL MUSCLE ENZYME BY CYCLIC 3’:5’-AMP-DEPENDENT PROTEIN KINASE

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SUMMARY

Complete conversion of skeletal muscle glycogen synthetase from the I form to the D form requires incorporation of 2 mol of phosphate per enzyme subunit (90,000 g). Incubation of synthetase I with low concentrations of adenosine 3’:5’-monophosphate (cAMP)-dependent protein kinase (10 units/ml) and ATP (0.1 to 0.3 mM) plus magnesium acetate (10 mM) results in incorporation within ½ hour of 1 mol of phosphate per subunit concomitant with a decrease in the synthetase activity ratio (minus glucose-6-P/plus glucose-6-P) from 0.85 to 0.25. Further incubation for 6 hours does not greatly increase the phosphate content of the synthetase or promote conversion to the D form. This level of phosphorylation is not increased by raising the concentration of protein kinase to 150 units/ml and is not influenced by the presence of glucose-6-P, UDP-glucose, or glycogen. However, at protein kinase concentrations of 10,000 to 30,000 units/ml a second mol of phosphate is incorporated per subunit, and the synthetase activity ratio decreases to 0.05 or less.

In addition to the 2 mol of phosphate per subunit which are required for formation of synthetase D, further phosphorylation can be observed which is not associated with changes in synthetase activity. This phosphorylation occurs at a slow rate, is increased by raising the ATP concentration to 2 to 4 mM, and is not blocked by the heat-stable protein inhibitor of cAMP-dependent protein kinase.

These data indicate that skeletal muscle glycogen synthetase contains multiple phosphorylation sites only two of which are involved in the synthetase I to D conversion.

Conversion of skeletal muscle glycogen synthetase from the physiologically active I form to the less active D form involves phosphorylation of the enzyme (1-4) catalyzed by the cAMP-dependent protein kinase (5, 6) as depicted in Equation 1.

\[
\text{Synthetase (I form)} + n\text{ATP} \rightarrow \text{cAMP, Mg}^2+ \rightarrow \text{Synthetase-nP (D form)} + n\text{ADP}
\]

Skeletal muscle synthetase I contains essentially no alkali-labile phosphate (5, 7). Conflicting values for the phosphate content of synthetase D have appeared in the literature. In one investigation (5) phosphorylation was performed at 30° with 0.1 to 0.6 mM ATP, and the reaction was allowed to proceed until synthetase I activity reached a minimum (about 30 min). Phosphate incorporation of 1 mol of phosphate per mol of subunit correlated with the synthetase I to D conversion. In the other study (7) phosphorylation was performed in the presence of glycogen and 10 mM ATP for 3 days at 3° with incorporation of 6 mol of phosphate per mol of subunit. The extent of synthetase D formation at intermediate values of phosphorylation was not given. The subunit molecular weight of glycogen synthetase is approximately 90,000 (5, 7).

This investigation was undertaken to examine in more detail the relationship of phosphate incorporation to formation of synthetase D. It was reasoned that phosphorylation of glycogen synthetase may be analogous to phosphorylation of phosphorylase kinase by this same protein kinase. Phosphorylase kinase contains several different phosphorylation sites some of which are specific for and others unrelated to activation of the enzyme (8, 9). The experiments reported in this paper indicate that glycogen synthetase also contains specific and nonspecific sites of phosphorylation. Some of these results have been summarized elsewhere (10, 11).

EXPERIMENTAL PROCEDURES

Methods

Purification and Assay of Glycogen Synthetase—Glycogen synthetase I was purified from rabbit skeletal muscle as described earlier (5) with the modification that potassium phosphate buffer was substituted for the glycerol-P buffer. Glycogen synthetase

1 The abbreviations used are: cAMP, adenosine 3’:5’-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N’-tetraacetic acid.
was assayed essentially as described by Thomas et al. (12) with 0.07-ml reaction mixtures containing 36 mM Tris buffer (pH 7.8), 3.6 mM EDTA, 4.8 mM UDP-[γ-32P]glucose (0.030 nM/mCi), 7.1 mg/ml of glycogen, and (when added) 4.8 mM glucose-6-P. The synthetase activity ratio is defined as activity measured in the absence of glucose-6-P divided by activity measured in the presence of glucose-6-P.

Phosphorylation and Conversion of Synthetase I—Unless indicated otherwise, phosphorylation of synthetase I was performed at 30° and at pH 7.0 in 0.1 ml reaction mixtures containing variable concentrations of [γ-32P]ATP and protein kinase, 10 mM magnesium acetate, 0.2 to 1.6 mg/ml of synthetase I, 5 μM cAMP, 10 to 20 mM NaF, and 20 mM potassium phosphate. Aliquots were spotted on 1-cm filter paper squares and washed with 10% trichloroacetic acid as described elsewhere (13) for the protein kinase assay.

Conversion of synthetase I to the D form was performed under identical reaction conditions except that nonradioactive ATP was used. Aliquots of the reaction mixture were diluted 10- to 100-fold in cold 50 mM Tris (pH 7.8), 5 mM EDTA, 50 mM NaF, 40 mM β-mercaptoethanol, and 1 mg/ml of bovine serum albumin. These dilutions were then assayed in duplicate for glycogen synthetase activity in the absence and presence of glucose-6-P.

Other Methods—Protein was determined by the method of Lowry et al. (14) with crystalline bovine albumin as the standard. [γ-32P]ATP was prepared as described elsewhere for the protein kinase assay. Disc gel electrophoresis of synthetase was performed in the presence of glucose-6-P as described earlier (5).

Materials

Protein Kinase—Homogeneous catalytic subunit from bovine liver was kindly supplied by Drs. P. S. Sugen, E. M. Reimann, and J. D. Corbin. Homogeneous bovine skeletal muscle catalytic subunit was a generous gift from Drs. J. A. Deavo and E. G. Krebs. Units of protein kinase activity were determined with the use of reaction conditions identical with those described for phosphorylation of synthetase I except that type II-A histone at 3 mg/ml was substituted for the synthetase I. One unit of protein kinase activity is that amount of enzyme which catalyzed incorporation of 1 pmol of 32P per min into histone.

Other Materials—ATP, UDP-glucose, glucose-6-P, and histone type II-A were purchased from Sigma Chemicals. UDP-[14C]glucose was obtained from New England Nuclear. Protein kinase inhibitor was partially purified through the heating, trichloroacetic acid precipitation, and dialysis steps as detailed elsewhere (15).

RESULTS

Phosphorylation of Synthetase I Using Low ATP and Low Protein Kinase—In confirmation of earlier work (5), it can be seen in Fig. 1 that in the presence of 0.23 mM ATP and 10 units/ml of protein kinase the phosphorylation of glycogen synthetase proceeds rapidly to a level of about 1 mol of phosphate per subunit (90,000 g) within 1/2 hour. Phosphorylation then either stops or continues only at a greatly diminished rate. This cessation of the synthetase phosphorylation is not due to loss of any reaction components other than synthetase I. Thus, addition of histone (3 mg/ml) to the phosphorylation reaction either at zero time or after 1 hour results in a nearly linear rate of phosphorylation for at least 6 hours (Fig. 1).

It was of interest to know if ligands which are known to bind to glycogen synthetase would alter its level of phosphorylation.
Fig. 2, it appears that at the higher concentrations of ATP there are two distinct phases of phosphorylation. Phosphorylation occurs rapidly to a level of about 1 mol of phosphate per subunit and then proceeds at a second linear but much slower rate for a period of at least 6 hours.

To verify that all the \( ^{32}P \) was incorporated into glycogen synthetase, the following experiment was performed. Synthetase I was phosphorylated with either 0.25 mM or 3 mM \([γ-^{32}P]ATP\) resulting in phosphate incorporation of 0.85 and 2.7 mol of \( ^{32}P \) per subunit, respectively. These two samples of \( ^{32}P \)-synthetase and a sample of synthetase I were subjected to disc gel electrophoresis. As shown in Fig. 3, essentially all the \( ^{32}P \)-protein in both samples coincided with the glycogen synthetase.

It has been noted (16) that under certain assay conditions the formation of a trichloroacetic acid-insoluble complex involving \([γ-^{32}P]ATP\), magnesium ion, and fluoride ion can interfere with determinations of protein phosphorylation. Under the assay conditions used in these experiments, formation of such a complex could not be detected. Assay blanks, determined by omission of synthetase I, were independent of the presence or absence of sodium fluoride or bovine serum albumin (1.5 mg/ml) or reaction time up to 6 hours.

**Effect of ATP Concentration on Conversion of Synthetase I to**

**Synthetase D**—It had previously been shown that phosphorylation of glycogen synthetase I to a level of 1 mol of phosphate per subunit correlated closely with formation of synthetase D (5). It was important to determine if the increased phosphorylation seen with high ATP also correlated with the synthetase I to D reaction. Accordingly, the time course of the I to D conversion at varying concentrations of ATP was measured (Fig. 4). Increasing the ATP concentration from 0.1 to 8 mM did not increase the extent of synthetase D formation. These results indicate that the additional phosphorylation above 1 mol of phosphate per subunit observed using high ATP and low protein kinase concentrations is not associated with changes in synthetase activity.

This conclusion is substantiated by the results of Fig. 5. Phosphorylation and I to D conversion were measured in the same

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**Fig. 3. Disc gel electrophoresis of glycogen synthetase.** Glycogen synthetase I containing endogenous protein kinase was incubated as described under "Experimental Procedures" with 0, 0.25, or 3 mM \([γ-^{32}P]ATP\) (0.007 pmol/cpm) for 1 hour resulting in incorporation of 0, 0.85, or 2.7 mol of \( ^{32}P \)/90,000 g, respectively. Duplicate aliquots of each reaction were subjected to disc gel electrophoresis on 5% polyacrylamide gels in the presence of 1 mM glucose-6-P as described elsewhere (5). One gel of each duplicate was stained for protein, and the other duplicate of the gels containing \( ^{32}P \)-labeled synthetase was sliced and counted for \( ^{32}P \). The duplicate of the gel containing synthetase I was assayed for synthetase activity as described elsewhere (5).

**Fig. 4. Effect of ATP concentration on synthetase I to D reaction.** Conversion of synthetase from the I to the D form was accomplished in reaction mixtures containing endogenous protein kinase, 90 mM potassium phosphate, 64 mM NaF, 11 mM magnesium acetate, 0.93 mg/ml of synthetase I, and the indicated concentrations of ATP. At each time point 0.01-ml aliquots were diluted 100-fold and assayed as described under "Experimental Procedures."

**Fig. 5. Phosphorylation and I to D conversion of synthetase at high ATP.** Reaction conditions were as described under "Experimental Procedures" with 1.05 mg/ml of synthetase I containing endogenous protein kinase (10 units/ml) and 0.0 mM ATP (□), 3.6 mM ATP (×, ▲), or 3.6 mM \([γ-^{32}P]ATP\) (0.028 pmol/cpm) (○). Synthetase activity in the presence of glucose-6-P is expressed as counts per min × 10^3 (▲).
experiment using 3.6 mM $\gamma$-ATP or 3.6 mM ATP. The synthetase activity ratio decreased from 0.85 at zero time to 0.26 at 30 min coincident with incorporation of 1 to 1.1 mol of $32P$ per subunit. From $\frac{2}{3}$ hour to 6 hours phosphorylation increased linearly to 2 mol of $32P$ per subunit without any further decline in the synthetase activity ratio. Total synthetase activity was essentially unchanged.

**Phosphorylation of Synthetase I in Presence of Protein Kinase Inhibitor or EGTA**—Since the $K_m$ for ATP of cAMP-dependent protein kinase is about 0.01 mM (13), the observation that increasing the ATP concentration from 0.2 mM to 2 to 4 mM stimulates the phosphorylation of synthetase (Figs. 2 and 5) is suggestive of the presence of a different kinase. A possible candidate would be phosphorylase kinase which has a $K_m$ for ATP of 0.2 to 0.4 mM (17) and a stringent requirement for calcium ion (18, 19). Phosphorylation of synthetase at 3.5 mM ATP is not affected, however, by the presence of 1.4 mM EGTA (data not shown).

Conversion of synthetase from the I form to the D form is blocked by the heat-stable kinase inhibitor (5). Likewise, the initial rate of synthetase phosphorylation at low ATP is reduced 85% or more by the protein kinase inhibitor (Table I). If the second, slow phase of phosphorylation seen with high ATP is catalyzed by the cAMP-dependent protein kinase, it should also be blocked by the protein kinase inhibitor. However, addition of protein kinase inhibitor after 1 mol of phosphate per subunit has been incorporated does not affect the subsequent slow phosphorylation (data not shown).

**Effect of Protein Kinase Concentration on Phosphorylation and Conversion of Synthetase I**—The synthetase I used in these studies does contain a small amount of endogenous CAMP-dependent protein kinase. From the results of Fig. 1 and other experiments, it can be calculated that the synthetase preparation contains approximately 24 units/mg of protein kinase activity (based on phosphorylation of histone). Assuming a specific activity of 4 x $10^6$ units/mg for homogeneous protein kinase, the endogenous protein kinase in the synthetase preparation would represent 0.0006% of the protein. This protein kinase is sufficient, however, to catalyze the rapid incorporation of 1 mol of $32P$ per subunit as shown in Figs. 1, 2, 5, and 6. During the course of this study variable amounts of exogenous, partially purified protein kinase were added to the phosphorylation reaction mixtures containing low ATP with little if any effect on the plateau value of 1 mol of phosphate per subunit.

With the availability of homogeneous liver protein kinase catalytic subunit from the laboratory of Dr. J. D. Corbin, it was decided to test whether a very high concentration of protein kinase would affect the stoichiometry of synthetase phosphorylation. The results of such an experiment are shown in Fig. 6. In the presence of 72,000 units/ml of protein kinase, synthetase phosphorylation proceeded within 1 hour to a value between 2 to 3 mol of $32P$ per subunit. This increased phosphorylation observed with low ATP (0.25 mM) and high protein kinase was associated with a decrease in the synthetase activity ratio to 0.05 or less. This is in contrast to the increased phosphorylation seen with high ATP (2 to 4 mM) and low protein kinase (Fig. 5).

The time course of the phosphorylation and synthetase conversion reactions over a period of 6 hours in the presence of 72,000 units/ml of protein kinase, synthetase phosphorylation proceeded within 1 hour to a value between 2 to 3 mol of $32P$ per subunit. This increased phosphorylation observed with low ATP (0.25 mM) and high protein kinase was associated with a decrease in the synthetase activity ratio to 0.05 or less. This is in contrast to the increased phosphorylation seen with high ATP (2 to 4 mM) and low protein kinase (Fig. 5).

**FIG. 5.** Time course of phosphorylation and I to D conversion of glycogen synthetase with low and high protein kinase activity. Reaction conditions were as given under "Experimental Procedures" with 0.05 mg/ml of synthetase I, 0.25 mM ATP (---) or 0.25 mM $\gamma$-ATP (0.035 pmol/cpm) (-- --), and 10 units/ml (○) of endogenous protein kinase or 72,000 units/ml (△) of liver protein kinase catalytic subunit.

**FIG. 6.** Dependence of the I to D reaction and synthetase phosphorylation on protein kinase concentration. Phosphorylation and I to D conversion were performed for 1 hour as described under "Experimental Procedures" with 0.42 mg/ml of synthetase I and 0.23 mM ATP (△) or $\gamma$-ATP (0.018 pmol/cpm) (○).

With the availability of homogeneous liver protein kinase catalytic subunit from the laboratory of Dr. J. D. Corbin, it was de-
Fig. 8. Correlation of synthetase activity ratio with phosphate content. Values for the $^{32}$P content are plotted versus the corresponding synthetase activity ratios obtained from experiments with 0.1 to 0.3 mmoles ATP or [γ-$^{32}$P]ATP and reaction times of 1 hour or less.

150 units/ml did not alter the synthetase phosphate content (1 mol/subunit) or the activity ratio (0.26). Only when much higher amounts of protein kinase were added did the phosphate content increase above 1 mol per subunit and the activity ratio decrease below 0.26. Essentially identical results with those shown in Fig. 7 were obtained when skeletal muscle catalytic subunit was used in place of liver catalytic subunit (results not shown).

**Correlation of Synthetase Activity Ratio with Phosphate Content of Synthetase**—Although skeletal muscle glycogen synthetase can be phosphorylated to a level of about 6 mol of phosphate per subunit (7), the results of this study indicate that not all of these phosphates are associated with formation of synthetase D. In an attempt to define the stoichiometry of the I to D reaction, synthetase was phosphorylated to varying degrees and the activity ratio determined. In order to minimize “nonspecific” phosphorylation (i.e. phosphorylation not associated with the I to D reaction), low ATP concentrations were used and only values obtained at reaction times of 1 hour or less were used. The results of this compilation are given in Fig. 8. There appeared to be a nearly linear correlation (coefficient of correlation = 0.97) up to 1 mol of phosphate per subunit and a second linear correlation (coefficient of correlation = 0.90) between 1 to 2 mol per subunit. Beyond 2 mol of phosphate per subunit there was little if any decrease in the activity ratio.

**DISCUSSION**

The data presented in this paper indicate that conversion in vitro of skeletal muscle glycogen synthetase from the I form to the D form occurs as depicted in Equation 2.

\[
\text{Synthetase (I monomer)} + 2 \text{ATP} + \text{protein kinase} \rightarrow \text{cAMP, Mg}^{2+} \rightarrow \text{synthetase-2 phosphate} \rightarrow \text{(D monomer)} + 2 \text{ADP}
\]

Equation 2 describes phosphate incorporation in terms of the monomer of synthetase. The native form of skeletal muscle glycogen synthetase I appears to be a tetramer (5, 10).

I is catalyzed by a low concentration of cAMP-dependent protein kinase (2 x 10^{-10} m) whereas phosphorylation of the second site requires roughly 0.5 μm protein kinase. The reason for this difference has not been established, but two possibilities are being studied. Protein kinase may have a much lower $K_m$ for the first phosphorylation site relative to the second, or the $V_{max}$ may be much higher for the first site. Alternatively, both phosphorylation sites in synthetase I may be kinetically about equivalent, but there may be strong negative cooperativity, i.e. phosphorylation of one site may provoke a conformational change in the synthetase rendering the second site kinetically unfavorable for phosphorylation.

A somewhat similar situation appears to occur during phosphorylation of pyruvate dehydrogenase (20). Phosphorylation of one serine residue in one of the α chains of pyruvate dehydrogenase results in inactivation of the tetramer. A second serine residue in the same α chain can be phosphorylated but only after phosphorylation of the first seryl residue.

A third alternative is that the purified liver catalytic subunit contains a small amount of some undefined kinase which catalyzes phosphorylation of the second site associated with the I to D conversion. This possibility seems unlikely since the results of Fig. 7 were duplicated with the use of purified skeletal muscle catalytic subunit. Thus, both preparations of catalytic subunit which were purified by different methods from different tissues would have to be contaminated to the same degree by this hypothetical kinase.

It is not known whether the synthetase I to D reaction in vitro occurs in two phases. The intracellular concentration of protein kinase, 0.23 μm (21), is high enough to catalyze phosphorylation of the second site. A more critical question is whether the concentration of protein kinase catalytic subunit ever becomes low enough to make the phosphorylation-dephosphorylation of the first site feasible. Even though the basal intracellular concentration of cAMP in muscle is about 0.25 μm, it has been estimated (21) that under these conditions protein kinase may be essentially inactive. At the high intracellular concentrations of protein kinase and ATP, 0.25 μm cAMP would produce dissociation and activation of only about 20% of the total protein kinase (21). Much of this catalytic subunit may be inactive, however, since there is sufficient protein kinase inhibitor in muscle to inhibit approximately 20% of the total protein kinase (22). Other factors that will determine whether phosphorylation-dephosphorylation of the first site is operative in vitro include (a) the activity of the synthetase phosphatase, and (b) competitive inhibition of the reaction by phosphorylase kinase (23) and other possible substrates of the cAMP-dependent protein kinase.

Values in the literature for the synthetase activity ratios which occur in vivo in muscle under various physiological conditions differ significantly. Some of this variation probably reflects differences in preparation of the muscle extracts and in assay conditions. Shen et al. (24) reported mean synthetase activity ratios in rat diaphragm of 0.49 for control, 0.26 after epinephrine treatment, and 0.57 after insulin treatment. It should be noted that these ratios were determined with 10 mM sodium sulfate (25) in the synthetase assay whereas the synthetase assays reported in this paper did not contain sodium sulfate. Goldberg et al. (20) reported activity ratios (μm sulfates) in control rat diaphragm of about 0.1, and after insulin treatment the activity ratio was about 0.20 to 0.25.

If the synthetase I to D conversion occurs as a two-step process in vivo, this would provide a unique regulatory mechanism. Very small increases in cAMP which cause a partial activation of pro-
tein kinase may produce sufficient catalytic subunit to catalyze incorporation of 1 mol of phosphate per synthetase subunit. This would allow only partial inactivation of glycogen synthetase. Complete conversion of synthetase to the D form could be accomplished only by much higher levels of cAMP which cause complete activation of protein kinase.

The physiological significance of the slow phosphorylation of synthetase (Fig. 5, 1/2 to 6 hours) observed at high concentrations of ATP is not clear. This phosphorylation is not associated with changes in synthetase activity. It is of interest that phosphorylation of phosphorylase kinase also continues at a slow rate for many hours after complete activation of the enzyme (8, 9). This slow phosphorylation of synthetase at 1 to 4 mM ATP may be catalyzed by some kinase other than cAMP-dependent protein kinase since the reaction is not blocked by the heat-stable inhibitor (15) of CAMP-dependent protein kinase. Phosphorylase kinase without conversion to the 11 form will require its purification.

Complete conversion of synthetase to the 11 form could be achieved only by much higher levels of CAMP which cause a conformation change in the synthetase due to ATP binding. It is known that ATP binds to synthetase and inhibits the enzyme (27). No results were obtained which indicated that this may be the mechanism of the ATP effect. Preliminary evidence did indicate, however, that the quaternary structure of glycogen synthetase did affect its phosphorylation. The preparations of synthetase used in this study exhibited a single peak of activity on sucrose gradients corresponding to a sedimentation coefficient of approximately 13.3 S (5). When the method of synthetase purification was altered, some preparations were obtained which gave two to three peaks of activity on sucrose gradients. Non-specific phosphorylation of these preparations appeared to occur much more rapidly even at low concentrations of ATP.

Even though the additional phosphorylation observed with high ATP does not affect the synthetase activity, it might affect the synthetase-phosphatase reaction in a manner analogous to that which has been suggested for phosphorylase kinase. Cohen and Antoniv (28) have presented evidence which suggests that the phosphate which is incorporated into phosphorylase kinase after complete activation of the enzyme in some manner increases phosphorylase kinase phosphatase activity. Experiments are being conducted to determine the specificity of synthetase-phosphatase for the two phosphate sites responsible for the interconversion of the enzyme as well as for the several phosphates not directly involved in the I to D reaction. This is important since some investigators base the synthetase-phosphatase assay on release on 32P from synthetase containing 6 mol of 32P/90,000 g.

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Note Added in Proof—Nimmo and Cohen (29) have recently observed “nonspecific” phosphorylation of glycogen synthetase when using high concentrations of ATP.

REFERENCES

Regulation of glycogen synthetase. Specificity and stoichiometry of phosphorylation of the skeletal muscle enzyme by cyclic 3':5'-AMP-dependent protein kinase.

T R Soderling