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 1/2 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.

phosphorylation of the enzyme (1-4) catalyzed by the cAMP-dependent protein kinase (5, 6) as depicted in Equation 1.

\[ \text{Synthetase (I form)} + n\text{ATP} \xrightarrow{\text{protein kinase}} \text{Synthetase-nP (D form)} \]

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 glyceral-P buffer. Glycogen synthetase

Conversion of skeletal muscle glycogen synthetase from the physiologically active I form to the less active D form involves

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1 The abbreviations used are: cAMP, adenosine 3':5'-monophosphate; EGTA, ethyleneglycol 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-[14C]glucose (0.030 mCi/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 indi-
cated otherwise, phosphorylation of synthetase I was performed 
at 30° and at pH 7.0 in 0.1 ml reaction mixtures containing vari-
able concentrations of [γ-32P]ATP and protein kinase, 10 mM 
magnesium acetate, 0.2 to 1.6 mg/ml of synthetase 1, 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 un-
der 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 al-
bumin. These dilutions were then assayed in duplicate for gly-
cogen 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 stand-
ard. [γ-32P]ATP was prepared as described elsewhere (5). Disc 
egel 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. Sveden, E. M. Reimann, 
and J. D. Corbin. Homogeneous bovine skeletal muscle catalytic 
subunit was a generous gift from Drs. J. A. Deaven and E. G. 
Krebs. Units of protein kinase activity were determined with the 
use of reaction conditions identical with those described for phos-
phorylation 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 in-
corporation 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 Pro-
tein 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 sub-
unit (90,000 g) within ½ 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. 1. Time course of phosphorylation of synthetase I and 

histone. Reaction conditions were as described under "Experi-

mental Procedures" with 0.42 mg/ml of synthetase 1, 0.23 mM 

[γ-32P]ATP (0.016 pmol/cpm) (○), and 10 units/ml of protein 

kinase endogenous in the synthetase preparation. Other additions 

were 3 mg/ml of histone at zero time (■) or at 1 hour (▲).

Fig. 2. Effect of ligands on phosphorylation of synthetase I. 
Synthetase phosphorylation was performed in reaction mixtures 
containing endogenous protein kinase, 67 mM potassium phos-
phate (pH 7.0), 47 mM NaF, 0.17 mM [γ-32P]ATP (0.10 pmol/cpm),
10 mM magnesium acetate, 1.6 mg/ml of synthetase I, and 4.2 μM 
cAMP. Other additions included the following: none (○), 7.5 
mg/ml of glycogen (▲), 7.5 mM glucose-6-P (●), 3.8 mM UDP-
glucose (■), or all three ligands (▲). One reaction mixture which 
did not contain any of the ligands had 3.5 mM [γ-32P]ATP (0.10 
pmol/cpm).

Phosphorylation of Synthetase I Using High ATP and Low Pro-
tein Kinase—In the study by Smith et al. (7) where synthetase 
D contained 6 mol of phosphate per subunit, 10 mM ATP was 
utilized in the phosphorylation reaction. As shown in Fig. 2, 
increasing the ATP concentration from 0.17 mM to 3.5 mM does 
not affect the stoichiometry of phosphate incorporation even though 
the Kₐ for ATP of the cAMP-dependent protein kinase is about 
0.01 mM (13). From a number of experiments similar to that of
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 $\gamma^3P$ was incorporated into glycogen synthetase, the following experiment was performed. Synthetase I was phosphorylated with either 0.25 mM or 3 mM $[\gamma-3^3P]ATP$ resulting in phosphate incorporation of 0.85 and 2.7 mol of $3^3P$ per subunit, respectively. These two samples of $\gamma^3P$-synthetase and a sample of synthetase I were subjected to disc gel electrophoresis. As shown in Fig. 3, essentially all the $\gamma^3P$-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 $[\gamma-3^3P]ATP$, 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 correlates 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
experiment using 3.6 mM [γ-32P]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 1/4 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 10^5 units/mg for homogeneous protein kinase, the endogenous protein 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 and 2.7 mM ATP was investigated (data not shown). At the 6-hour time point the phosphate content of synthetase was 5 mol per mol of subunit. It should be noted, however, that the I to D reaction was essentially complete when 2.3 mol of phosphate per subunit were incorporated. Total synthetase activity was stable over the 6 hours of incubation.

Synthetase phosphorylation and I to D conversion were studied at low ATP (0.23 mM) as a function of protein kinase concentration. The 1-hour values obtained are shown in Fig. 7. Increasing the protein kinase concentration from 10 units/ml to

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**TABLE I**

Effect of CAMP and protein kinase inhibitor on synthetase phosphorylation

<table>
<thead>
<tr>
<th>Additions</th>
<th>32P Content at 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.15</td>
</tr>
<tr>
<td>CAMP, 2.4 μM</td>
<td>0.65</td>
</tr>
<tr>
<td>CAMP, 2.4 μM plus inhibitor, 0.04 ml</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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*Dr. J. D. Corbin, personal communication.*
I is catalyzed by a low concentration of cAMP-dependent protein kinase (2 × 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 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 vivo 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 vivo 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 (no sulfate) in control rat diaphragm of about 0.1, and after insulin treatment the activity ratio was about 0.90 to 0.95.

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-

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**Fig. 8.** Correlation of synthetase activity ratio with phosphate content. Values for the 32P content are plotted versus the corresponding synthetase activity ratios obtained from experiments with 0.1 to 0.3 mM ATP or [γ-32P]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 of phosphate 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 vivo of skeletal muscle glycogen synthetase from the I form to the D form occurs as depicted in Equation 2.

Synthetase (I monomer)

\[ + 2 \text{ ATP} \xrightarrow{\text{protein kinase, cAMP, Mg}^{2+}} \text{synthetase-2 phosphate} \]  

\[ \xrightarrow{\text{PM}} \text{synthetase-D phosphate} \]  

\[ \text{(D monomer)} + 2 \text{ ADP} \]

Incorporation of the first mole of phosphate into synthetase

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).
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, ½ 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 occurs 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 protein kinase since the reaction is not blocked by the calcium chelator EGTA does not affect the reaction rate. Proof of the existence of another kinase which catalyzes the phosphorylation of synthetase without conversion to the D form will require its purification.

This slow phosphorylation of synthetase at 1 to 4 mM ATP may affect changes in synthetase activity. It is of interest that phosphorylation of synthetase (Fig. 5, z to 6 hours) observed at high concentrations of ATP is not clear. This phosphorylation is not associated with the quaternary structure of glycogen synthetase (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.

Acknowledgments—The author wishes to express his appreciation to Miss Martha Buss for her excellent technical assistance and to Drs. C. R. Park, J. D. Corbin, and J. H. Exton for their helpful suggestions during this investigation and in the preparation of this manuscript.

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

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T R Soderling


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