Rabbit Skeletal Muscle Glycogen Synthase

I. RELATIONSHIP BETWEEN PHOSPHORYLATION STATE AND KINETIC PROPERTIES*

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Nine samples of purified rabbit skeletal muscle glycogen synthase (UDP-glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) were obtained with alkali-labile phosphate contents ranging from 0.27 to 3.49 residues per 85,000 molecular weight subunit. The enzyme samples appeared essentially homogeneous when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had relatively constant specific activity under standard conditions with saturating UDP-glucose and glucose-6-P concentrations (37.1 ± 1.0 μmol of glucose incorporated/mg/min).

When the UDP-glucose concentration was varied, deviations from Michaelis-Menten kinetics were observed for all samples (Hill slopes of 0.79 ± 0.02), but these deviations were virtually abolished by the presence of 5 mM glucose-6-P. Glucose-6-P decreased the S₅₀ (concentration required for half-maximal rate) for UDP-glucose. The plots of activity increase caused by glucose-6-P versus glucose-6-P concentration became progressively more sigmoid in shape with enzyme samples of higher phosphate content.

Both the S₅₀ for UDP-glucose and the M₅₀ for glucose-6-P (concentration for half-maximal activation) were sensitive functions of the alkali-labile phosphate content of glycogen synthase. The M₅₀ increased from 2.3 μM at 0.27 phosphate/subunit to 2.7 mM at 3.5 phosphates/subunit, and the S₅₀ varied from 0.75 mM (0.27 phosphate/subunit) to at least 61 mM (2.3 phosphates/subunit). Both parameters increased continuously with phosphate content, with the greatest absolute changes occurring at values greater than 2 residues of phosphates/subunit. The effects of both phosphorylation and activation by glucose-6-P appeared to be mediated primarily through alteration of the apparent affinity for UDP-glucose. With the phosphorylating conditions used, the results suggested that phosphate could not be introduced into the glycogen synthase molecule without altering its kinetic properties. Further, either the different phosphorylation sites were not equivalent, or else identical sites interacted in determining the kinetic properties of glycogen synthase.

Glycogen synthase (UDP-glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) is one of the enzymes that is regulated in the cell by covalent modification of the protein molecule. For glycogen synthase, this modification takes the form of phosphorylation and dephosphorylation (for a review, see Ref. 1), leading to enzyme species that differ particularly in their activation by glucose-6-P. The dephosphorylated Z form of the enzyme, at high UDP-glucose concentration, is scarcely activated by this effector, whereas the phosphorylated D form has very low activity in the absence of glucose-6-P. As was originally reported by Smith et al. (2), and as is becoming increasingly clear, both from further work in this laboratory (3, 4) and from the work of other investigators (5–7), the subunit of glycogen synthase can be multiply phosphorylated. For the rabbit muscle enzyme the number of phosphorylation sites per subunit is between three and six. In the work presented here, the relationship between the phosphorylation state of the enzyme and some of its catalytic properties was determined by studying a series of glycogen synthase samples of different phosphate contents.

EXPERIMENTAL PROCEDURE

Materials—[U-¹⁴C]UDP-glucose was prepared by the enzymic conversion of [U-¹⁴C]glucose (New England Nuclear) into UDP-glucose (8). Using the standard assay conditions described below, 99% of the radioactivity in [U-¹⁴C]UDP-glucose so prepared could be incorporated into glycogen on incubation with purified glycogen synthase. Contamination by radioactive glucose-1-P and glucose-6-P was estimated by measuring labeled glycogen formation after incubation with glycogen, phosphorylase b, AMP, and phosphoglucomutase. ¹⁴C]Sulfate phosphate contamination of [¹⁴C]UDP-glucose was determined as less than 0.05%.

Because relatively high UDP-glucose concentrations were used, and because of the serious implications of glucose-6-P contamination,
UDP-glucose (Sigma) was carefully analyzed for glucose-6-P content using glucose-6-P dehydrogenase (9). Internal glucose-6-P standards were also run. No glucose-6-P was detectable in a 60 mM UDP-glucose solution, indicating less than 0.0025% (mole/mole) of the sugar phosphate in the UDP-glucose.

Rabbit liver glycogen (Sigma) was purified by passage through a column of Amberlite MB-3 ion exchange resin and precipitation with ethanol before use in enzyme assays (10). Purified cAMP-dependent protein kinase (EC 2.7.1.37) from rabbit muscle was the generous gift of Dr. L. C. Huang (University of Virginia School of Medicine).

Purification of Glycogen Synthase—Glycogen synthase I and D forms were purified by the method of Smith et al. (10), as modified by Takeda et al. (3). The enzyme samples numbered 1 (I form) and 8 (D form) were so prepared. Samples 7 and 9 (both D form) were similar. Twenty-three milligrams of enzyme were used for the gel filtration step with Sepharose 4B, a second chromatography with Sepharose 6B was added. To prepare glycogen synthase with intermediate phosphorylation state, the normal purification scheme was adjusted slightly. The procedure for the isolation of I form was followed up to the conversion of the enzyme to the I form, and one portion of the enzyme was continued through this preparation scheme (Sample 2). For the rest of the enzyme, the protocol was switched to that for the purification of D form and the enzyme was incubated at 7°C with 5 mM ATP, 10 μM cyclic AMP, and 12 mM MgCl₂. After various times of incubation, a portion of the sample was removed and taken through the purification steps for the D form, thus giving rise to Samples 3 to 6. Purified protein kinase was added before the final sample (Sample 6) was taken. The yield for each of these samples was 5 mg. In this preparation (that giving Samples 2 to 6) only fresh rabbit muscle was used, in contrast to the mixture of frozen and fresh muscle employed by Takeda et al. (6, 7).

All nine glycogen synthase samples showed no detectable loss of activity over the period of this work when stored at −75°C.

Assay for Glycogen Synthase Activity—The method of Thomas et al. (8) was used as a standard assay and is referred to as such below. The specific activity of [U-14C]UDP-glucose was normally 200 cpm/nmol. The term "%I activity" refers to the ratio of enzyme activity in the absence of glucose-6-P to that in the presence of 7.2 mM sugar phosphate, expressed as a percentage. In all other cases, the following conditions (taking into account the contributions of the buffer containing the enzyme) were used. The reaction mixture (0.1 ml) contained 50 mM Tris-HCl, pH 7.8; 10 mM EDTA; 2 mM EGTA; 50 mM mercaptoethanol; 2 mg/ml of rabbit liver glycogen, (U-14C]UDP-glucose; and other additions as indicated; and from 15 ng to 2.6 μg of purified glycogen synthase. The specific activity of the UDP-glucose was varied according to the reaction conditions, and up to 20,000 cpm/nmol were used at low UDP-glucose concentrations. Enzyme was diluted immediately before use in cold buffer containing 50 mM Tris-HCl, pH 7.8; 5 mM EDTA; 2 mM EGTA; 50 mM mercaptoethanol; and 1 mg/ml of rabbit liver glycogen, and stored on ice. It was found in these experiments that incubation of the enzyme at 30°C before use caused no increase in activity. The reaction was started by the addition of enzyme, and after an appropriate time (usually between 5 and 10 min), 75 μl of the reaction mixture were placed on a filter paper square washed, dried, and counted as for the standard assay (10). The reaction was started by the addition of UDP-glucose (Sigma) was purified by passage through a column of Amberlite MB-3 ion exchange resin and precipitation with ethanol before use in enzyme assays (10). Purified cAMP-dependent protein kinase (EC 2.7.1.37) from rabbit muscle was the generous gift of Dr. L. C. Huang (University of Virginia School of Medicine).

Other Methods—Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard. Determinations of alkali-labile phosphate of glycogen synthase were carried out as described by Smith et al. (2). Polycrylamide gel electrophoresis (7.5% gel) in the presence of sodium dodecyl sulfate was done according to Weber et al. (12), with 7.5 μg of protein and a constant current of 8 mA per tube. Errors are given as the standard error of the mean.

RESULTS

Characterization of Glycogen Synthase Samples—Nine separate glycogen synthase samples were studied. Of these, four were different enzyme preparations of either the I (Sample 1) or the D forms (Samples 7 to 9). The remaining five (Samples 2 to 6) originated from the same preparation but diverged in their purification as under "Experimental Procedure. These five samples separately underwent two ethanol precipitations and a chromatography step on Sepharose 4B. All nine samples had similar elution profiles from the Sepharose 4B column. So far as we can judge, those enzyme samples that were phosphorylated by incubation with ATP, Mg⁺², and cAMP in the presence of endogenous protein kinase activity (namely, Samples 3 to 9) were subject to very similar phosphorylating conditions, with the reservation that purified protein kinase was added for one sample (Sample 9).

After polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, one main band was observed for all the samples corresponding to the subunit of molecular weight 85,000 reported by Takeda et al. (3, 4). The same authors also described a proteolytic breakdown product, of molecular weight 81,000, present in normal preparations of the I form of glycogen synthase. The 81,000 molecular weight species was clearly visible in Sample 1, but only very faintly so in the other samples. This difference appeared to depend on whether or not frozen muscle was included in the starting material, the use of frozen muscle increasing the proportion of the 81,000 molecular weight species.

Differences in the enzyme purification for these samples of glycogen synthase are detailed under "Experimental Procedure."

TABLE I

| Enzyme sample | Alkaline labile phosphatase (U/mg) | Specific activity (V₉₅) | % I Activity (±SO₄⁻) |
|---------------|-----------------------------------|-------------------------|---------------------|
| mol/85,000g   | μmol/mg/min                       |                         |                     |
| 1             | 0.39 ± 0.01                       | 77.6                    | 106 ± 80            |
| 2             | 0.27 ± 0.09                       | 36.6                    | 103 ± 86            |
| 3             | 0.41 ± 0.00                       | 33.2                    | 101 ± 85            |
| 4             | 0.80 ± 0.02                       | 35.3                    | 95 ± 64             |
| 5             | 1.89 ± 0.01                       | 37.7                    | 61 ± 19             |
| 6             | 2.29 ± 0.02                       | 37.5                    | 45 ± 11             |
| 7             | 3.25 ± 0.04                       | 35.6                    | 5.8 ± 2.3           |
| 8             | 3.48 ± 0.06                       | 41.5                    | 3.8 ± 1.3           |
| 9             | 3.49 ± 0.09                       | 41.3                    | 6.4 ± 2.3           |

* Determined as described under "Experimental Procedure" with between 300 and 500 μg of protein per tube. Averages and standard errors are for triplicate determinations.

† Determined by the standard assay procedure in the absence of sulfate and the presence of 7.2 mM glucose-6-P. Enzyme was from 74 to 175 ng per assay.

‡ Ratio, expressed as percentage, of enzyme activity in the absence of glucose-6-P to that in its presence at 7.2 mM using the standard assay. Where indicated, SO₄⁻ was present at 10 mM. Enzyme was between 74 and 300 ng per assay.

§ Average and standard error of all nine specific activities.
The %I activity was a function of the phosphorylation state of samples consistently showed deviation from Michaelis-Menten kinetics, resulting in Eadie-Hofstee plots convex to the origin. The %I activity was calculated from Hill plots as in Fig. 2. Values of S0.5, the UDP-glucose concentration required for half-maximal rate, were calculated from Hill plots as in Fig. 2. The %I activity was determined from Hill plots of data such as those in Fig. 5. The data were displayed as Eadie-Hofstee plots. The circled numbers identify the different enzyme samples. The amount of enzyme per assay ranged from 30 ng to 2.6 µg depending on the phosphorylation state of the enzyme.

**Summary of kinetic parameters of glycogen synthase**

Glycogen synthase activity was measured as under "Experimental Procedure." The activity of the glycogen synthase samples was measured as a function of UDP-glucose concentration in the range 25 µM to 25 mM as described under "Experimental Procedure." The data are displayed as Eadie-Hofstee plots. The circled numbers identify the different enzyme samples. The amount of enzyme per assay ranged from 30 ng to 2.6 µg depending on the phosphorylation state of the enzyme.

![Graph showing variation of glycogen synthase activity with UDP-glucose concentration](http://www.jbc.org/)

**Table II**

| Enzyme sample | UDP-Glucose varied | Glucose-6-P varied |
|---------------|---------------------|---------------------|
| No glucose-6-P | Vmax, S0.5 | Vmax, S0.5 |
| 5 mM glucose-6-P | 0.2 mM UDP-glucose | 0.2 mM UDP-glucose |

| Vmax | S0.5 | 5 mM | S0.5 |
|------|------|------|------|
| µmol/min | mM | µmol/min | mM |
| 1 | 38 | 1.3 | 32 | 65 |
| 2 | 34 | 0.75 | 31 | 56 |
| 3 | 30 | 0.67 | 25 | 45 |
| 4 | 30 | 1.3 | 38 | 67 |
| 5 | 36 | 42 | 35 | 78 |
| 6 | 27 | 61 | 32 | 74 |
| 7 | 35 | (6500) | 33 | 150 |
| 8 | 36 | (9100) | 43 | 250 |
| 9 | 39 | (5500) | 39 | 180 |

*Vmax was calculated by extrapolation of Eadie-Hofstee plots such as in Fig. 1. Values of S0.5, the UDP-glucose concentration required for half-maximal rate, were calculated from Hill plots as in Fig. 2.*

**Kinetic parameters for UDP-glucose in the presence of 5 mM glucose-6-P.** Both Vmax and S0.5 were estimated from Eadie-Hofstee plots such as in Fig. 4.

*The Mmax for glucose-6-P, concentration for half-maximal activation, was determined from Hill plots of data such as those in Fig. 5.*

*Not determined.

The ordinate of Hill plots arranged for enzyme kinetic experiments, log(u/Vu) = m log(v/Vu) + m log(Vu), tends to log(u/Vu) as v becomes very small compared with Vmax. In this situation, the selection of Vmax will contribute little to the evaluation of the slope of the Hill plot. Because the observed reaction rates for Samples 7 to 9 were still increasing steadily at the highest substrate levels used (as exemplified by the shallow slope of the Eadie-Hofstee plot in Fig. 1), we assumed the velocities to be small compared with Vmax and have used Vn as a somewhat arbitrary approximation to Vmax. The term log(u/Vn) then has been used as the ordinate of those plots in Fig. 2. Values of S0.5 derived from Hill plots will of course depend strongly on the selection of Vmax, and for this reason we have not emphasized the reliability of the estimates of S0.5 for Samples 7 to 9. It is sufficient for our general argument to point out that the values were certainly very large.
UDP-glucose saturation curves, as judged by the Hill plots, appeared not to depend on phosphorylation state. By contrast, the values of $S_{a.s}$ derived from such plots showed a very clear dependence on phosphorylation state, increasing with increasing phosphate content (Table II). For the three most phosphorylated samples, precise knowledge of $S_{a.s}$ values was not possible, although they were certainly much higher than 25 mM. If, by analogy to enzyme of lower phosphorylation, the maximal rates were in fact close to those in the presence of glucose-6-P, the high values for $S_{a.s}$ in Table II would be calculable. In spite of these difficulties the important point is nonetheless clear, namely that the phosphorylation of glycogen synthase had a very marked influence on the $S_{a.s}$ for UDP-glucose, while the Hill coefficient, and the $V_{max}$, where measurable, were less influenced.

Effect of NaCl Concentration on Glycogen Synthase Activity—Interpretation of the deviations from Michaelis-Menten kinetics is in any event not straightforward, and is further complicated here by the fact that the highest substrate levels used significantly altered the ionic strength of the reaction mixture. Experiments to show the effect of salt concentration on reaction rate are shown for enzyme of high and low phosphorylation in Fig. 3. For both enzymes, increasing NaCl concentration caused a decrease in activity, although the more phosphorylated enzyme was more sensitive. Glucose-6-P, 5 mM, afforded protection to this decrease in activity. The influence of salt concentration was such that, for the more phosphorylated enzymes, the rates measured at high UDP-glucose concentrations were probably underestimates, and this may have contributed to the shape of the UDP-glucose saturation curves. With enzyme of low phosphorylation, however, this is unlikely, judging from Fig. 3. Thus, we feel that the observed anomalies in the kinetic behavior of glycogen synthase with respect to UDP-glucose did not result primarily from changes in salt concentration.

Another explanation for non-Michaelis-Menten kinetic behavior would be the occurrence of UDP-glucose-dependent changes in the association state of glycogen synthase. Such behavior should be detected as a variation in kinetic properties with enzyme concentration. For a glycogen synthase sample of somewhat intermediate phosphorylation state (Sample 4), little change in the kinetics with respect to UDP-glucose was observed over an 8-fold range of enzyme concentration. We therefore have no evidence for this possibility.

Kinetic Behavior of Glycogen Synthase with Respect to Glucose-6-P—Glucose-6-P is a well known activator of glycogen synthase from many sources (1), and it was of interest to study this activation as a function of phosphorylation state. In the presence of 5 mM glucose-6-P, the deviations from Michaelis-Menten kinetics for the variation of UDP-glucose concentration were virtually abolished (Fig. 4), leading to Hill slopes close to unity, irrespective of phosphorylation state. Inhibition of the enzyme at high UDP-glucose levels was seen for enzyme of low phosphate content (Fig. 4). For all enzyme samples, the $S_{a.s}$ for UDP-glucose was greatly reduced by glucose-6-P, the greatest decrease occurring with enzyme of high phosphate content (Table II). On the other hand, there appeared to be little evidence for such a strong effect of glucose-6-P on the $V_{max}$, at least for Samples 1 to 6, where comparisons could be made with the $V_{max}$ in the absence of activator (Table II). The $S_{a.s}$ for UDP-glucose increased by a factor of 3 in passing from enzyme of low to high phosphorylation state, but the variation of $S_{a.s}$ with phosphate content of the enzyme was much less than in the absence of glucose-6-P (Table II).

At nonsaturating UDP-glucose concentrations glucose-6-P can activate glycogen synthase of any phosphorylation state. A substrate concentration of 0.2 mM was selected to study the dependence of this activation on glucose-6-P concentration (Fig. 5). Slight inhibition at high glucose-6-P levels was consistently seen for enzyme of low phosphorylation (Samples 1 to 5). As the phosphorylation state of the enzyme increased, there was a tendency for the relation between the velocity increase caused by the sugar phosphate and its concentration to become more sigmoid (Samples 6 to 9), as evidenced by Eadie-Hofstee plots concave to the origin (Fig. 5). The Hill plots in such cases were generally nonlinear, with slopes varying from 2 at low glucose-6-P to unity at high concentration. When UDP-glucose was increased to 1 mM, the $M_{a.s}$ for glucose-6-P was reduced (Table II), but the shape of the saturation curves was little altered (not shown). Values of $M_{a.s}$ for glucose-6-P, estimated from Hill plots are listed in Table II. This parameter varied continuously with the phosphorylation state of the enzyme.
state, and, in fact, changed by almost 3 orders of magnitude in passing from the least to the most phosphorylated enzyme. Increasing phosphorylation of glycogen synthase has usually been regarded as increasing the susceptibility of the enzyme to activation by glucose-6-P, which may seem initially rather opposed to the large increase in $M_{n}$, for glucose-6-P reported here. The parameter $M_{n}$, however, relates only to the increase in rate above that in the absence of activator. Thus, for highly phosphorylated enzyme (D form), exhibiting a very low rate without activator, even a very low saturation with respect to glucose-6-P can cause a manifold increase in velocity. A similar absolute increase in rate for the nonphosphorylated enzyme (I form) could represent only a small fractional increase over the nonactivated rate.

**DISCUSSION**

The rationale for these studies was to obtain purified samples of rabbit muscle glycogen synthase that were phosphorylated to varying degrees. This allowed comparison of the chemically determined alkali-labile phosphate content with some enzymic properties and enabled a more detailed description of the effect of phosphorylation on glycogen synthase than is possible by comparing only the extreme (I and D) forms of the enzyme. A basic assumption in this investigation has been the validity of comparing the different enzyme samples described. In justification are the following points. The enzyme samples appeared to be almost homogeneous when analyzed by gel electrophoresis, and had similar specific activities when assayed under standard saturating conditions (Table I). The enzymes were prepared by the same basic purification procedure, save for the adjustments to produce samples of different phosphorylation state. The reproducibility of column elution patterns and yields was good. Most importantly perhaps, the conditions for phosphorylation were comparable (with the single exception of Sample 6, to which purified protein kinase had been added). We feel then that these glycogen synthase samples could be compared constructively on the basis of their alkali-labile phosphate content. It was in fact one of the most striking features of this work that enzymic properties varied monotonically with the phosphate content.

The analyses of alkali-labile phosphate for the various enzyme preparations indicated that the I form could contain as little as 0.27 mol of phosphate/85,000 g. The D form could contain up to 3.5 mol/subunit. We also feel that the determina-

**FIG. 4.** Effect of glucose-6-P on glycogen synthase kinetics with varied UDP-glucose. The activity of glycogen synthase (Samples 9 (•) and 8 (○)) was measured as described under "Experimental Procedure" in the presence of 5 mM glucose-6-P and [U-14C]UDP-glucose in the range 25 µM to 25 mM. The amount of enzyme per assay was 15 and 24 ng for Samples 2 and 8, respectively.

**FIG. 5.** Activation of glycogen synthase by glucose-6-P. Glycogen synthase activity was measured as described under "Experimental Procedure" with 0.2 mM UDP-glucose (8500 cpm/nmol) and glucose-6-P as indicated: enzyme Samples 3 (•), 5 µM to 1 mM; 6 (○) and 7 (△), 5 µM to 10 mM; and 9 (□), 50 µM to 10 mM. From 17 to 30 ng per assay were used. The results are shown as Eadie-Hofstee plots using the velocity increase, $v_{o}$, caused by glucose-6-P (activated rate minus rate in the absence of sugar phosphate) in place of velocity. The rates for the different samples in the absence of glucose-6-P were 8.2 units/mg (Sample 3), 0.24 units/mg (Sample 6), and 0.0 units/mg (Samples 7 and 9).
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to deviate least from Michaelis-Menten kinetics. The Hill slope, m, is an index of the extent of the departure from hyperbolic kinetics, so that the results here indicated little change in the nature of the kinetic behavior for UDP-glucose in passing from a minimally to a highly phosphorylated enzyme. We also know that in the presence of saturating glucose-6-P, all the UDP-glucose sites behaved as though they were identical, since hyperbolic kinetics were found. The final observation relevant to these considerations is the finding of positive cooperativity with respect to glucose-6-P activation, as has been noted previously (20, 26, 27). This is difficult to accommodate on a model of heterogeneous sites. On balance, the results here can be economically explained if glycogen synthase exhibited subunit interactions, but other explanations cannot be rigorously excluded.

Irrespective of the detailed interpretation of the shapes of the kinetic curves, it is clear that the properties of glycogen synthase with respect to variation of both UDP-glucose and glucose-6-P concentrations varied extensively with the phosphorylation state of the enzyme. The $M_{ss}$ for glucose-6-P, which is a well defined parameter in an operational sense varied by 800 fold over the phosphorylation range studied. The $S_{ss}$ for UDP-glucose, though poorly defined for the three most phosphorylated samples (Samples 7 to 9), nonetheless showed considerable variation as a function of the alkali-labile phosphate content. With both parameters, the greatest change occurred above 2 phosphates/subunit. For enzyme Samples 1 to 6 neither phosphorylation state nor the presence of 5 mM glucose-6-P greatly altered the $V_{max}$. It has generally been suggested that glucose-6-P acts on muscle glycogen synthase by altering the $V_{max}$ of the phosphorylated D form (22, 28, 29), although in liver this question is less certain (30-33). It is not obvious why the results here differ from earlier studies with the muscle enzyme, although we point out that the evaluation of $V_{max}$ may depend on the substrate range used (Fig. 2). For Samples 7 to 9, where evaluation of $V_{max}$ in the absence of glucose-6-P was not possible, effects on $V_{max}$ were unknown, but phosphorylation or glucose-6-P binding had large effects on the apparent affinity for UDP-glucose. Thus, we envisage an enzyme in which the pronounced effects of phosphorylation and glucose-6-P on enzymic activity are mediated primarily through correspondingly pronounced effects on the apparent affinity of the enzyme for its substrate, UDP-glucose.

As preface to a discussion of the multiple phosphorylation of the glycogen synthase subunit in relation to the enzymic properties reported here, a recent investigation of Cohen et al. (34) is of interest. These workers demonstrated, using purified cAMP-dependent protein kinase from rabbit muscle, that, of 22 proteins tested, only phosphorylase kinase (α and β subunits), glycogen synthase, and histone F1 could be phosphorylated to any significant extent (greater than 0.05% compared with the β subunit of phosphorylase kinase). Under their conditions, there was a high degree of specificity for phosphorylation. It therefore seems a reasonable working assumption that the observed multiple phosphorylation of the glycogen synthase subunit does not represent the gratuitous introduction of phosphate residues. It follows then to ask whether the present report contains any evidence for function of the different phosphorylation sites. We can say unequivocally that, with the phosphorylating conditions used, a detectable change in glycogen synthase properties was observed as up to 3.5 phosphates/55,000 molecular weight subunit were introduced, and in fact such changes were most pronounced after about 2 phosphates had been incorporated. In a recent paper (5), Soderling contended, on the basis of enzyme activities measured in the presence and absence of glucose-6-P, that complete conversion of glycogen synthase was effected by the introduction of 2 mol of 32P/90,000 g of enzyme. The reason for the disparity between his results and our alkali-labile phosphate determinations is not entirely clear, although differences in phosphorylating conditions may be important. We would point out, however, that %I activity is a useful but not necessarily the most sensitive parameter by which to monitor "conversion." For example, at a very low substrate concentration, a 10-fold increase in $K_c$ causes a 10-fold decrease in an enzymic rate, although both rates may be very close to zero. In our studies, we have clear evidence for further kinetic changes at alkali-labile phosphate contents greater than 2.

In studies of the phosphorylation of glycogen synthase, we as others, have measured the average number of phosphate residues per enzyme subunit (although in 32P incorporation experiments the starting phosphate content is not usually

![Fig. 6. Two site model for multiple phosphorylation of an enzyme. Two sites of phosphorylation, designated as a circle and a square, are assumed per enzyme molecule. In A, both are assumed to be occupied with equal probability. The curves show the dependence of the fraction of given classes of enzyme species on the average phosphorylation state. I, all enzyme molecules with at least 1 phosphate per molecule; II, enzyme molecules with one particular site, the circle, occupied regardless of the state of the other site. A similar curve would result if the other site was considered. III, enzyme molecules with both sites phosphorylated. In B, phosphorylation probability is assumed unequal, the circular site being phosphorylated with 10 times the probability of the square site. Once the fast site is saturated, all subsequent increases in average phosphorylation were assumed to fill the slower, square site. Curve I then shows the variation in fractional concentration of enzyme with the faster, circular site filled as a function of average phosphorylation. Curve II is analogous for the slower, square site.](http://www.jbc.org)
known). Clearly, the relationship between the level of phosphorylation at a specific site and the average phosphorylation could be quite complex. From the work presented here, it is evident that the behavior of the enzyme samples of intermediate phosphorylation was not deducible from the appropriate combination, on the basis of phosphate content, of the properties of the extreme forms of the enzyme. Several explanations for this nonadditivity are possible, some of which are illustrated by the model of Fig. 6. Here, two phosphorylation sites per enzyme molecule have been assumed, and the relative concentrations of various phosphorylated species of enzyme are related to the average phosphorylation state. A property such as the reaction rate under fixed conditions will be proportional to the concentration of the relevant enzyme species. With equal probability of phosphorylation at each site (Fig. 6A), a nonlinear relationship between an enzymic property can only arise if both sites are relevant to changing this property. This could be the interaction of two identical sites (curve III) or the equal but independent effect of the two sites (curve I). With unequal phosphorylation probabilities at the different sites (Fig. 6B), such nonlinearity can result even if only one site is relevant to changing the property of interest. The data presented here cannot distinguish these possibilities, or others not considered, and the model is presented only to emphasize the sort of statistical interpretations necessitated for an enzyme subject to multiple phosphorylation.

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This point is in fact made by the results shown here although the representations of the data do not emphasize it. Also, in experiments not shown, velocities determined for mixtures of enzyme were those predicted from the individual action of the enzymes, not from the final average phosphate content.
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