Regulation of Rat Liver Glycogen Synthetase

EVIDENCE FOR A LYSYL RESIDUE ESSENTIAL FOR GLUCOSE 6-PHOSPHATE ACTIVATION*

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SUMMARY

Glycogen synthetase D was inactivated by treatment with trinitrobenzene sulphonate with the incorporation of 2.5 moles of trinitrophenyl group per mole of enzyme subunit. The sulphydryl content of the enzyme was unchanged during trinitrophenylation indicating that derivatization was through the amino group. Glucose-6-P and other phosphate activators of glycogen synthetase D offered protection against modification. Partially active, trinitrophenylated derivatives of the enzyme were characterized by a decreased $V_{max}$ without any change in the $K_m$ for the glucose-6-P or the $K_m$ for the substrate, UDP-glucose.

Pyridoxal-P was used to probe further these amino groups. As an inhibitor of glycogen synthetase D, pyridoxal-P showed competitive inhibition with respect to glucose-6-P. The unreactive analogues, pyridoxine-P and pyridoxamine-P, were weak activators of the enzyme by themselves, whereas pyridoxine-P was a competitive inhibitor with respect to glucose-6-P. The corresponding unphosphorylated forms of these analogues could not duplicate these effects, providing evidence that the site of action of pyridoxal-P was at or near the glucose-6-P-binding site involved in the activation of the enzyme.

Reduction of the Schiff's base complex between pyridoxal-P and glycogen synthetase D by NaBH₄ led to the irreversible inactivation of the enzyme. Inactivation was the result of the incorporation of 1.0 mole of phosphopyridoxal group per mole of enzyme subunit through the ε-amino group of a lysyl residue. Glucose-6-P offered some protection against inactivation. Reduction of pyridoxine-P or pyridoxamine-P-treated enzyme had no effect on enzyme activity.

These results suggest that there is a lysyl residue located at or near the glucose-6-P-binding site which is essential for the activation of glycogen synthetase D by glucose-6-P.

Glycogen synthetase (ruidine diphosphate glucose:α-1,4-glucosyltransferase, EC 2.4.1.11) is the rate-limiting enzyme for glycogen synthesis and is present in most tissues in two interconvertible forms. The dephosphorylated enzyme (glycogen synthetase I) is independent of glucose-6-P, whereas the phosphorylated enzyme (glycogen synthetase D) requires glucose-6-P for activity (1, 2). Besides the difference in glucose-6-P requirement, the two forms are differentially subjected to allosteric regulation by a variety of cellular metabolites (3-6). Amplification of this allosteric control is achieved by enzymatic interconversion between synthetase I and D which in turn may be influenced by various hormonal (2, 7) and metabolic states (8, 9). These mechanisms taken together exert a very sensitive control over the synthesis of glycogen.

A number of other phosphate esters including P₃ could partially substitute for glucose-6-P in activating glycogen synthetase D, suggesting that the binding site for these activators is cationic (10, 11). In this paper, we utilized two amino group-specific reagents to demonstrate the presence of an essential lysyl residue in glycogen synthetase D which is required for the activation of the enzyme by glucose-6-P.

MATERIALS AND METHODS

Chemicals—UDP-[U-¹⁴C]glucose (specific activity 228 mCi per mmole) was purchased from Schwartz-Mann. TNBS* was a product of the Aldrich Chemical Co. Pyridoxal-HCl and pyridoxal-P were obtained from Pierce Chemical Co. Pyridoxine and pyridoxamine-P-HCl were purchased from Nutritional Biochemical Corp. Pyridoxine-P was a product of Eastern Chemical Co. All other chemicals and biochemicals were purchased from Sigma Chemical Co. ε-Pyridoxyllysine was prepared by NaBH₄ reduction of the Schiff's base complex between ε-benzyloxy-carbonyl-L-lysine and pyridoxal-HCl followed by removal of the benzoyloxycarbonyl-protecting group by acid hydrolysis. The product was characterized as described by Forrey et al. (12).

Glycogen Synthetase D—Homogeneous, glycogen-free glycogen synthetase D was prepared from rat liver using the p-hydroxy-mercuribenzoate dissociation method of McVerry (13) which will be described elsewhere. The studies reported in this paper were all performed with the reconstituted glycogen-glycogen synthetase D complex containing 25.5 μg of 80,000 molecular weight subunit per mg of glycogen prepared as described previously (11). Glycogen synthetase D was assayed as usual (11), except in some cases where sodium glycicylglycinate buffer was replaced by MOPS-HCl. The sulphydryl content was determined by DTNB (11).

* The abbreviations used are: TNBS, 2,4,6-trinitrobenzene sulphonate; MOPS, morpholinopropane sulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); pyridoxal-P, pyridoxal-5'-P, and likewise for the other analogues.

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Kinetic Analysis—The activation of glycogen synthetase D by glucose-6-P is a cooperative phenomenon characterized by a Hill coefficient (n) of 2.5 under our assay conditions. As a result, double reciprocal plots of initial velocity versus glucose-6-P concentration yield concave upward curves from which it is difficult to extrapolate values for $K_v$. From the observation by Atkinson et al. (14) that the Hill equation written in terms of initial velocity is a slightly generalized version of the Michaelis equation, it was found that our data could be fitted to the equation

$$v = \frac{V_{\text{max}} [\text{glucose-6-P}]}{K_v + [\text{glucose-6-P}]}$$

and plots of $1/v$ versus $1/[\text{glucose-6-P}]$ were linear over the range of glucose-6-P concentrations used in the experiments reported in this paper. This type of double reciprocal plot was used to evaluate the effects of TNBS and pyridoxal-P on the $K_v$ for glucose-6-P. This was not necessary in determining the $K_v$ for UDP-glucose for which the data could be plotted directly as a Lineweaver-Burk plot. All initial velocities were expressed as micromoles of glucose incorporated into glycogen per min.

Inactivation by Trinitrobenzene Sulfonate—A typical inactivation mixture contained 7.5 amoles of MOPS-HCl, pH 8.0, 0.75 mM NaF, 5 amoles of glucose-6-P, and 1.05 amoles of glycogen synthetase D in a final volume of 0.25 ml. The reaction was initiated by the addition of various concentrations of TNBS followed by incubation at 37°C. Aliquots (5 μl) were removed from the inactivation mixture at various times, and activity was determined by the standard assay procedure. TNBS-4-fold dilution of these samples into the glycelycine buffer used in the assay effectively terminated the inactivation process.

Partially active, trinitrophenylated derivatives of glycogen synthetase D were prepared by incubating inactivation mixtures for various times after which the reaction was terminated by the addition of lysine in 16-fold excess over the concentration of TNBS. The mixtures were then quickly chilled and desalted on a column (0.7 x 3.5 cm) of Sephadex G-25 (fine), equilibrated, and eluted at 0-4°C with 30 mM MOPS-HCl, pH 8.0, 3.0 mM NaF, and 10% glycerol.

Quantitation of Trinitrophenyl Group Incorporation—The extent of trinitrophenylation was monitored directly in the inactivation mixture at 317 nm assuming a molar extinction coefficient of 10,800 for the trinitrophenyl group (15, 16). All spectrophotometric measurements were made in a Cary 14 spectrophotometer with an expanded scale recorder (0 to 0.1 absorbance full scale) against an appropriate blank.

Inactivation by Pyridoxal-P—All procedures involving pyridoxal-P or its analogues were performed at very low levels of room light or in the dark. Pyridoxal-P was used either directly in the assay mixture as an inhibitor of glycogen synthetase D, or in an inactivation mixture as for TNBS followed by NaBH₄ reduction and assay. When used directly, sodium glycelycinate buffer in the assay mixture was replaced by MOPS-HCl (5). For NaBH₄ reduction, the inactivation mixture described for TNBS was used. The reaction was initiated by the addition of pyridoxal-P and incubated for 5 min at 37°C. The mixture was quickly chilled, 2.5 μl of octyl alcohol were added followed by a fresh cold water solution of NaBH₄ in 100-fold excess over the concentration of pyridoxal-P present. Reduction proceeded for 10 min at 0-4°C after which a 5-μl aliquot was removed for assay by the standard procedure.

Partially active derivatives of glycogen synthetase D were prepared by incubating various concentrations of pyridoxal-P with enzyme followed by NaBH₄ reduction and gel filtration as described for the TNBS derivatives.

Quantitation of Phosphopyridoxyl Group Incorporation—The extent of incorporation of pyridoxal-P into the enzyme after NaBH₄ reduction was determined directly on the inactivation mixture after exhaustive dialysis against 50 mM sodium phosphate, pH 7.0, 5 mM EDTA. A molar extinction coefficient at 325 nm of 10,966 for t-phosphopyridoxyllysine was used to quantitate the mixture after exhaustive dialysis against 50 mM sodium phosphate, pH 7.0, 5 mM EDTA. A molar extinction coefficient at 325 nm of 10,966 for t-phosphopyridoxyllysine was used to quantitate the concentration of pyridoxal-P present. Reduction proceeded for 10 min at 0-4°C after which a 5-μl aliquot was removed for assay by the standard procedure.

RESULTS

Trinitrobenzene Sulfonate

TNBS is generally considered to be an amino-specific reagent although at high concentrations it will react with other residues, particularly thiol groups (17). The enzyme is quite susceptible to inhibition by TNBS as shown in Fig. 1. The TNBS to enzyme ratio varied from 2.4 for 10 μM TNBS to 24 for 100 μM TNBS. In all cases, the reaction appears to be pseudo-first order with respect to TNBS with apparent first order rate constants (k₁) from 9.9 x 10⁻³ min⁻¹ for 10 μM TNBS to 0.017 min⁻¹ for 100 μM TNBS. A plot of k₁ versus the concentration of TNBS was also linear and yielded a second order rate constant for TNBS in activation of 1.08 x 10⁻³ μM⁻¹ min⁻¹.

Protection by Glucose-6-P—Since glycogen synthetase D has an absolute requirement for glucose-6-P, it was of interest to determine whether this activator could protect against TNBS inactivation. These results are shown in Fig. 2 for inactivation by 50 μM TNBS. The apparent first order rate constant for inactivation of unprotected enzyme was 0.047 min⁻¹. This value was reduced by increasing concentrations of glucose-6-P up to 10.0 mM which completely protected against inactivation. P₄ can substitute for glucose-6-P in activating glycogen synthetase D.

![Fig. 1. TNBS inactivation of glycogen synthetase D. Glycogen synthetase D in the standard inactivation mixture in the absence of glucose-6-P was incubated with various concentrations of TNBS at 37°C. At the appropriate times, a 5-μl aliquot was removed for assay. Percent activity was calculated on the basis of activity obtained in the absence of TNBS. The concentrations of TNBS were: none ( ), 10 μM ( ), 25 μM ( ), 50 μM ( ), and 100 μM ( ).](http://www.jbc.org/)

1 M. J. Ernst and K.-H. Kim, unpublished experiments.

2 $K_v$ for glucose-6-P is the concentration required for one-half maximum velocity.
phenylation begins to increase faster than enzyme inactivation. Coincidentally, this decrease in sulfhydryl content takes place at the same stage of inactivation at which trinitrophenylation increases faster than inactivation. The loss of 1.5 groups upon a 7% further inactivation of the enzyme. The protection experiments were consistent with the ability of these compounds to activate glycogen synthetase D and thus were the first indication that trinitrophenylation may interfere with the activation of glycogen synthetase D by glucose-6-P.

Extent of Trinitrophenylation—It was important to determine the relationship between trinitrophenylation and enzyme inactivation and in particular to eliminate the possibility that derivitization involved the sulfhydryl groups of glycogen synthetase D which are essential for enzyme activity (10, 11). The protection offered by P$_i$ against TNBS inactivation is also shown in Fig. 2. P$_i$ did reduce the rate constant for inactivation by TNBS but the protection was not as great as that obtained at equimolar concentrations of glucose-6-P. When PP$_i$ was substituted for P$_i$, there was no protection, and inactivation proceeded with a rate constant for all PP$_i$ concentrations tested of 0.049 ± 0.005 min$^{-1}$ which was identical with unprotected enzyme. The protection experiments were consistent with the ability of these compounds to activate glycogen synthetase D and thus were the first indication that trinitrophenylation may interfere with the activation of glycogen synthetase D by glucose-6-P.

Glycogen synthetase D in a 3.0-ml standard inactivation mixture was treated with 50 μM TNBS in the absence of glucose-6-P at 37°C. The absorbance at 367 nm was monitored continuously against an appropriate protein blank. At various times, a 5-μl aliquot was quickly removed for assay while the absorbance at 367 nm was noted. Per cent activity was based on the activity obtained prior to the addition of TNBS after correction for the volume change. The moles of trinitrophenyl group incorporated per mole of enzyme subunit were calculated as described under "Materials and Methods."

### Table I

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<th>Percent activity</th>
<th>Sulfhydryl group/subunit</th>
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<td>100</td>
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<tr>
<td>51</td>
<td>8.3</td>
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The only effect was a reduction in $V_{max}$ that was proportional to the extent of inactivation of the enzyme. Fig. 5 is a Line-
FIG. 4. Effect of trinitrophenylation on the kinetic properties of glycogen synthetase D with respect to glucose-6-P. Partially active, trinitrophenylated derivatives of glycogen synthetase D were prepared as described under "Materials and Methods." The kinetic properties of these derivatives were then determined in the standard assay mixture (11) containing 4.0 mM UDP-glucose and a variable concentration of glucose-6-P. The data were plotted as a double reciprocal plot of initial velocity versus [glucose-6-P] (n = 2.5) for 100% (○), 63% (●), and 34% (▲) active enzyme.

FIG. 5. Effect of trinitrophenylation of the kinetic properties of glycogen synthetase D with respect to UDP-glucose. The procedure was the same as for Fig. 4 except the assay mixture contained 5.0 mM glucose-6-P and a variable concentration of UDP-glucose. The data were plotted as a Lineweaver-Burk plot for 100% (○), 63% (●), and 34% (▲) active enzyme.

Pyridoxal-P

The irreversible combination of TNBS with amino groups of glycogen synthetase D prevented studies on the reversal of the inhibition by the substrate and activator which could more closely define the role of these residues in enzyme activity. Since reaction of an amino group with an aldehyde is reversible, we chose to use pyridoxal-P to probe further the amino groups of glycogen synthetase D.

Enzyme Inhibition by Pyridoxal-P—It was found that pyridoxal-P reversibly inhibited glycogen synthetase when added directly to the assay mixture in the presence of low concentrations of glucose-6-P. The inhibited glycogen synthetase D-pyridoxal-P complex was formed in less than 5 s, and this level of inhibition was not increased further by preincubation prior to assay. The inhibition was reversed by dilution or by the addition of excess glucose-6-P. The concentration dependence for a pyridoxal-P inhibition of the enzyme in the presence of 1.0 mM glucose-6-P is shown in Fig. 6. It should be noted that pyridoxal can also inhibit glycogen synthetase D, but comparable inhibition was achieved only with concentrations 250- to 500-fold greater than the corresponding phosphorylated form. This indicated that the phosphate group enhanced the reactivity of this reagent.

After establishing the conditions necessary for inhibition of glycogen synthetase D by pyridoxal-P, the effects of UDP-glucose and glucose-6-P on inhibition were investigated. Fig. 7 is a Lineweaver-Burk plot showing the effect of UDP-glucose on pyridoxal-P inhibition of glycogen synthetase D. In this case, inhibition was complex noncompetitive with changes in both the K_m for UDP-glucose and V_max. However, when the effect of glucose-6-P on pyridoxal-P inhibition was examined (Fig. 8), it was found that pyridoxal-P was a true competitive inhibitor with respect to glucose-6-P. This result provided the first evidence that pyridoxal-P was inhibiting glycogen synthetase D by interfering with the glucose-6-P activation of the enzyme, and that this inhibition could be overcome by high concentrations of glucose-6-P.

Further evidence that pyridoxal-P was interfering with glucose-6-P activation was provided by studies with the analogues pyridoxine-P and pyridoxamine-P. In pyridoxine-P, the reactive aldehyde group is reduced to an alcohol, whereas in pyridoxamine-P it is replaced by an amine. In either case, these analogues cannot form a Schiff's base and so they should not inhibit glycogen synthetase D. Table II shows that in fact pyridoxine-P and pyridoxamine-P were actually weak.

![Pyridoxal-P inhibition of glycogen synthetase D.](image-url)
TABLE III

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<td>Pyridoxamine-P</td>
<td>9</td>
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Fig. 7. Kinetic properties of pyridoxal-P-inhibited glycogen synthetase D with respect to UDP-glucose. Glycogen synthetase D was assayed in the presence of several concentrations of pyridoxal-P. The standard assay mixture contained MOPS-HCl, pH 8.0, 1.0 mM glucose-6-P, and a variable concentration of UDP-glucose. The data were plotted in the form of a Lineweaver-Burk plot. The concentrations of pyridoxal-P were: 0.0 mM ( ), 0.2 mM ( ), 0.4 mM ( ), 1.0 mM ( ), 2.0 mM ( ), and 4.0 mM ( ).

Fig. 8. Kinetic properties of pyridoxal-P-inhibited glycogen synthetase D with respect to glucose-6-P. The procedure was the same as for Fig. 7 except the assay mixture contained 4.0 mM UDP-glucose and a variable concentration of glucose-6-P. The concentrations of pyridoxal-P were the same as Fig. 7. The data were plotted as a double reciprocal plot of initial velocity versus [glucose-6-P]$^n$ with $n = 2.5$.

activators of the enzyme when tested by themselves with 15% and 9% activity, respectively, compared to glucose-6-P. The corresponding unphosphorylated forms did not activate the enzyme at all. This indicated that the phosphate group of these analogues was responsible for the activation of the enzyme, and thus directed these pyridoxyl analogues to the activation site. Evidence that this site may be the glucose-6-P binding site was provided when pyridoxine-P and glucose-6-P were tested together for activation (Table III). In the presence of saturating glucose-6-P (20 mM), pyridoxine-P caused a slight decrease in enzyme activity compared to 20 mM glucose-6-P alone. However, in the presence of a subsaturating concentration of glucose-6-P (1.0 mM), 20 mM pyridoxine-P reduced the enzyme activity by 36% when compared to 1.0 mM glucose-6-P alone. When the effect of increasing concentrations of pyridoxine-P on glucose-6-P activation of the enzyme was examined (Fig. 9), pyridoxine-P behaved as a true competitive inhibitor with respect to glucose-6-P. Thus, pyridoxine-P, which by itself is a weak activator of the enzyme, will compete with glucose-6-P when it serves as the activator of glycogen synthetase D. Since pyridoxine did not show these effects, it appears that pyridoxine-P is preferentially directed by its phosphate group to a site on the enzyme which can activate glycogen synthetase D, and this site may be the glucose-6-P binding site.

TABLE IV

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<tr>
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<td>93</td>
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Enzyme Inactivation by Pyridoxal-P—If pyridoxal-P inhibits glycogen synthetase D by formation of a Schiff's base with lysyl residues on the enzyme, then reduction of this complex to the secondary amine should lead to irreversible modification of the enzyme. NaBH₄ reduction of pyridoxal-P-treated glycogen synthetase D resulted in the formation of a stable, inactivated enzyme whose activity could not be restored by dilution or high concentrations of glucose-6-P, as was the case before reduction. These results are summarized in Table IV. In these experiments, glycogen synthetase D was treated with pyridoxal P followed by NaBH₄ reduction and a 100-fold dilution for assay. Although 5.0 mM pyridoxal-P in the inactivation mixture was more than enough for complete enzyme inhibition, dilution of the sample for assay in the presence of glucose-6-P before reduction completely dissociated the Schiff's base complex leading to restoration of 97% of the enzyme activity. However, when
Glycogen synthetase D was incubated in the standard inactivation mixture at 37°C for 5 min with 3.0 mM pyridoxal-P or one of its analogues in the presence or absence of 20 mM glucose-6-P. The mixtures were then either diluted and assayed directly in the standard assay mixture, or reduced with NaBH₄ and then diluted and assayed as described under "Materials and Methods." Percent activity was calculated on the basis of the activity of untreated enzyme.

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<td>NaBH₄</td>
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<tr>
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<tr>
<td>Pyridoxine-P ± glucose-6-P</td>
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Fig. 9. Effect of pyridoxine-P on the kinetic properties of glycogen synthetase D with respect to glucose-6-P. The conditions were the same as in Fig. 8 except pyridoxine-P replaced pyridoxal-P. The concentrations of pyridoxine-P were: 0 ( ), 10 mM ( ), 20 mM ( ), and 40 mM ( ). The data were plotted as a double reciprocal plot of initial velocity versus [glucose-6-P] with n = 2.5.

this inactivation mixture was reduced prior to dilution and assay, 95% of the enzyme activity was abolished. Glucose-6-P offered some protection against this inactivation since the enzyme retained 22% of its activity when 20 mM glucose-6-P was included in the inactivation mixture. Reduction of pyridoxine-P or pyridoxamine-P-treated glycogen synthetase D, even in the absence of glucose-6-P, failed to inactivate the enzyme, providing further evidence that inactivation by pyridoxal-P was through Schiff's base formation since neither of these analogues contains the reactive aldehyde group. Glucose-6-P protection against inactivation was further studied by examining the concentration dependence of pyridoxal-P for inactivation after

NaBH₄ reduction. These results appear in Fig. 10. Significant protection was offered by 20 mM glucose-6-P which increased the concentration of pyridoxal-P required for 50% inactivation of the enzyme from 0.02 mM for unprotected enzyme to 0.89 mM.

Kinetic Properties of Pyridoxal-P-Modified Glycogen Synthetase D—It was felt that an investigation of the kinetic properties of derivitized glycogen synthetase D might be useful, particularly in comparison to TNBS-inactivated glycogen synthetase D and pyridoxal-P-inhibited glycogen synthetase D. Partially active derivatives of glycogen synthetase D were prepared and the effect of increasing concentrations of UDP-glucose and glucose-6-P on their kinetic properties was examined. Fig. 11 is a double reciprocal plot for glucose-6-P using 100%, 65%, and 30% active glycogen synthetase D. It was clear that modification had no effect on the Km for glucose-6-P, while Vₘₐₓ was reduced in proportion to the extent of inactivation of the enzyme. These results are identical with those obtained for TNBS inactivation of glycogen synthetase D (Fig. 4), but in complete contrast to those for pyridoxal-P inhibition of the enzyme (Fig. 8), which was competitive with respect to glucose-6-P. Fig. 12 is a Lineweaver-Burk plot for UDP-glucose also showing only a proportional reduction in Vₘₐₓ without any effect on the Km for UDP-glucose (0.45 mM). Once again, these results are identical with those obtained for TNBS inactivation (Fig. 5), but in contrast to these for pyridoxal-P inhibition (Fig. 7), which affected both the Vₘₐₓ and Km. Thus, when the Schiff's base between glycogen synthetase D and pyridoxal-P was reduced by NaBH₄, the inhibition pattern of pyridoxal-P changed from competitive to pure noncompetitive with respect to the activator, glucose-6-P, and from complex noncompetitive to pure noncompetitive with respect to the substrate, UDP-glucose.

Extent of Pyridoxal-P Modification and Isolation of ε-Pyridoxyllysine—Reduction of a Schiff's base involving pyridoxal-P to the secondary amine results in the appearance of a characteristic absorption peak at 325 nm (12). The absorption spectrum of the NaBH₄-reduced complex between glycogen synthetase D and pyridoxal-P showed such a peak which was not present in native enzyme, NaBH₄-reduced enzyme, or enzyme treated with pyridoxal-P but not reduced with NaBH₄. Using a molar
FIG. 11. Effect of pyridoxal-P modification on the kinetic properties of glycogen synthetase D with respect to glucose-6-P. Partially active, pyridoxylated derivatives of glycogen synthetase D were prepared as described under "Materials and Methods." The kinetic properties of these derivatives were then determined in the standard assay mixture containing 4.0 mM UDP-glucose and a variable concentration of glucose-6-P. The data were plotted as a double reciprocal plot of initial velocity versus [glucose-6-P] (n = 2.5) for 100% (○), 65% (□), and 30% (△) active enzyme.

FIG. 12. Effect of pyridoxal-P modification on the kinetic properties of glycogen synthetase D with respect to UDP-glucose. The procedure was the same as for Fig. 11 except the assay mixture contained 5.0 mM glucose-6-P and a variable concentration of glucose-6-P. The data were plotted as a Lineweaver-Burk plot for 100% (○), 65% (□), and 34% (△) active enzyme.

Fig. 13. Enzyme activity as a function of the extent of pyridoxal-P modification. Glycogen synthetase D was incubated in a 3.0-ml inactivation mixture containing 1.0 mM glucose-6-P at 37° for 5 min in the presence of several concentrations of pyridoxal-P. These mixtures were then reduced with NaBH₄, and assayed for activity. Per cent activity was based on the activity of enzyme that had been reduced by NaBH₄ in the absence of pyridoxal-P. The moles of phosphopyridoxyl group incorporated per enzyme subunit were determined as described under "Materials and Methods."

DISCUSSION

In this paper we have shown that glycogen synthetase D was inactivated by TNBS with the incorporation of 2.5 moles of trinitrophenyl group per mole of enzyme subunit. Partially active, trinitrophenylated enzyme was characterized by a decreased maximum velocity but with a $K_m$ for glucose-6-P and a $K_v$ for UDP-glucose identical with native enzyme. These results may be explained in at least two different ways. First, there may be an amino group either at the active site or at an allosteric site which is necessary for enzyme activity. Trinitrophenylation of this residue would block the catalytic activity of the enzyme, leading to a reduction in the maximum velocity of the reaction. Alternatively, since glycogen synthetase D has an absolute requirement for glucose-6-P, if there were an amino group either at the glucose-6-P binding site or at an allosteric site which is necessary for glucose-6-P binding, then modification of this residue would effectively eliminate the enzyme molecule from catalysis, again leading to a reduction in the maximum velocity of the reaction. In the former case, trinitrophenylation
may be an all-or-none process, or it may produce a mixture of enzyme molecules with variable $V_{\text{max}}$, although all species must still have the same $K_m$ and $K_v$ values as native enzyme. In the latter case, modification must be an all or none process with partially active derivatives of glycogen synthetase D composed of varying proportions of fully active and completely inactive enzyme molecules. In other words, trinitrophenylation must completely prevent glucose-6-P binding and not simply decrease the affinity of the binding site for the activator, since the $K_v$ for glucose-6-P is not affected by modification, and high concentrations of glucose-6-P cannot reverse inactivation by TNBS. Evidence that trinitrophenylation interferes with glucose-6-P activation of the enzyme comes from the observation that glucose-6-P could protect against inactivation as could P1, a weak activator of glycogen synthetase D by itself and a competitive inhibitor with respect to glucose-6-P (10). On the other hand, PP1, which could not activate the enzyme, failed to protect against inactivation.

In an attempt to distinguish between the two mechanisms of action for TNBS suggested above, we chose to use pyridoxal-P as another reagent to probe further the amino groups of glycogen synthetase D for a number of reasons. Besides its specificity (18), its reaction with amino groups via Schiff's base formation is a reversible process (19, 20), the Schiff's base can be reduced with NaBH4 leading to irreversible modification (21), the spectral properties permit quantitation of the number of pyridoxyl groups incorporated into the enzyme (12), various useful pyridoxal-P analogues are available (22), and most important, the phosphate group might serve to direct pyridoxal-P to the glucose-6-P binding site (23), which is known to bind a wide variety of phosphate esters (10, 11).

The evidence from the pyridoxal-P experiments favoring a lysyl residue essential for the binding of glucose-6-P by glycogen synthetase D is summarized below. Pyridoxal-P was a true competitive inhibitor with respect to glucose-6-P, suggesting that they both bind to the same site on the enzyme. On the other hand, the unreactive analogues, pyridoxine-P and pyridoxamine-P, were weak activators of glycogen synthetase D alone. Their corresponding unphosphorylated forms failed to activate the enzyme, demonstrating that activation was through the phosphate group, which in turn, directed these reagents to the activation site on the enzyme. Evidence that this was the glucose-6-P binding site was provided when pyridoxine-P and glucose-6-P were tested together for activation. Pyridoxine-P behaved as a competitive inhibitor with respect to glucose-6-P, suggesting that pyridoxine-P and, presumably, pyridoxal-P bind to the glucose-6-P site rather than to an allosteric site which would not be expected to mediate both activation and inhibition of the enzyme depending upon the pyridoxyl analogue bound there. The fact that the unphosphorylated analogue, pyridoxine, could not duplicate the effects described above demonstrated the phosphate group of the pyridoxal-P reagents served to direct them to a site which was indistinguishable from the glucose-6-P binding site. At this site, the phosphate group of the unreactive analogues was capable of activating the enzyme as do other phosphate esters while the reactive aldehyde of pyridoxal-P formed a Schiff's base complex which reversibly blocked activation by glucose-6-P. Reduction of the Schiff's base complex led to the irreversible inactivation of the enzyme and the incorporation of 1.0 mole of phosphopyridoxyl group per mole of enzyme subunit through the ε-amino group of a lysyl residue. This finding, together with the evidence favoring pyridoxal-P action at the glucose-6-P binding site, suggested that there was only one glucose-6-P binding site per enzyme subunit. Moreover, the kinetic properties of partially active, pyridoxal-P-modified glycogen synthetase D were identical with TNBS-modified enzyme, indicating that one of the 2.5 amino groups derivatized by TNBS during inactivation was probably the lysyl residue modified upon NaBH4 reduction of pyridoxal-P-treated enzyme.

The question of the role of this lysyl residue in glucose-6-P activation of glycogen synthetase D is difficult to answer. Since a small proportion of glucose-6-P exists as the free aldehyde in solution, an attempt was made to determine whether glucose-6-P activation of the enzyme was through a Schiff's base. However, NaBH4 reduction of glucose-6-P-treated glycogen synthetase D neither activated nor inhibited the enzyme when assayed in the absence or presence of glucose-6-P, respectively. It was noted before that a variety of phosphate esters can partially replace glucose-6-P in activating glycogen synthetase D probably by substituting for glucose-6-P at its binding site since they are also competitive inhibitors with respect to glucose-6-P (10, 24). Therefore, it is possible that the binding of glucose-6-P and other phosphate ester activators may involve the ionic interaction of their phosphate group with the lysyl residue at the glucose-6-P binding site, and modification of this residue would then prevent activation of the enzyme. Nevertheless, one cannot exclude the possibility that this lysyl residue is located near the glucose-6-P binding site and derivitization merely blocks access of the activator to this site.

One final point worth noting is that glycogen phosphorylase, the enzyme that catalyzes the reverse reaction of glycogen synthetase leading to glycogen degradation, has an absolute requirement for pyridoxal-P as a structural element rather than as a coenzyme (25, 26). Thus, changes in the intracellular level of free pyridoxal-P might exert a sensitive control over the metabolism of glycogen by regulating the enzyme required for its synthesis and the enzyme required for its degradation.

REFERENCES

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