The Regulation of Glycogen Phosphorylase $\alpha$ by Nucleotide Derivatives

KINETIC AND X-RAY CRYSTALLOGRAPHIC STUDIES*

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Initial reaction rate studies of rabbit muscle phosphorylase $\alpha$ (1,4-\alpha-glucan:orthophosphat$\alpha$-glucosyltransferase, EC 2.4.1.1) were carried out as a function of variable concentrations of nucleotide activators and derivatives. These kinetic data are correlated with the results of crystallographic binding experiments. Two separate binding sites for nucleotide are identified in the phosphorylase monomer. These sites are progressively saturated as the nucleotide concentration is raised and may be distinguished both functionally and physically.

The site having a stronger affinity for nucleotide corresponds to that previously reported to bind the allosteric activator AMP. It also binds the substrates inorganic phosphate, glucose 1-phosphate, and other phosphoryl compounds. The second site has weaker affinity for nucleotides, but binds nucleoside derivatives more tightly. Kinetic and crystallographic experiments indicate that binding of nucleotide derivatives at this second site (nucleoside site) inhibits phosphorylase activity via allosteric competition with glucose 1-phosphate. Both the positive and negative effector sites are distant from the active site. The former is about 30 Å removed, whereas the latter is about 10 Å away.

The virtual substrate 5-thio-\alpha-D-glucopyranosyl phosphate is a competitive inhibitor with respect to glucose 1-phosphate. Crystallographically, at low concentration (10 mM), binds to the enzyme at two sites per monomer. These are the AMP and glucose sites reported previously. In double ligand binding experiments, allosteric competition between inosine (bound at the nucleoside site, above) and 5-thio-\alpha-D-glucopyranosyl phosphate (bound at the glucose site) is demonstrated. This competition is consistent with the kinetic results and the conclusion that the latter glucose 1-phosphate site adjacent to the pyridoxal 5'-phosphate coenzyme provides the binding locus for substrate in the active site of phosphorylase.

Nucleosides and analogues, such as adenosine and caffeine, are effective at less than 1 mM concentration in preventing substrate binding at the active site, presumably by stabilizing an inactive conformer of the enzyme. The synergistic inhibition of phosphorylase $\alpha$ by glucose and ligands binding at the nucleoside site suggests that this inactive conformer may be the glucose "T" state (Helmreich, E., Michaelides, M. C., and Cori, C. F. (1967) Biochemistry 6, 3695-3710).

The effects of nucleotides on muscle glycogen phosphorylase (EC 2.4.1.1) have been discussed in the recent review of Graves and Wang (1). The requirement of phosphorylase $b$ for the activator AMP is well known (2-5). In addition, Lowry et al. (6) have demonstrated a possible physiological role for AMP activation with the $\alpha$ form of the enzyme. Other nucleotides, notably IMP, are also capable of activating phosphorylase $b$ (7-11). However, these activators differ from AMP, since they do not cause association of the enzyme to a tetramer in the absence of substrate (7, 10). This fact was exploited by Johnson et al. (12) to obtain tetragonal crystals of phosphorylase $b$ in the dimeric state, which were suitable for high resolution x-ray diffraction studies.

Recent reports from our laboratory (13, 14) have indicated that the allosteric activator AMP and substrate glucose-1-P may bind at the same site in phosphorylase $\alpha$. Such a result suggested that kinetic studies of nucleotide binding with respect to variable concentration of glucose-1-P should be undertaken to determine the consequences of this finding. Since IMP has never been reported to activate phosphorylase $\alpha$, our initial studies were with this nucleotide.

In the present communication, we describe the effects of nucleotide and nucleoside derivatives on phosphorylase $\alpha$. Kinetic and x-ray crystallographic methods were used in an attempt to assign both functional and structural roles for the sites associated with nucleotide and glucose-1-P binding. By the use of the virtual substrate 5-thio-D-glucose-1-P, a competitive inhibitor of phosphorylase $b$ activity (15), we also investigated glucose-1-P binding to crystals of phosphorylase $\alpha$. The kinetic effects of the various nucleotide derivatives with respect to glucose-1-P are correlated with crystallographic studies using this substrate analogue.

1 The abbreviations used are: glucose-1-P, $\alpha$-D-glucopyranose-1-phosphate; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; 5-thio-D-glucose-1-P, 5-$\alpha$-D-glucopyranosyl phosphate; 5-thio-D-glucose pentaacetate, 1,2,3,4,5-penta-O-acetyl-5-thio-$\alpha$-D-glucopyranosyl phosphate.

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Nucleotide Binding and Regulation of Phosphorylase

MATERIALS AND METHODS

Rabbit muscle phosphorylase \( b \) was prepared by the method of Fischer and Krebs (16) and recrystallized at least three times. Phosphorylase \( a \) was prepared from phosphorylase \( b \) with phosphorylase \( b \) kinase (EC 2.7.1.38) (17). Rabbit liver glycerol-3-P dehydrogenase, purchased from Sigma, was applied to a Dowex 1-Cl column and assayed by the method of Diestel as described by Ashwell (19). The concentration of glycogen is expressed as the molar equivalent of its glucose residues. Adenosine, adenine, AMP, ATP, hypoxanthine, inosine, IMP, caffeine, and theophylline were obtained from Sigma Chemical Co. No contaminants could be identified in these compounds by high voltage paper electrophoresis at pH 3.5, 0.1 M sodium citrate, 1500 V, 1 h.

The virtual substrate 5-thio-D-glucose-1-P was synthesized according to the published procedure of Whistler and Stark (20). The starting material, a syrup of 2,3,4,5-tetra-O-acetyl-6-thio-D-glucopyranosyl bromide, was obtained by reaction of 4.0 g of 3-thio-D-glucose pentaacetate with hydrogen bromide in glacial acetic acid for 18 h at 4°C (21). Acetic acid and HBr were removed by distillation under reduced pressure at no more than 35°C. Residues of these compounds were removed by co-distillation with toluene (22). The 3-thio-D-glucose pentaacetate was synthesized by acetylation of 3-thio-D-glucose (Pfanstiehl Laboratories, Inc.) in acetic anhydride with sulfuric acid catalyst by a modification of the method of Redemann and Niemann (23). Since the product is heat-labile (24), the reaction was kept at room temperature and the reaction time was extended to 72 h. The pentaacetate was isolated from an ice/water mixture as described by Ashwell (19).

The second method utilized the Fiske-Subbarow phosphate analysis as described previously (18, 27). Reaction mixtures were 100 \( \mu \)l in volume and contained 10 mM Bes (pH 6.7), 10 mM magnesium acetate, 1 mM EDTA, 0.5 mg/ml of bovine serum albumin, 5 mM dithiothreitol, 56 mM glycogen, and about 0.5 \( \mu \)M of phosphorylase. Method II utilized the Fiske-Subbarow phosphate analysis as described by Engers et al. (28). Reaction mixtures were 0.5 ml and contained 3 mM sodium \( \beta \)-glycerophosphate (pH 6.8), 0.15 mM EDTA, 1 mM dithiothreitol, 28 mM glycogen, and 3 to 6 \( \mu \)M of phosphorylase. Enzyme and glycogen were preincubated for 15 min at 30°C before initiating the enzymatic reaction with glucose-1-P in both methods. These assays were used interchangeably without noticeable differences in results. Method I, however, is considerably more sensitive than Method II.

X-ray Crystallography - Single crystals of phosphorylase \( a \), suitable for diffraction studies, were prepared as previously described (29). Glutaraldehyde cross-linking of the crystals, when desired, was according to Kunitzsky and Manabe (18). They observed no effect on the crystal structure of the enzyme. Standard buffer for ligand binding studies contained 10 mM Bes, 10 mM magnesium acetate, and 1 mM EDTA, pH 8.0. In most cases, the inhibitory glucose, which is required for the formation of tetragonal crystals, was removed prior to initiating the binding studies. Difference electron density maps at 3.0-Å resolution indicate that this removal does not result in any alteration of the protein structure.

Crystals of phosphorylase \( a \) were soaked in solutions of the various ligands for 3 h. Diffraction data were measured with a Syntex P21, diffractometer using data acquisition techniques as described before (27, 29). The basis for calculation of the 4.5- and 3.0-Å resolution difference Fourier maps utilizing about one-half the observable data has been reported previously (27). All 6- and 4.5-Å resolution analyses by difference Fourier maps utilize a single crystal for each ligand. The 3.0-Å AMP and 3.0-Å 5-thio-D-glucose-1-P experiments were measured and analyzed in three independent experiments. The Fourier map revealing AMP bound at the nucleotide site was sectioned along the crystallographic Z axis and placed in a Richards box (30) for fitting a Labquab model (1 cm/Å) of AMP. The coordinates of the carbon, oxygen, and nitrogen atoms were measured from this model.

RESULTS

Kinetics - Heterotropic cooperativity between AMP and glucose-1-P has been reported previously for phosphorylase \( a \) (6, 28, 31, 32). These papers show that phosphorylase \( a \) is a "K" system, with the activator having only a slight effect on \( V_{\text{max}} \) but causing a pronounced decrease in the \( K_m \) for the substrate. Similar results were obtained with IMP. Using the treatment of Segel (33) for nonessential activation, the dissociation constant for IMP from the enzyme-glycogen complex was calculated. In the absence of glucose-1-P, it is 0.2 mM; however, in the presence of saturating substrate, it is 5 \( \mu \)M. This latter value is similar to that for AMP (2 \( \mu \)M) determined by physical methods (32). It is interesting that essentially no effect of IMP on glycogen binding to phosphorylase \( a \) was noted (Fig. 2), even though studies with AMP suggest that these heterotropic interactions should have been observed (28).

Depending on its concentration, IMP acts either as an activator or an inhibitor. When the IMP concentration used in activation studies of phosphorylase is raised into the millimolar range (Fig. 3), competitive inhibition of glucose-1-P binding is noted. As indicated in Fig. 4, the inhibitory effects of IMP are only on \( K_m \); the limiting \( V_{\text{max}} \) is that of the IMP-activated form of phosphorylase \( a \). Thus, it is the nucleotide-activated conformer which is inhibited. Such a result is consistent with the existence of a second site for IMP in addition to that at the activator site. A replot of the primary data (Fig. 3, inset) gives a \( K_i \) of 1.5 mM for IMP at the second site. Although these kinetic data do not allow us to distinguish the second IMP binding site from the binding site for glucose-1-P at the catalytic site, the X-ray crystallographic results in the following section show that this second IMP site is, in fact, the nucleoside binding site reported previously (13). Similar experiments (primary data not presented) were carried out with AMP and gave results which are qualitatively identical with those obtained with IMP. However, the \( K_i \) for AMP binding to the nucleoside site is 0.6 mM (Table I).

Purine nucleosides and bases were also examined for their ability to inhibit glucose-1-P binding to phosphorylase \( a \). These experiments were performed in the absence of AMP or IMP so that any inhibition would be with respect to the "unactivated" state. The data for inosine inhibition are presented in Fig. 5. All other compounds tested gave similar results which are shown in Table I. Caffeine is the best inhibitor with a \( K_i \) of 0.1 mM. The competitive inhibition observed with these compounds indicates that nucleotide activation is not required for the effect of binding at the nucleoside site to be observed. However, when we examined the effect of caffeine on the inhibition of glucose-1-P binding in the presence of AMP (Table I), we found that caffeine binding was considerably diminished (\( K_i = 3.6 \) mM). Conversely, the binding of AMP is diminished in the presence of glucose-1-P. A similar result was obtained when the effects of IMP on glycogen binding to phosphorylase \( b \) were examined (data not shown). Primary kinetic plots showed no homotropic cooperativity for IMP, AMP, or glucose-1-P. The apparent dissociation constant for glycogen from the phosphorylase \( b \) glucose-1-P complex varies from 3.9 to 0.5 mM as AMP increases from zero to "infinity." However, when IMP is used, the apparent \( K_m \) for glycogen is invariant at 2.6 mM even though \( V_{\text{max}} \) increases nearly 4-fold. We observed a second inhibition constant of 0.15 mM for AMP in the absence of glucose. This may be compared to the value 3.7 mM for IMP under similar conditions. Previous studies (7) indicate a \( K_i \) of about 2.5 mM IMP for phosphorylase \( b \) over a wide range of glucose-1-P concentrations. The results obtained here by the use of variable glucose concentrations are consistent with the data of Black and Wang who used variable glucose-1-P (7).
Fig. 1. The activity of phosphorylase a as a function of glucose-1-P concentration at variable activating concentrations of IMP. Assays were carried out by Method II (see "Materials and Methods") in the presence of 28 mM glycogen. Concentrations of IMP and corresponding apparent Michaelis constants for glucose-1-P are as follows: O, no IMP, 0.8 mM glucose-1-P; □, 3.0 mM IMP, 0.5 mM glucose-1-P; O, 10 mM IMP, 0.3 mM glucose-1-P; ■, 30 mM IMP, 0.1 mM glucose-1-P; O, 0.1 mM IMP, 0.06 mM glucose-1-P. Inset, secondary replot of 1/a slope, O, and 1/n intercept, O, versus 1/(1[IMP]) according to Segel (33). The lines extrapolate to the same point on the ordinate.

Fig. 2. The effect of IMP on the binding of glycogen by phosphorylase a. Assays were carried out by Method II (see "Materials and Methods") in the presence of 55 mM glucose-1-P. The apparent K_m for glycogen was 0.7 mM in the absence of IMP (O) and 0.6 mM in the presence of 10 mM IMP (□).

Table II shows the effect of glucose and caffeine, individually and together, on the activity of phosphorylase a in the presence and absence of AMP. Caffeine and glucose were synergistic with respect to their inhibitory properties in all cases except that of high glucose 1-P (30 mM) in the presence of AMP. Presumably, in this case, the activating effect of high substrate and AMP (which are both competitive with glucose and caffeine) cannot be overcome by the two inhibitors at the concentrations used.

Since 5-thio-D-glucose-1-P has been shown to be a strong competitive inhibitor of glucose-1-P binding to phosphorylase b, we examined the effects of this compound on phosphorylase a. We found that this glucose-1-P derivative was competitive with that substrate (Fig. 6) and had a K_i equal to 0.3 mM 5-
TABLE I

Effects of purine derivatives on glucose-1-P binding to phosphorlyase α

| Ligand         | Concentration Kᵢ | Kᵢ,          |
|----------------|------------------|--------------|
| None           | 1.2 mM           | 0.41 mM      |
| AMP            | 6.0 mM           | 1.1 mM       |
| IMP            | 1.5 mM           | 0.44 mM      |
| Inosine        | 0.9 mM           | 0.25 mM      |
| Hypoxanthine   | 1.1 mM           | 0.44 mM      |
| Adenosine      | 1.0 mM           | 0.25 mM      |
| Adenine        | 1.1 mM           | 0.25 mM      |
| Theophylline   | 1.0 mM           | 0.25 mM      |
| Caffeine       | 0.6 mM           | 0.25 mM      |
| Caffeine*      | 3.5 mM           | 0.25 mM      |

* These Kᵢ values were obtained from replots of the primary data as shown in Fig. 3, inset.

** In the presence of 50 μM AMP.

The conformation of the AMP molecule as determined from the difference Fourier in Fig. 8 is c(Z')-endo, anti, tram using the nomenclature of Sundaralingam (34). The approximate values for the dihedral angles (34) were determined from the atomic coordinates measured with a Richards box (30). The values for x, $, o, and 4' are 54°, 163°, 157°, and 143°, respectively. This conformation is a usual one found for 5'-nucleotides bound to enzymes where the trans conformation may predominate rather than the preferred gauche + conformation found for the free nucleotides. The NMR studies of Morange et al. (35) indicate the conformation of AMP when bound to phosphorylase b is anti.

A summary of the results from the ligand binding studies is given in Table III. The estimation of the ligand occupancy from the difference Fourier analysis is from analysis of the background-corrected electron density maxima at the various subsites. We have assumed that the nucleoside site is fully occupied with 5 mM inosine (Fig. 7b). The division of the nucleotide binding site into three subsites for the base, sugar, and phosphate rests on the 3-A resolution difference Fourier maps for AMP and 5-thio-D-glucose-1-P which clearly define these subsites. The detailed structural analyses will be postponed until atomic coordinates are available for the enzyme.

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TABLE III

| Ligand                  | Concentration | Glucose | Cross-linked | Resolution | Lattice constants | Root mean square ΔP/F | Sites occupied by ligand* |
|-------------------------|---------------|---------|-------------|------------|-------------------|------------------------|---------------------------|
|                         | mM           | (50 mm) |             |            |                   |                        |                           |
| AMP                     | 4.4          | -       | +           | 4.5        | 128.1, 117.1      | 9.0                    | +++ + + + -                |
| AMP                     | 0.5          | +       | -           | 3.0        | 128.2, 117.0      | 9.0                    | +++ + + + -                |
| IMP                     | 5.0          | -       | -           | 4.5        | 128.1, 117.0      | 6.7                    | +++ + + + -                |
| ATP                     | 10.0         | -       | -           | 4.5        | 128.2, 116.6      | 7.0                    | +++ + + + -                |
| Adenine                 | 15.0         | -       | -           | 6.0        | 128.1, 116.6      | 5.1                    | - - - + + +                |
| Adenosine               | 2.0          | -       | -           | 6.0        | 128.3, 116.6      | 4.5                    | - - - + + +                |
| Inosine                 | 5.0          | -       | -           | 4.5        | 128.2, 116.6      | 3.7                    | - - - + + +                |
| Cibicron, F3GA          | 1.0          | +       | -           | 6.0        | 128.2, 116.6      | 4.2                    | + + + + + +                |
| Theophylline            | 20.0         | -       | +           | 4.5        | 128.1, 116.6      | 4.8                    | + + - + + +                |
| 5-Thio-D-glucose-1-P    | 10.0         | -       | +           | 3.0        | 128.5, 117.6      | 14.0                   | - ++ - + + +                |
| UDP-Glc                 | 20.0         | -       | +           | 4.5        | 128.3, 117.6      | 10.0                   | + + - - - + +              |
| Inosine + 5-Thio-D-glucose-1-P | 40.0 | - | + | 4.5 | 128.4, 116.9 | 6.0 | - ++ - + + + |
| UDP-Glc                 | 10.0         | -       | +           | 4.5        | 128.3, 117.6      | 10.0                   | + + - - - + +              |

* Occupancy (relative to background) at the various sites is indicated by plus and minus. The latter indicates no ligand at the site; +, less than 25% occupied; ++, approximately 50% occupied; and ++++, nearly 100% occupied.

Inconsistent with kinetic results for AMP or IMP activation, which show no competition between nucleotide and glucose-1-P (Fig. 1). However, the existence of a second glucose-1-P site at the glucose binding locus (recall that glucose is a competitive inhibitor with respect to glucose-1-P (32) is consistent with the latter site being part of the active site of phosphorylase a. Binding of glucose-1-P, P, and other anionic substrate analogs at elevated concentration (300 mM) in the crystal has been shown at both the AMP and glucose-1-P (glucose) sites. Difference Fourier maps for some of these binding studies have been published (36). It is of great interest that glucose-1-P bound in the active site is adjacent to the pyridoxal 5'-phosphate coenzyme (36).

The most striking demonstration that the nucleoside site blocks the active site and a unique demonstration of the location of the active site itself is shown in Fig. 7d where, in the presence of inosine, 5-thio-D-glucose-1-P (Fig. 6a) no longer binds to the glucose-1-P (glucose) binding site. This result is consistent with the kinetic results (Figs. 3, 5, and 6) which indicate that inosine and the nucleoside site to which it binds allosterically exclude glucose-1-P from the active site. Note that 5-thio-D-glucose-1-P still remains firmly bound at the AMP activator site as shown by the positive density peak at the phosphate subsite (Fig. 7d, site P). However, no inosine is bound at the adenine subsite (compare Fig. 7, d with a).

Phosphorylase a crystals, obtained in the presence of glucose (39), contain glucose in the active site and are most likely in the inhibited "T" conformation as described by Helmreich et al. (32). As pointed out above, in the crystal, glucose-1-P is capable of binding at the active site only at elevated concentration where ionic strength could effect the crystal. The virtual substrate 5-thio-D-glucose-1-P, however, at low concentration (10 mM) is capable of binding to the active site and partially induces a transition from T → R. This transition is macroscopically manifested by severe cracking followed by annealing of the crystals. X-ray analysis indicates that the protein dimer has lengthened (Table III) and the difference Fourier map shows considerable changes in the NH,-terminal region near the nucleotide activator site, Fig. 7c, NT (the changes are less apparent in the 5-thio-D-glucose-1-P map at...
FIG. 7. Difference electron density maps of ligand binding to phosphorylase \( a \) at 4.5-Å resolution. X and Y coordinates at 0 and 0.25 are indicated by +. Dotted lines are shown about the molecular outlines at the two regions along Z that include the nucleotide, glucose-1-P, and nucleoside binding sites (Z sections are 0.08 Å thick, centered on 0.4 for the nucleotide site and 0.24 for the latter sites). The horizontal bar represents 10 Å. Changes in electron density which are positive from the native enzyme's are represented by white contour lines; negative by black. a, binding of 5 mM IMP-A, purine subsite; S, sugar subsite; P, phosphate subsite; N, nucleoside site. b, binding of 5 mM inosine. Symbol as for a. c, binding of 10 mM 5-thio-D-glucose-1-P. Symbols are as in a with G-1P indicating the glucose-1-P binding site and NT the NH\(_2\)-terminal region. d, binding of 10 mM 5-thio-D-glucose-1-P plus 40 mM inosine. Symbols are as previously stated.
4.5-Å resolution than in a 6-Å resolution map). In the presence of both substrates maltotetraose and glucose-1-P (or 5-thio-glucose-1-P), the entire α helix (amino acids -50 to 75) in the NH₂-terminal region moves inward toward the body of the monomer (data to be presented elsewhere). We consider this movement to be a prelude to activation of the enzyme and formation of the "R" state.

**DISCUSSION**

The presence of two classes of AMP binding sites per monomer of phosphorylase b has been suggested previously by Wang and collaborators (8, 37). Similar conclusions were made more recently by Morange et al. (11). The present study verifies the interpretation of these authors and by the use of kinetic and x-ray crystallographic methods assigns a functional role and physical location to each of the nucleotide sites in the phosphorylase α monomer.

The high affinity AMP binding site (8, 11, 37) is probably equivalent to our AMP activator site (13). As pointed out earlier, the kinetic and crystallographic results are consistent with two sites per monomer of phosphorylase α. The site having the highest affinity for IMP (or AMP), which is responsible for enzyme activation (Fig. 1), should be fully occupied by nucleotide at relatively low concentrations. We find, at 0.5 mM AMP, that one of the two binding sites (the AMP site previously reported (13)) is essentially fully occupied by ligand (Table III). The second, low affinity nucleoside site (13) is, however, only partially occupied. Kinetic and crystallographic experiments indicate that binding of ligand to the nucleoside site inhibits glucose-1-P binding. The apparent Kₜ values for AMP and IMP binding to the nucleoside site are, respectively, 6.0 and 1.5 mM (Table I). Wang et al. (37) have reported similar values (Kₜ = 3 mM) for the calorimetrically determined binding of AMP to the low affinity site of phosphorylase b.

Two classes of binding sites have also been suggested for adenine and adenosine binding to phosphorylase b (11). Again the kinetic and crystallographic results presented here are in accord with this finding. It is interesting and not unexpected that the sites which bind the nucleoside or purine bases (Table III) are the same as those for the nucleotides. The occupancy data for the purine bases at these sites, however, is reversed with respect to those obtained with the nucleotides. Thus, the inhibitory site is preferred by the nucleosides or free bases. The Kₜ obtained by Morange et al. (11) for adenine binding (0.2 mM) and the Kₜ for adenosine binding (1.1 mM) to phosphorylase b is similar to our Kₜ values for these and analogous compounds listed in Table I. It is interesting that Buc and collaborators (11) found adenine and adenosine inhibitory with respect to the residual activity of phosphorylase b, which is present in the absence of AMP. Such a result is consistent with the conclusion that the inhibitory site is one other than that for AMP allosteric activation, especially since these compounds do not release radioactive AMP or glucose-6-P bound at the allosteric activator site until the low affinity nucleoside site is saturated (11).

The important unanswered question remains the physiological role for these effector sites and what ligands function in vivo. Effects of purine derivatives on muscle phosphorylase

**Fig. 8.** A photograph of an AMP model fit into the 3.0-Å difference electron density map. The sections are calculated perpendicular to the Z direction. The conformation of the molecule is c(2')-endo, anti, trans.

**Fig. 9.** Stereo diagram of the polypeptide chain drawn through the positions of the 829 α carbons for one monomer of phosphorylase α. Positions identified are: N, NH₂ terminus; A, phosphate of AMP; G, glucose; IP, phosphate moiety of glucose-1-P and P; PL, ring of pyridoxal phosphate; 5P, phosphate of pyridoxal phosphate; I, nucleoside binding site; GGGGG, glucose residues of maltotetraose.
were known as early as 1955, when a report by Kihlman and Overgaard-Hansen (38) indicated that the methylated oxypurines, such as caffeine and theophylline, were inhibitory in nature. Since this initial report, it has been assumed that the effect of the purine bases on phosphorylase is the result of direct competition with the allosteric activator AMP (39, 40, 41). Although this conclusion was justified at the time, our finding that the nucleoside site is most specific for caffeine or theophylline (Tables I and III) indicates that the effects of the methylated oxypurines are mediated via binding at the new locus. That the nucleotide and nucleoside sites are linked allosterically is clear, since we have demonstrated the negative heterotropic effects of ligand binding at the respective sites (Table I). In fact, ligand binding at the nucleoside site appears to function in a manner analogous with that observed for glucose binding to phosphorylase a (32). For example, both glucose-1-P and AMP binding are decreased in the presence of inhibitor. The competition with respect to glucose-1-P, however, is of a direct nature in the case of glucose but is allosteric with the nucleosides or purine bases.

The involvement of various metabolites with the regulation of glycogen metabolism is well documented (42). The specific effects of AMP, glucose, and glucose-6-P on phosphorylase phosphatase has been thoroughly investigated by Martensen et al. (43, 44). Methylated oxypurines were reported by Wosiek and Sutherland (45) to have an activating effect on phosphorylase phosphatase. This effect, which is exerted via the substrate, has been examined with muscle phosphorylase phosphatase (46, 47) and more recently with purified liver phosphorylase (48). The similarity between the activation of phosphorylase phosphatase by glucose and theophylline (48) and inhibition by AMP (43-48) suggests that the nucleoside site also functions as a regulatory site of phosphorylase activity in muscle. This function might be analogous to the glucose regulation of phosphatase in liver tissue (42). The synergistic relationship between the glucose and nucleotide inhibition of phosphorylase (Table II) might support this hypothesis. Structural changes in the NH₂-terminal-serine 14 PO₂ region of the phosphorylase a monomer, which are present in the 5-thio-D-glucose-1-P (glucose-free) crystal (Fig. 7c, NT) are blocked in the presence of nucleoside (compare Fig. 7c and d). These structural changes will be the subject of another manuscript; however, it will suffice to say here that they are consistent with a role similar to that which has been proposed for glucose. As intriguing as this suggestion of a physiological function for the nucleoside site may be, we can provide no information as to the identity of the ligand(s) which physiologically binds to this site. Investigations in this area are now underway.

In summary, we have used kinetic and crystallographic studies to identify the location and function of three ligand binding sites in the glycogen phosphorylase a monomer. The first site is shared between the two monomers, adjacent to the serine 14 phosphate (13), binds nucleotides preferentially, and is likely to be the activator site. About 30 A away, near the center of the monomer and adjacent to the pyridoxal phosphate coenzyme is a site which binds the inhibitor glucose, or the substrate, glucose-1-P, and which evidence in this paper (and in Ref. 36) identifies as the catalytically active site. The location of these sites is shown on a stereo diagram of the high resolution structure. Finally, the third site is 10 A away from the glucose-1-P site, in the nucleotide-binding fold, and was identified as the adenine site in our earlier paper (13). This site binds nucleosides and purine bases preferentially and inhibits phosphorylase activity via allosteric competition with glucose-1-P.

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Note Added in Proof—A recent paper by Witters and Avruch (1978) Biochemistry 17, 406-410 suggests that insulin activation of liver glycogen synthetase in vivo is solely the result of its ability to inactive phosphorylase a. Furthermore, the effect of insulin is synergistic with respect to glucose. Recent kinetic experiments in our laboratory with bovine liver phosphorylase a (unpublished) have demonstrated that the nucleoside site also functions in the liver enzyme. Since this is the only negative allosteric regulator site of phosphorylase activity which has a synergistic relationship to glucose inhibition, it is possible that an insulin induced effector may act to inhibit phosphorylase a activity by binding at this locus.

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