Location of pyridoxal phosphate in glycogen phosphorylase a
(glycogen metabolism/x-ray diffraction)

J. SYGUSCH, N. B. MADSEN, P. J. KASVINSKY, AND R. J. FLETTERICK

Department of Biochemistry and the Medical Research Council Group on Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Communicated by Edmond H. Fischer, August 15, 1977

ABSTRACT The pyridoxal 5'-phosphate cofactor of glycogen phosphorylase a (1,4-α-D-glucanorthophosphorylase α-glucosyltransferase, EC 2.4.1.1) has been positioned on the protomer with x-ray diffraction data, chemical markers, and sequence information. The electron density was computed from 3.0-Å resolution phases calculated from four heavy-atom derivatives. The cofactor is buried inside the protomer adjacent to the glucose-binding site.

The phosphorl substrates P3 and glucose-1-P each bind at two sites on the protomer. At low concentrations, P3 and glucose-1-P bind in the same location as does the allosteric effector AMP, near the monomer–monomer interface and some 30 Å from the glucose site. At high concentrations glucose-1-P also binds strongly at the glucose site, with its phosphate only 7.2 Å from that of the cofactor. Inorganic phosphate can also bind at this site. Implications for the participation of the pyridoxal phosphate in the catalytic mechanism are discussed in the light of these structural findings as well as the wealth of indirect evidence in the literature.

The role of the vitamin B₆ cofactor, pyridoxal 5'-phosphate (PLP), in rabbit muscle glycogen phosphorylase (1,4-α-D-glucan:orthophosphorylase α-glucosyltransferase, EC 2.4.1.1) has been a tantalizing enigma since its discovery in 1957 (1). The cofactor is found in all known α-glucan phosphorylases (2) and is absolutely required for their enzymic activity. In rabbit muscle phosphorylase it is covalently bound to a lysine residue (3). A variety of spectroscopic, chemical and model compound studies suggest that PLP in phosphorylase is buried in a hydrophobic environment as an imine (4, 5). Fluorescence quenching studies show, however, that it is accessible to the aqueous environment and that substrates or glucose make it less accessible (6). The discovery that the imine double bond between the PLP and lysine residue could be reduced by sodium borohydride with little loss of enzymic activity (3) demonstrated a striking contrast with other PLP-requiring enzymes. Reduction of the imine bond in these enzymes results in a complete loss of enzymic activity (7). Because the PLP cofactor in phosphorylase does not function via its aldehyde group, this posed the question of why it is required in the catalytic process.

Considerable evidence has accumulated to clearly demonstrate at least a conformational role for the PLP cofactor in phosphorylase (8, 9). Although removal of the cofactor does not lead to a general disordering of the native protein (10, 11), the quaternary structure of the apoprotein is significantly different from the native enzyme, because it shows a greater tendency to dissociate and is less stable (8, 12). In contrast, there is no definitive evidence for a catalytic function of the PLP cofactor, although a high degree of primary sequence homology about the PLP site between two otherwise dissimilar phosphorylases, rabbit and yeast, certainly suggests this possibility (13).

Numerous studies on the reconstitution of phosphorylase with analogues of PLP have ruled out all functional groups of the cofactor except for the phosphate on the 5' position and possibly the ring nitrogen as requirements for enzymic activity (12, 14, 15). A critical role in catalysis for the 5'-phosphate has been supported recently by nuclear magnetic resonance spectroscopic studies, which have shown that a proton transfer from the phosphate is triggered in phosphorylase b by arsenate, an alternate substrate, and AMP, the allosteric activator (15). Studies on pyridoxal-reconstituted phosphorylase b showed that phosphate could restore activity while pyrophosphate was competitive with both phosphate and glucose-1-P, thus supporting the hypothesis of a catalytic role for PLP. Lacking in all the experiments done to date, however, is definitive knowledge of the spatial relationship of the PLP cofactor to the various ligand-binding sites. Knowledge of this PLP location would greatly facilitate interpretation of the large body of experimental data in the literature.

We have recently published the 3.0-Å resolution structure of phosphorylase a with a preliminary study of its ligand-binding sites (16). A binding site for the allosteric effector AMP, the substrates glucose-1-P, phosphate, or arsenate, and substrate analogues such as UDP-glucose was found at the subunit interface of the dimer. A subsequent study suggested that the anionic moiety of all these compounds binds to a specific arginine residue (17). The phosphoserine is located on the outside of the molecule, 15 Å from this binding site. Some 25 Å away is the binding site for the glycogen analogue maltopentose. In the interior crevice near the center of the monomer is the binding site for the negative allosteric effector glucose. Since publication of the 3.0-Å resolution structure, which was based on two heavy-atom derivatives (16), data have been measured for two more derivatives. The resultant electron density map is greatly improved and allows us to compare known partial amino acid sequences and carry out more definitive ligand binding studies. In this communication we present the location of the pyridoxal 5'-phosphate in glycogen phosphorylase a and its spatial relationships to the various substrate-binding sites.

MATERIALS AND METHODS

Crystallization conditions for phosphorylase a and data collection techniques have been reported (18). The 3.0-Å resolution map for this study was calculated using 17,300 protein phases determined from four heavy-atom derivatives. A least-squares procedure (19) was employed as before to simultaneously refine both the heavy-atom parameters and protein phases. Table 1 presents a summary of the data measurement and refinement statistics pertaining to the heavy-atom derivatives used for the protein phase calculation. Since the previous communication we have remeasured the entire parent data set in order to sig-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: PLP, pyridoxal 5'-phosphate.

7F. Parrish and D. J. Graves, unpublished data.
The optical monomer fitting about carried out in a solution of standard buffer with added ligands. Our standard buffer is 10 mM N,N,N-tris(2-hydroxymethyl)-2-aminomethane sulfonic acid/0.1 mM EDTA/10 mM magnesium acetate, pH 6.7.

Approximately half of the observable intensities at 4.5-Å resolution were measured. These reflections were taken from a list of reflections that had been sorted according to the product of figure-of-merit, magnitude of parent structure amplitude, and resolution (sin θ) squared. This procedure gives in this case the best measured and best phased data consistent with a constant distribution with respect to resolution. Only the top half of the list, 2765 reflections in all, was used for actual data collection from a single crystal. The P, binding experiment required two crystals due to severe radiation damage to the crystals. Difference Fourier maps on the same scale as the parent electron density map were prepared for the interpretation of the ligand-binding experiments. All difference electron density map sections have been calculated perpendicular to the crystallographic c-axis direction.

Chemical marker experiments with pyridoxal 5'-phosphate were carried out on crystals of phosphorylase a using previously published conditions as a guide (21). Each crystal in standard buffer was incubated with 2 mM PLP for 70 min at room temperature, followed by reduction with 10 mM sodium borohydride for 15 min, then washed with standard buffer. The reagents 5'-AMP, glucose-1-P, and pyridoxal 5'-phosphate were all obtained from Sigma Chemical Co.

**RESULTS**

**Electron Density Map.** The quality of our present Fourier map is sufficient to permit verification of partial amino acid sequences in many regions of the protomer. In some places the electron density map still needs improvement to verify the identity of sequenced side chains. Fig. 1 is a stereo drawing of the phosphorylase a monomer prepared from the 829 α-carbon coordinates that were graphically measured from the minimap. The connectivity of amino acids is the same as that of the map previously calculated from two heavy-atom derivatives (16). Fig. 1 also includes the location of various ligand-binding sites on the enzyme which will be discussed.

**Table 1. Refinement parameters and diffraction statistics at 3.0-Å resolution with 17,300 reflections**

<table>
<thead>
<tr>
<th>Data set</th>
<th>No. of reflections (crystals)</th>
<th>No. of sites (merging)</th>
<th>Rs (merging)</th>
<th>ΔF/F</th>
<th>Root mean square of E</th>
<th>Root mean square of f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>53,000 (34)</td>
<td>4</td>
<td>0.038</td>
<td>0.18</td>
<td>6.4</td>
<td>17.2</td>
</tr>
<tr>
<td>Hg</td>
<td>41,000 (39)</td>
<td>4</td>
<td>0.046</td>
<td>0.13</td>
<td>6.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Pb</td>
<td>40,000 (41)</td>
<td>4</td>
<td>0.038</td>
<td>0.20</td>
<td>6.4</td>
<td>18.8</td>
</tr>
<tr>
<td>Hg + Pb</td>
<td>59,000 (66)</td>
<td>6</td>
<td>0.034</td>
<td>0.20</td>
<td>5.6</td>
<td>13.0</td>
</tr>
<tr>
<td>Yb + Ir*</td>
<td>28,500 (18)</td>
<td>5</td>
<td>0.026</td>
<td>0.11</td>
<td>6.8</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Average figure of merit = 0.73, Rs = \(\frac{\sum|I - I_0|}{\sum I}\), I is the average corrected intensity for all crystals; Rs = \(\frac{\sum|F_{obs} - F_{cal}|}{\sum|F_{obs}|}\), for centric reflections; \(F = \) protein structure amplitude; \(\Delta F = \) change in \(F\) on binding heavy atom; \(E = \) lack of closure error; \(f = \) heavy-atom structure amplitude.

*Note Added in Proof. On a more recent electron density map at 2.5-Å resolution, this peak was the strongest.*
lysine residue at a second site having the partial sequence Tyr-Lys-Asn-Pro-Arg-Glu (21, 25). The sequencing work of the Seattle laboratory places this lysine 112 residues (NH$_2$ → COOH) from that of the native PLP site. When crystals of phosphorylase a are reacted with PLP, a strong new peak is located in the electron density map at the end of a long side chain on the outside of the protein. This probable lysyl side chain is eight or nine residues distant from the cysteine residue and 111 or 112 residues (NH$_2$ → COOH) from the original PLP site, again in good agreement with the sequence of phosphorylase. Thus, we feel confident that our location of the PLP cofactor is indeed the correct one.

Relation of the Coenzyme to the Ligand-Binding Sites. Preliminary ligand-binding studies on glycogen phosphorylase a at 6-Å resolution have been reported for glucose-1-P, AMP, glucose, and maltoheptaose (16). These binding studies have now been extended to 4.5 Å for numerous additional ligands and will be the subject of another report (P. J. Kasvinsky et al., unpublished). We will discuss here only those studies necessary for interpretation of the significance of the location found for the PLP. Difference electron density maps for ligand-binding studies with 4 mM AMP, 50 mM glucose-1-P, 300 mM glucose-1-P, and 300 mM P$_i$ are presented in Figs. 3a, b, c, and d, respectively. In each case the structure amplitudes from glucose-free phosphorylase a were used for calculation of the electron density.

The quality of all difference maps is such that nearly all significant density features are confined to the protein regions. A comparison of the four difference maps shows a single, almost round, positive peak at the same location, labeled A in Figs. 1 and 3. Examination of the electron density map of the bound AMP in Fig. 3a allows us to assign this peak to the phosphate moiety, the ribose and purine rings also being clearly distinguishable. This assignment has been confirmed at 3-Å resolution (P. J. Kasvinsky et al., unpublished).

Examination of the peaks in the remaining panels of Fig. 3 shows that P$_i$ and glucose-1-P bind at this same phosphoryl-binding site, there being no sign of the glucose moiety of glucose-1-P. Arsenate also binds here (not shown). The anionic parts of the substrate molecules therefore bind to the same site as does the anionic part of AMP.

At higher substrate concentration, 300 mM glucose-1-P or

![Fig. 1. Stereo diagram of the polypeptide chain of phosphorylase a drawn through the positions of the 829 α-carbon atoms assigned from the 3.0-Å resolution electron density map. Positions identified are: N, NH$_2$-terminus; A, phosphate of AMP; G, glucose; 1P, phosphate moiety of glucose-1-P and P$_i$; PL, ring of PLP; 5P, phosphate of PLP; CY, cysteine residue 103 residues from lysine of PLP; L, lysine residue 112 from lysine of PLP; G G G G G, glucose residues of maltoheptaose. The lower chain in black is the partial sequence to which PLP has been shown to be attached (22). The upper chain in black is the α helix containing the arginine to which the phosphate of AMP is bound (17), and it continues through a loop that is adjacent to the glucose-1-P site.](image1)

![Fig. 2. Stereo representation of the electron density map about the PLP region of native phosphorylase. The computed map was contoured at a single density level per section and fitted with a stick-ball model representing the PLP cofactor and seven adjacent amino acids (Gly-Thr-PLP Gly-Asn-Met-Lys-Phe). The glucose of glucose-1-P was fitted to density corresponding to bound glucose in native phosphorylase a while the phosphate moiety has been positioned from consideration of bound glucose-1-P and P$_i$ in Fig. 3c and d. The symbol X denotes part of the tentatively assigned Lysine-289 residue.](image2)
The substrates bind only at the phosphoryl site of AMP but at an additional site, labeled G and 1P in Figs. 1 and 3 c and d, which corresponds to the previously identified glucose-binding site near the center of the monomer (16). The glucose moiety of the glucose-1-P in fact overlaps the bound glucose position, although we have not determined whether the glucose interacts with the enzyme in the identical fashion. The phosphate moiety of glucose-1-P and the inorganic phosphate anion can be shown to bind to the same site by the appropriate difference Fouriers. A comparison of electron density peak heights at both the glucose and the phosphoryl site of AMP indicates that glucose-1-P binds more tightly to the glucose site.

The paired positive and negative density features near the coordinate origins of Fig. 3 c and d correspond to structural changes in the long α helix near the NH₂-terminus. This α-helix starts near A on Fig. 1 and runs from left to right. Similarly, bindings of the substrates at the glucose site have induced structural changes in the turn of the β loop adjacent to the glucose site (darkened section of main chain in Fig. 1). At this time interpretation of these structural changes and others is not unequivocal due to insufficient resolution and lack of isomorphism induced by the high substrate concentrations.

**DISCUSSION**

Because the two substrates P₃ and glucose-1-P have been shown to bind to two distinct regions of the protomer we are presented with the task of defining the active site and assigning a role to the second site. The crystals, which are grown in the presence of glucose, presumably contain only the inactive conformer of the enzyme, which cannot be converted to an active conformation by AMP or 50 mM glucose-1-P. However, 300 mM concentrations of either substrate induce a conformational change that is coupled to binding of substrates at the glucose site.

The phosphoryl-binding site, located near the interface of the phosphorylase dimer, binds the allosteric activator AMP and...
the substrates glucose-1-P, P₈, and arsenate. Although the AMP molecule is clearly discernible at 4.5-Å resolution, the glucose-1-P is not, even when its concentration is well in excess of its Kₘ in the crystalline state (26). Instead, only a large round peak, similar to that produced by the P₈ anion, can be seen on the difference map, suggesting no recognition of the glucose moiety. The allosteric activator AMP binds preferentially to the phosphoryl site and, in solution, has a dissociation constant several hundredfold smaller than that for either substrate (27). A variety of physical and kinetic studies have established that AMP exhibits not only positive homotropic cooperativity upon binding to phosphorlyase a (28, 29) but also positive heterotropic cooperativity with respect to the substrates P₈ and glucose-1-P (27, 28). Under routine kinetic assay conditions of 1 mM AMP, with a dissociation constant of less than 2 μM, will fully saturate the phosphoryl-binding site. If this site were in fact part of the catalytic site, as we had previously postulated (16), then consideration of our current binding studies would suggest that AMP should be competitive with respect to the substrates, which it is not. This assumes the active conformation of the enzyme binds AMP in the same way as the glucose-inhibited enzyme in the crystalline state. To reconcile this inconsistency with the kinetic data for phosphorlyase a, we propose that the active site region is located at the glucose-binding locus and that the phosphoryl-binding locus is simply a regulatory binding site.

The close proximity of the glucose-1-P, glucose, and P₈ binding site to the PLP cofactor location in glycogen phosphorylase suggests a strong possibility for an involvement of the cofactor in catalysis. The phosphoprotein of the PLP is sufficiently close that protons exchanging from the 5'-phosphate (15) could either participate directly in general acid–base catalysis or transfer via the aforementioned side chain (lysine-289). Studies with 5'-deoxypyridoxal-reconstituted phosphorylase have established the absolute requirement of the 5'-phosphate for catalysis (9, 14, 30, 31). That the role of the 5'-phosphate was not simply one of maintaining structural integrity of the protein was eliminated because these studies established that the 5'-deoxypyridoxal enzyme retained its allosteric properties and substrate and activator binding capabilities (9).

A more recent series of experiments has elegantly addressed this question of involvement of the 5'-phosphate in catalysis.* In the presence of noncovalently bound phosphate, pyridoxal-reconstituted phosphorlyase b was shown to possess enzymic activity. This restoration of activity, which occurred in a concentration range of phosphate that had no effects on the native enzyme, suggests that phosphate occupied the 5'-phosphate site of the PLP cofactor. When these kinetic studies were carried out in the presence of pyrophosphate, again in a concentration range where no effect by pyrophosphate is seen on the native enzyme, the studies indicated that pyrophosphate was competitive with respect to both substrate and phosphate. Radioactive binding studies showed one molecule of pyrophosphate binding per phosphorlyase monomer. In considering the results of our structural studies, the conclusion reached by Parrish and Graves that pyrophosphate probably shares both the phosphate site and the substrate-binding site would tend not only to substantiate our location of the PLP cofactor with respect to the glucose site but also argue very strongly in favor of the location of the catalytic site adjacent to the 5'-phosphate of the PLP cofactor.

We thank K. Titani and K. A. Walsh and their colleagues for providing us with the amino acid sequence of phosphorlyase before publication and for several helpful discussions. We thank D. J. Graves for permission to quote unpublished material. The assistance of N. Murray, H. Semple, and S. Schechosky is gratefully acknowledged. This research was supported by grants from the Medical Research Council of Canada, from whom also a Fellowship is gratefully acknowledged by J.S.