Crystals of glucose-inhibited phosphorylase α shatter when exposed to substrates because the latter stabilize an active conformer. Cross-linked crystals can reanneal slowly without an alteration in the space group and can then be compared with the native crystals by x-ray diffraction. The subunits in the protein dimer move apart by about 2 Å. Several long segments of polypeptide chain in the NH2-terminal half of each subunit show significant shifts in position. These chain movements tend to link the four major ligand binding sites, either directly or via intersubunit excursions. Helix 49-75, which is near the interface between the two subunits and forms part of the binding site for the allosteric activator, AMP, shifts inward by 5 Å along its entire length. Ser-14-phosphate is associated with the outer surface of this helix and it, together with adjacent protein, moves inward in concert with the aforementioned shift. These changes extend across the subunit interface and link the two AMP binding sites of the dimer. All these structural changes bear obvious relationships to the well documented homotropic and heterotropic cooperativities observed upon ligand binding to this enzyme.

The effect of these allosteric transitions on the position of the Ser-14-phosphate provides a molecular explanation for the kinetics of phosphorylase α phosphatase and suggests a mechanism for glucose control of glycogen metabolism in the liver. Phosphorylase α, in vivo, is complexed with AMP and substrates so that its Ser-14-phosphate is tucked into a fold in the protein and is unavailable to the phosphatase, which nevertheless still binds tightly. This conformational inhibition is relieved by glucose which, in promoting the inhibited T state, causes the Ser-14-phosphate to be exposed to the action of the phosphatase. When the phosphatase has converted the 10-fold excess of phosphorylase α to b, to which it binds poorly, it becomes free to activate glycogen synthetase.

The extensive studies of several laboratories have built up a large body of knowledge on the properties of the enzyme concerned with glycogen metabolism and led to the hypothesis that phosphorylase α is the glucose receptor in the liver. The allosteric structural changes reported here provide a common molecular basis for this area of metabolic regulation.

While numerous physical and chemical studies suggest that protein conformational changes accompany the binding and interaction of various ligands, no detailed molecular picture of the structural basis for these allosteric interactions is available for any enzyme with effector sites in addition to active sites. X-ray crystallographic studies of allosteric conformational changes have been reported for some systems. Hexokinase B has been shown to bind nucleotide at an inter-subunit site which regulates the ligand binding at the glucose binding sites but no structural mechanism for the interaction in the crystals is available (2). Biesecker et al. (3) have described a structural change in the NAD binding domain of the R-glyceraldehyde-3-phosphate dehydrogenase subunits lacking co-enzyme for the tetrameric enzyme partially saturated with NAD. Lactate dehydrogenase has also been shown to exhibit more than one conformational state for the arginine loop involved in binding NAD (4, 5).

Using x-ray crystallography, we describe a major molecular structural transformation which accompanies activation of glycogen phosphorylase α (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). We can relate this to the well known allosteric phenomena and suggest a structural basis for a mechanism for glucose and hormonal control of glycogen metabolism in the liver. This work rests on our recent crystallographic and kinetic studies which have located the active site and three major effector sites, and demonstrated their interactions (6-8). Included among these sites is a previously unrecognized negative effector site which promotes the T state synergistically with glucose and likely has a role in hormonal regulation of glycogen metabolism.

The high resolution structure of phosphorylase α (T conformation) was obtained from crystals obtained in the presence of the allosteric inhibitor glucose. Since we know these crystals are active (9), we can study the structural change in the crystals after adding ligands which will promote the T → R conversion. Extensive studies from several laboratories (10-13) have demonstrated the effects of the T → R interconversion on the activity of the specific phosphorylase phosphatase. We show below that the observed structural changes agree with the biochemical data (in vivo and in vitro) concerning the phosphatase’s role in regulation of glycogen metabolism.

**EXPERIMENTAL PROCEDURES**

Tetragonal crystals of phosphorylase α from rabbit muscle were grown in the presence of 50 mM glucose, 10 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid, 10 mM magnesium acetate, and 2 mM dithiothreitol, as previously described (14). Crystals for this study were lightly cross-linked by treating with 0.03% glutaraldehyde for 1 h. This chemical treatment has been shown by difference Fourier to leave the structure of the protein unaltered while stabilizing the crystal (9). The cross-linked crystals exhibit catalytic activity (9).

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†To whom requests for reprints should be addressed.

1 W. B. Bennett and T. A. Steitz, personal communication.
Glucose was removed before soaking the crystals in solutions of various ligands for 3 to 4 h. Synthesis of the 5-thio-a-D-glucopyranosyl phosphate has been described previously (8). The techniques employed in collecting the diffraction data and calculating the difference Fourier maps have been described in previous publications (8, 14).

The difference map of 5-thio-a-D-glucopyranosyl phosphate plus maltoheptaose was calculated with difference coefficients using structure amplitudes for the enzyme with bound maltoheptaose as the parent structure. More than 90% of the possible data to 6 Å were observable. The map was calculated for one subunit with a 1.5 Å sampling interval. The gradient in electron density at each of the α carbon positions was calculated from this map using a step size of 2 Å in each of the three coordinate directions.

RESULTS AND DISCUSSION

Crystallographic Analysis of the Structural Transitions—When a crystal of glycogen phosphorylase a is soaked in a solution of its substrates, or substrate analogues, it cracks and would shatter completely if it were not cross-linked with glutaraldehyde (Fig. 1). This phenomenon is reminiscent of that observed by Haurowitz (15), that crystals of deoxyhemoglobin shatter when they are oxygenated because of tertiary and quaternary structural changes (16). We expect an analogous interpretation to apply to crystals of phosphorylase since they are known to have the presence of the allosteric inhibitor, glucose, and therefore show the dimeric enzyme in its inhibited (T) conformation. The virtual substrate, 5-thio-a-D-glucopyranosyl phosphate, or the natural substrates, cause conformational changes leading toward an active (R) state which cannot be confined within the existing crystal lattice. However, as shown in Fig. 1, the cross-linked crystals reanneal slowly and by 4 h are suitable for x-ray diffraction analysis and comparison of the two structures. Unlike the hemoglobin case, the R and T states of phosphorylase can both exist in nearly isomorphic crystal lattices of the same space group. Unfortunately, the crystallographic analysis of crystals which have been soaked in ligands which cause these large conformational changes is difficult because the lattice constants are variable and the radiation-sensitive crystals do not diffract well. Furthermore, as the lattice is not isomorphic, we cannot present a structure of the putative "R" form at 2.5 Å resolution without a sophisticated analysis. The results at 6 Å resolution provide a good quality difference Fourier which can be interpreted with the knowledge of the 2.5 Å resolution structure. Blake and Steita et al. (18) have both emphasized the importance of studying protein-ligand interactions, and accompanying conformational changes, by collecting data on co-crystallized binary or ternary complexes, in order to overcome the interference caused by the crystal lattice forces. While acknowledging the correctness of their views, we have not yet been able to overcome technical obstacles to this approach, and would add that a very conformationally mobile allosteric enzyme may not even have a single, final and stable, putative R state.

Fig. 2 is an α carbon drawing of one subunit showing the locus of binding of various ligands that we have determined from difference Fourier analysis. This drawing is based on a study at 3 Å resolution which has since been extended to 2.5 Å (6). Atomic coordinates for the NH2-terminal half of the subunit have been measured and show portion of the α carbon backbone drawing to be essentially correct.7

There are three general regions of the subunit of 641 amino acids in which we might expect and indeed find significant changes in tertiary structure (indicated by the shading in Fig. 2). The first of these is the subunit interface of the dimer where the two NH2 termini juxtapose and where Ser-14-phosphate, the site of covalent modification, is located. Only 15 Å away from this crucial control site is a binding site for the positive effector, AMP. The second is the saccharide binding lobe which we presume forms a docking or storage site for glycogen in vivo. An extensive kinetic-crystallographic analysis demonstrates that this site activates the enzyme but is spatially distinct from the active site (7). Finally, the third is the active site region which binds glucose and glucose-1-P and inorganic phosphate at essentially the same locus, near the pyradoxal-β-phosphate (6). Nearly 10 Å from the binding site for glucose, a second negative effector locus has been found and studied with kinetics and x-ray crystallography (8).

We are presenting here portions of two similar difference Fourier maps at 6 Å resolution. Fig. 3 shows the map calculated for 10 mM 5-thio-a-D-glucopyranosyl phosphate + 200 mM maltoheptaose. The α carbon positions and pseudobonds are superposed in this stereo photograph. Fig. 4 shows a map for 50 mM glucose-1-P + 200 mM maltoheptaose for those sections corresponding to the position of the helix from Pro 49 to Tyr 75.

Inspection of these two figures shows the structural changes to be complex and extensive so that their exact interpretation is clearly a demanding proposition. We precede the visual accounting of the conformational changes with a simple unbiased identification of the regions of greatest change. Fig. 5 highlights these regions along the sequence in a plot of the "moving average" (over 10 α carbon positions) of the absolute value of the difference electron density gradient. We visually estimate the maximum noise level by the two dotted lines which are drawn from the maximum amplitudes observed above and below the average value in the COOH-terminal half of the plot. No significant structural changes can be seen in the corresponding regions of the difference Fourier maps. This procedure clearly demonstrates the largest contiguous changes in tertiary structure to be in the segments 1-75, 102-123, 242-297, and 385-400, all in the NH2-terminal half of the protein.

We know from the similarity of lattice constants that the change in quaternary structure on adding ligands to the crystal is small. Judging from a gradient analysis, as used to relate the several crystalline hexokinase structures (20), the phosphorylase subunits seem to move about 2 Å further apart (in the crystallographic C direction) without a complementary rotation. Thus, we can focus on the extensive changes in tertiary structure.

Inspection of Fig. 3 with the overlay of a carbon markers clearly shows a major shift of protein chain in the NH2-terminal region which forms the subunit interface. This shift is most apparent for the helix from Pro 49 to Tyr 75 in the plane of the sections. That this shift inward of the helix by about 5 Å also involves residues 1 to 48 is apparent from careful study of the map. This can be seen from markers at 10, 30, and 40 which are seen to be located in negative density which is adjacent to positive density in Fig. 3. Indeed, the 2.5 Å electron density map for the glucose-induced form of the enzyme shows that Ser-14-phosphate is interacting with Arg 69 on this helix.

Fig. 4 should clarify and illustrate in a different way the movement of helix 49 to 75, together with the associated NH2-terminal region. There can be no doubt that the same structure

7 The abbreviations used are: glucose-1-P, D-glucopyranose 1-phosphate; thiglucose-1-P, 5-thio-a-D-glucopyranosyl phosphate.

7 This numbering system is for the model of 829 a carbon positions shown in Fig. 2, as published previously. Fitting of the 841 amino acids of the completed sequence (19) to the electron density map has proceeded through the first half of the molecule and confirms the connectivity and general secondary structure, but some corrections will be needed, usually at loops on the surface. The segments quoted here would correspond to the following residues determined by theSeattle group: 1-75, 103-124; 240-287 and 383-397; respectively.

R. J. Fletterick and S. Sprang, unpublished observations.
Fig. 1. Effect of a substrate analogue on a crystal of phosphorylase a. A tetragonal crystal of phosphorylase a was cut to approximately 0.4 x 0.4 x 0.4 mm, placed in a capillary tube, and photographed between partially crossed polarizing filters. A, without ligand; B, 10 min after adding 5 mM thioglucose-1-P; C, 4 h after adding the thioglucose-1-P.

Fig. 3. Stereo photograph of the difference Fourier between an electron density map at 6Å resolution from a crystal soaked in 10 mM thioglucose-1-P + 200 mM maltoheptaose. The native phosphorylase plus maltoheptaose is subtracted from the former to yield negative density, depicted as yellow areas, and positive density, depicted as areas enclosed by solid lines. X and Y coordinates at 0 and 0.20 are depicted by +, while the Z sections are 2Å thick and extend from 0.3 to 0.5. The dotted lines indicate the molecular outlines at Z = 0.25 and 0.4. Superimposed on the map are the Cα positions, linked by straight lines, and their numbers, with red circles for the lower subunit of the dimer, and black stars for its symmetry-related mate. The root mean square change in structure amplitude compared to the native protein is 25%.

Fig. 4. Difference Fourier at 6 Å resolution of the electron density changes caused by soaking a crystal in 50 mM glucose-1-P + 200 mM maltoheptaose. Only the portion of the map centered on Z = 0.4 is shown. Negative changes are red; positive are blue. The density due to glucose-1-P itself is adjacent to X = 0.25. The root mean square change in structure amplitude compared to the native protein is 28%.

Structural changes seen in Fig. 3 can be discerned here. Similar conformational changes, though less pronounced, are caused by glucose-1-P alone at 300 mM and were illustrated in Fig. 7C of Ref. 8.

To return to our consideration of Fig. 3, another segment of peptide chain which is seen to move in concert with the NH2-terminal rearrangement is in the oligosaccharide lobe. The analysis shown in Fig. 5 indicates that residues 385 to 400 show significant movement, while visual inspection of Fig. 3 suggests that this movement extends down part of the helix which begins at residue 400. This conformational change thus extends from the maltoheptaose binding site toward the glucose-1-P binding site, and one may speculate that it is concerned with the heterotropic cooperativity observed between these two sites (7).

Finally, there is another long segment of polypeptide chain
Allosteric Transitions of Phosphorylase a

Fig. 2. Stereo diagram of the polypeptide chain drawn through the positions of 829 a carbons for one monomer of phosphorylase a (6). Positions identified are: N, NH-terminus; SP, serine 14-phosphate; 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 (inhibitor) binding site; GGGGG, glucose residues of maltoheptaose (glycogen storage site). Shaded chain identifies the portions observed to move upon addition of substrate(s) to the crystals.

Fig. 5. “Moving average” analysis of the difference electron density gradient data of Fig. 3. The significant structural changes are confined to the NH2-terminal half of the polypeptide chain. The quantity plotted is the absolute value of the gradient in arbitrary units but averaged over 10 consecutive a carbon positions.

which changes position when substrate is bound; this runs from residue 242 to 297. This was indicated by the analysis in Fig. 5, and much of it can be seen clearly in Fig. 3. This segment includes a loop which is near the glucose-1-P binding site and adjacent to the site of nucleoside binding (9). The binding site for negative effectors is in fact partially constructed from residues 286 to 297 of this loop. As noted before (8), binding of nucleosides or derivatives such as caffeine prevents the movement of this loop, which accounts structurally for the competitive inhibition of glucose-1-P binding. Movement can also be seen extending up from this loop throughout a chain which noncovalently links the other protein subunit and then returns to form part of the b sheet below the AMP binding site.

Movement of protein described by this long segment, as well as by that seen in the NH+ terminal region, extends across the subunit association contact surfaces and may reflect the homotropic cooperativity of ligand binding (both AMP and glucose-1-P). The mechanism for heterotropic cooperativity between the AMP and active sites may proceed directly through a single monomer or via the intermonomeric contacts.

Physiological Implications of the Structural Rearrangement—Even without an adequate structural description of the R conformation, the NH+ terminal rearrangement which we have outlined has important consequences in regulating the enzymic activity and interaction with the phosphatase enzyme. Phosphorylase a does not normally exist in vivo exclusively in the inhibited or T form, as in our crystals, but to a large extent in the R form because of complexes with AMP and various organic phosphates (21). The effect of adding glucose to the enzyme would be to position the serine 14-phosphate out from its location on the surface of the enzyme and to expose it to the solvent and to the action of phosphorylase phosphatase, resulting in the formation of phosphorylase b. We thus have a molecular basis for the observation that glucose causes a 3-fold increase in the maximal velocity of the phosphatase action on phosphorylase a while AMP causes a 95% inhibition, the K, in all three situations remaining approximately the same, 5 µM (11). These remarkable effects on the kinetics of phosphorylase phosphatase are thus brought about by the allosteric conformational changes in its substrate, phosphorylase a. The product of the reaction, phosphorylase b, binds poorly to the phosphatase with a K, of 144 µM (11) whereby, we may assume, increasing the free phosphatase pool in vivo about 25-fold.

The physiological significance of these observations and their molecular mechanism remains uncertain in skeletal muscle which is not considered to contain intracellular glucose under normal conditions (22). The situation is further complicated by our discovery of a second negative effector locus which functions synergistically with glucose. We have demonstrated in vitro that in order to generate an appreciable population of the T conformation a second ligand may be required and that perhaps in vivo glucose may not be acting alone.

For the liver cells which are freely permeable to glucose (23), Hers and Stalmans have proposed a mechanism for glucose control of glycogen metabolism (24, 25). The level of liver phosphorylase a is normally poised at about 50% of maximal (26). An influx of glucose reduces the level of phosphorylase a by the substrate-induced activation of phosphatase. After a lag period, which allows the phosphorylase a level to decrease below 5% of its starting value, there is an increase in the level of glycogen synthetase a due

to the action of glycogen synthetase phosphatase. The latter enzyme has been shown to be inhibited by phosphorylase a (R form), not phosphorylase b. Two laboratories have presented evidence that phosphorylase phosphatase and synthetase phosphatase are identical (27, 28).

Several lines of evidence suggest similarities between the liver and muscle systems. For example, the kinetics of phosphorylase a from liver (29, 30) shows a close resemblance to that of muscle (21, 31). Structural homology is demonstrated by the construction of active hybrids (32) and the fact that muscle phosphorylase kinase and phosphatase can act on the liver enzyme (33). If we assume that the liver and muscle systems are similar, we can propose the scheme shown in Fig. 6 to rationalize the physiological and enzymatic observations. Phosphorylase a, being in 10-fold molar excess over the phosphatase, would bind all of it and prevent its action on glycogen synthetase. Glucose would release this inhibition by means of the allosteric changes described above and cause the formation of phosphorylase b, but the phosphatase would continue to be bound to successive molecules of phosphorylase a until nearly all the latter was converted. Only then would the phosphatase be released to act on the glycogen synthetase to form synthetase a and finally glycogen. The conformational changes involving the serine 14-phosphate demonstrate how the allosteric interplay between positive and negative effectors has evolved into a mechanism for control of covalent interconversion between two chemically different forms of the enzyme. This provides the molecular basis for the role of phosphorylase a as the glucose receptor of the liver, permitting a feedback control of far greater sophistication than that afforded by direct inhibition of the responsible enzyme.

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