on which research is being conducted at present.

I thank the Army Research Office/Durham and Materials Research Division of the NSF for the partial support of this research (the latter under Contract DMR-79-23575 with the Northwestern Materials Research Center). I am grateful to the Northwestern University Ion Conductivity Group, particularly S. Jacobson, B. Papke, J. McOmber, S. Topiol, T. Wong, R. Dupon, D. F. Shriver, M. Brodwin, A. Nitzan, and D. Whitmore, for their generous help and patience. It is a pleasure to thank B. R. Sundheim and C. A. Angell for incisive and greatly helpful advice.

Glycogen Phosphorylase Structures and Function

ROBERT J. FLETTERICK* and STEPHEN R. SPRANG

Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, California 94143

Received October 31, 1981 (Revised Manuscript Received May 19, 1982)

The carbohydrate reserve of most metabolically active cells in the animal kingdom is glycogen, a polymer of glucose. The cellular demands to convert glycogen and orthophosphate (P_i) to glucose 1-phosphate (G-1-P) are met by glycogen phosphorylase, one of the most complex and finely regulated enzymes yet encountered. The observation by Cori and Cori[1,2] that the catalytic activity of phosphorylase *b* could be triggered by AMP provided the first example of enzyme regulation by a ligand which is not a substrate. In their classic treatise defining the fundamental concepts of allostery, Monod, Jacob, and Changeux[3] interpreted this phenomenon as a "concerted" transition between conformational states. Phosphorylase, like the archetypical allosteric protein hemoglobin, assumes at least two conformations, of which only one is catalytically active. It is accepted that the equilibrium among these is modulated by small molecules, "effectors", which, by interacting at specific sites on the protein, favor a particular conformation. Thus, the positive allosteric effectors of phosphorylase promote a catalytically active "R" state while negative effectors are inhibitors and bind to stabilize the inactive "T" conformation. In general, the effector is not a substrate nor is the effector site the active site, although substrates that are also effectors at the active site are common.

The complexity of phosphorylase admits a variety of allosteric mechanisms, involving both the catalytic site and specific loci elsewhere on the molecule. Phosphorylase is no exception to the general rule that allosteric proteins are multimeric, such that the binding of effectors and substrates is cooperative among the subunits. The best characterized and physiologically important species of phosphorylase is the dimer, although in the absence of its glycogen substrate, the "R" state enzyme tends to form tetramers.[4] The activity of glycogen phosphorylase is promoted not only by effectors but also by covalent modification: the addition of a phosphate at the hydroxyl group of a serine residue located near the N terminus of the molecule. Thus phosphorylase *b* (predominantly T) is converted to phosphorylase *a* (predominantly R) by phosphorylase kinase, a very complex enzyme, which is itself regulated by phosphorylation in response to nervous or hormonal regulation. The *dephosphorylation* of Ser-14-P, which inactivates phosphorylase, is catalyzed by yet another enzyme, phosphorylase phosphatase. This enzyme is regulated, at least in part, by phosphorylase itself. The system is designed such that a *functioning* phosphorylase, promoted by positive effector ligands or substrates at high concentration, inhibits the phosphatase, while an idling phosphorylase, resulting from a lack of substrate or high concentrations of negative effectors, is a phosphatase substrate.[5] Conceptually, the covalent effector site is similar to the other effector sites. Functionally, the difference is that covalent modification allows a precise temporal control that is insensitive to short-term changes in effector fluxes. Even though the stoichiometric ratio of the kinases to their substrates is low, a relatively light hormonal signal can, by cascade amplification, effectively and rapidly activate phosphorylase. Figure 1 shows the relationship among the kinases, phosphatase, phosphorylase, and glycogen synthetase.

The phosphorylase molecule, then, can be imagined to be a molecular transducer that samples positive effector signals (nervous or hormonal stimulation, G-1-P, P_i, glycogen, AMP) and negative effector signals (insulin-induced signals, glucose, ATP, and some still unidentified ligands) and proceeds either to degrade glycogen for fuel or halt its degradation, allowing the cell to begin storing glucose as glycogen for a later time.

The details of this catalytic process have yet to be described at the molecular level although it is certain that the catalytic event requires the formation of a ternary enzyme·substrate complex[6-8] comprising the

Stephen R. Sprang is an Assistant Research Biochemist in the Department of Biochemistry and Biophysics at the University of California at San Francisco. He received his B.S. degree from the University of California, Los Angeles, and his Ph.D. from the University of Wisconsin for his studies of nucleotide conformation.

Robert J. Fletterick received his Ph.D. in Physical Chemistry from Cornell University. Following his work on the hexokinase system at Yale University, he was Assistant Professor of Biochemistry at the University of Alberta in Edmonton. The work on structure and function of glycogen phosphorylase continues at the University of California at San Francisco in the Department of Biochemistry and Biophysics where he is Professor of Biochemistry and Biophysics.

(1) Cori, C. F.; Cori, G. T. *Proc. Soc. Exp. Biol. Med.* **1936**, *34*, 702–705.
(2) Cori, G. T.; Cori, C. F. *J. Biol. Chem.* **1940**, *135*, 733–756.
(3) Monod, J.; Changeux, J.-P.; Jacob, F. *J. Mol. Biol.* **1963**, *6*, 306.
(4) Huang, C. Y.; Graves, D. J. *Biochemistry* **1970**, *9*, 660–671.
(5) Cohen, P. (London) *Nature* **1982**, *296*, 613–196.
(6) Gold, A. M.; Johnson, R. M.; Tseng, J. K. *J. Biol. Chem.* **1970**, *245*, 2564–2572.
(7) Engers, H. D.; Sheckosky, S.; Madsen, N. B. *Can. J. Biochem.* **1970**, *48*, 746–754.
(8) Gold, A. M.; Osber, M. P. *Arch. Biochem. Biophys.* **1972**, *153*, 784.

0001-4842/82/0115-0361$01.25/0 © 1982 American Chemical Society

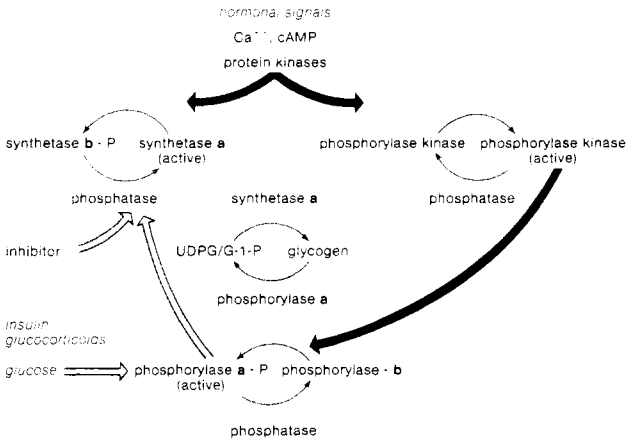

Figure 1. The phosphorylase cascade showing how hormonal signals activate phosphorylase through the two kinases and inactive glycogen synthetase. The inactivation (open arrows) is by the action of the phosphatase(s) that are regulated by phosphorylase and inhibitor protein(s). Glucose inactivates phosphorylase *a* directly. The mechanism by which insulin and other hormones do this is not known.

substrates P_i or G-1-P and glycogen within the "R" phosphorylase conformer. The reaction mechanism includes the participation of a coenzyme, pyridoxal phosphate (PLP), which is bound as a Schiff base to an active-site lysine.[9-11] Many enzymes require PLP in catalysis, commonly exploiting the electrophilic character of the pyridine ring. However, phosphorylase appears to be unique, involving the phosphate of the coenzyme as a functional group,[12,13] possibly as an electrophile in direct or enzyme-mediated contact with the substrate phosphate.[14,55] The most recent crystallographic studies of both the *a*[14] and *b*[15,16] enzymes show that when G-1-P is bound at the catalytic site, the substrate and PLP phosphate groups are close together (ca. 6–7 Å) even though the enzyme conformation in these crystals is essentially "T".

The long-term goals in the biochemical study of this enzyme are to define structurally and functionally the effector and the catalytic sites. X-ray diffraction analysis has defined the R and T states of hemoglobin, but not yet those of any enzyme. The situation for allosteric enzymes with multiple subunits and many types of effector sites may be that the interactions among these sites produce a spectrum of structures—the principal ones being the R and T states. However, even a complete X-ray structural and kinetic analysis may not fully describe the mechanisms by which catalytic activity is regulated, since the essence of the allosteric properties lies in the dynamic response of the molecule in its transition between at least two distinct conformations.

Only the T states of glycogen phosphorylase *b* and phosphorylase *a* have been investigated by X-ray crystallography. In this Account we review the results derived from this work and consider their implications for the allosteric response in this complex enzyme. There are a number of recent reviews of glycogen phosphorylase that address the details of its allosteric properties structurally,[17] functionally,[5,12,18,19] and physiologically.[5,20] Phosphorylase *b* structure and function are reviewed by Jenkins et al.[15]

Crystallographic Analysis

X-ray diffraction analysis of macromolecules or their oligomers in the range of 100 000 daltons and up has become straightforward with the recent advances in the technology of data measurement and processing. The current limiting step remains the preparation of high quality crystals. The crystal forms of both phosphorylase *a* and *b* are, except from the standpoint of radiation sensitivity, ideal.[21,22] Initial crystallographic work from Oxford on the *b* enzyme located the heavy atom positions for a mercurial derivative.[22] This information was then immediately applicable to the preparation of an electron density map of phosphorylase *a* at moderate resolution[23] following a typically thorough search for other suitable heavy-atom labels. Guided by the amino acid sequence determined by Walsh et al.,[24] interpretation of the electron density at 2.5-Å resolution[25] led to the construction of an atomic model; this analysis is being extended with 2.1-Å data by model building and least-squares refinement, a systematic and semiautomatic process that has reduced the conventional crystallographic *R* factor to 30% (Sprang and Fletterick, unpublished). The current coordinate set has some positional errors greater than 1 Å, but most of the nearly 14 000 non-hydrogen atoms of the M_r 200 000 dimer are well-determined. There are isolated regions of the molecule where errors are large and must be corrected by careful examination of difference electron-density ($F_{obsd} - F_{calcd}$) maps.

Difference Fourier analysis provides the most direct and fruitful approach to the description of ligand–protein interactions. The current atomic coordinates determined for phosphorylase *a* provide a reasonably accurate phasing model for the calculation of difference electron-density maps between the native enzyme and its ligand complexes at moderately high resolution. It is important to realize that difference Fourier maps are easily interpretable only for isomorphous structures. This is the case for ligands that do not promote activation of the enzyme. Activating ligands tend either to disintegrate the crystals or cause large conformation changes[26] such that the direct application of difference

(9) Baranowsky, T.; Illingworth, D. H.; Brown, D. H.; Cori, C. F. *Biochim. Biophys. Acta* **1957**, *25*, 16.
(10) Shaltiel, S.; Cortigo, M. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 594.
(11) Sygusch, J.; Madsen, N. B.; Kasvinsky, P. J.; Fletterick, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4757–4761.
(12) Helmreich, E. J. M.; Klein, H. W. *Angew Chem., Int. Ed. Engl.* **1980**, *19*, 441–455.
(13) Parrish, R. J.; Uhing, R. J.; Graves, D. J. *Biochemistry* **1977**, *17*, 4824.
(14) Withers, S. J.; Madsen, N. B.; Sprang, S. R.; Fletterick, R. J. *Biochemistry*, in press.
(15) Jenkins, J. A.; Johnson, L. N.; Stuart, D. I.; Stura, E. A.; Wilson, K. S.; Zanotti, G. *Philos. Trans. R. Soc. London, Ser. (B)* **1981**, *293*, 23–40.
(16) Johnson, L. N.; Jenkins, J. A.; Wilson, K. S.; Stura, E. A.; Zanotti, G. *J. Mol. Biol.* **1980**, *140*, 565–580.
(17) Fletterick, R. J.; Madsen, N. B. *Annu. Rev. Biochem.* **1980**, *49*, 31–61.
(18) Graves, D. J.; Wang, J. H. *3rd Ed. Enzymes*, **1972**, *7*, 435–482.
(19) Dombradi, V. *Int. J. Biochem.* **1981**, *13*, 125–139.
(20) Hers, H. G. *Annu. Rev. Biochem.* **1976**, *45*, 167–189.
(21) Fletterick, R. J.; Sygusch, J.; Madsen, N. B.; Johnson, L. N. *J. Mol. Biol.* **1976**, *103*, 1–13.
(22) Johnson, L. N.; Madsen, N. B.; Mosley, J.; Wilson, K. S. *J. Mol. Biol.* **1974**, *90*, 703–714.
(23) Fletterick, R. J.; Sygusch, J.; Semple, H.; Madsen, N. B. *J. Biol. Chem.* **1976**, *251*, 6142–6146.
(24) Titani, K.; Kordi, A.; Ericsson, L.; Kumar, S.; Hermann, J.; Wade, R. D.; Walsh, K.; Neurath, H.; Fischer, E. H. *Biochemistry* **1978**, *17*, 5680–5693.
(25) Sprang, S.; Fletterick, R. J. *J. Mol. Biol.* **1979**, *131*, 523–551.

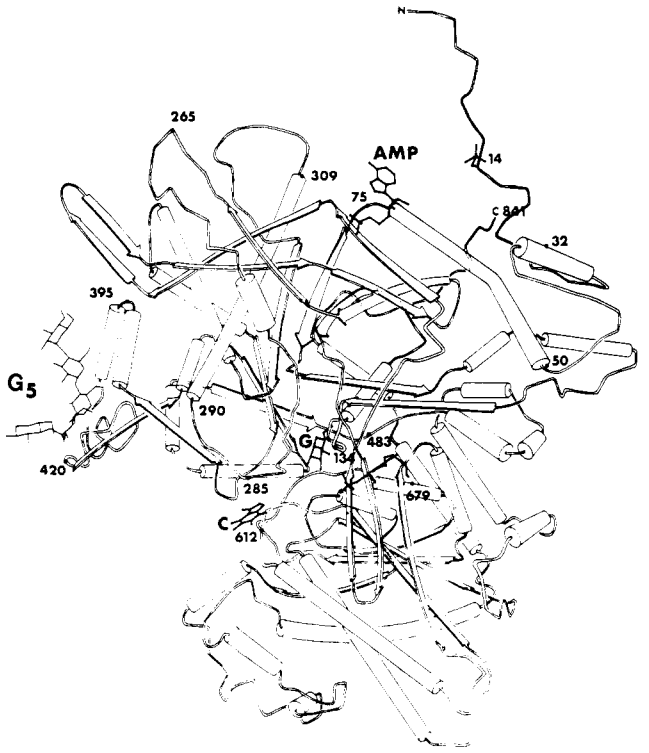

Figure 2. Schematic of the phosphorylase *a* monomer. The N- and C-terminal domains are bounded by residues 1–480 and 481–841, respectively. The latter is divided into the "regulatory" (1–320) and "glycogen binding" (321–480) subdomains. Residues near these positions are labeled. Skeletal representations of the allosteric ligands are shown at their binding sites: AMP, maltoheptaose (G5, an oligosaccharide which binds to the glycogen storage site), caffeine (C, at the purine-inhibitor site), and α-D-glucose (G, at the catalytic site). The side chains for Ser-14-P and lysylpyridoxal phosphate (PLP 679) are also shown. The helices are represented by cylinders and the β strands by arrows.

Fourier methods is not practicable.

Description of the Structure

A schematic representation of phosphorylase *a* is shown in Figure 2. The monomer is composed of two domains. The N-terminal domain (Ser-1 to Gly-480) includes the Ser-14-P, and AMP/ATP site, a glycogen storage site, and part of the active-site machinery. It also forms the whole of the intersubunit contact region, through which conformational changes are coordinated between the two monomers. The C-terminal domain (Tyr-481–Pro-841) contains the covalently bound pyridoxal phosphate coenzyme and part of the active site. The purine inhibitor or I site is also shared between the N and C domains. The spatial disposition of the active site with respect to the effector sites leaves no doubt that conformational states at distant regions of the enzyme can be tightly coupled; suggestive of an "action at a distance" view of allostery.

The tertiary structure of the two phosphorylase domains, i.e., β-sheet cores surrounded by α helices, is typical of the glycolytic enzymes.[27] The C-terminal domain (Figure 3) has a core of six β strands covered topside and bottom by five α helices. However, it is only in phosphorylase, so far, that we observe this classic "nucleotide binding fold"[27] to be itself covered

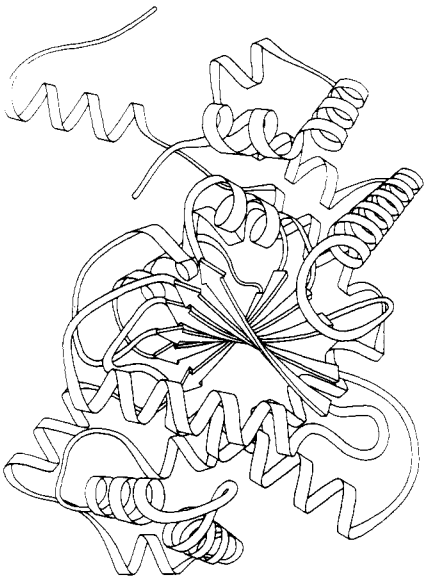

Figure 3. Drawing of the C-terminal domain (by Jane Richardson) in the orientation of Figure 2, showing the β-sheet core and surrounding α helices as a five-layer structure.

by two additional layers of α helices (top and bottom). Thus, the usual three-layer α/β/α structure seen in the glycolytic enzymes becomes a five-layer structure in phosphorylase: α/α/β/α/α.[25] The N-terminal domain is more complex. Functionally, and to a lesser degree structurally, it is divided, nearly through the middle of its β-sheet core, into two subdomains. The first (residues 1–320) appears to be involved principally in the subunit interaction, binding the effector AMP, and in recognition of the introconverting enzymes phosphorylase kinase and phosphatase. The second unit (residues 321–483) is a polysaccharide-binding domain at which phosphorylase is attached to ("stored" on) the glycogen particle. Glycogen is itself an allosteric effector at this site. It is important to note that both subdomains contribute residues to the substrate binding locus of the catalytic site. The β-sheet core that forms the whole of the N-terminal domain is a nonclassical α/α/β/α/α structure consisting of nine strands (predominantly parallel with a sheet twist of ~180°) surrounded by 15 helices. The units of secondary structure that eminate from the core are quite extensive, some forming long antiparallel β ribbons which drape over the molecular surface or define regions of the subunit interface.

The surface of phosphorylase is characterized by a deep groove at the interdomain boundary (Figure 4) and is modulated by the specialized structures which form the five effector, substrate, or enzyme (phosphatase/kinase) recognition sites. This highly invaginated molecule approaches the upper limit in accessible surface/volume exhibited by any proteins or their oligomers.[28] As a consequence of these requirements of tertiary organization, size, and surface accessibility, the relative tendency of individual residues, on the basis of their hydrophobicity, to be buried with the protein differs significantly from those in smaller proteins. For example, the fraction of solvent-accessible hydrophobic residues is greater than in the "average" globular protein of one-third the molecular weight of phosphorylase. On the other hand, two out of three hydrogen bond forming

(26) Madsen, N. B.; Kasvinsky, P. J.; Fletterick, R. J. *J. Biol. Chem.* **1978**, *253*, 9097–9101.
(27) Rossmann, M. G. *Philos. Trans. R. Soc. London, Ser. B* **1981**, *293*, 191–203.
(28) Sprang, S.; Yang, D.; Fletterick, R. J. *Nature (London)* **1979**, *280*, 333–335.

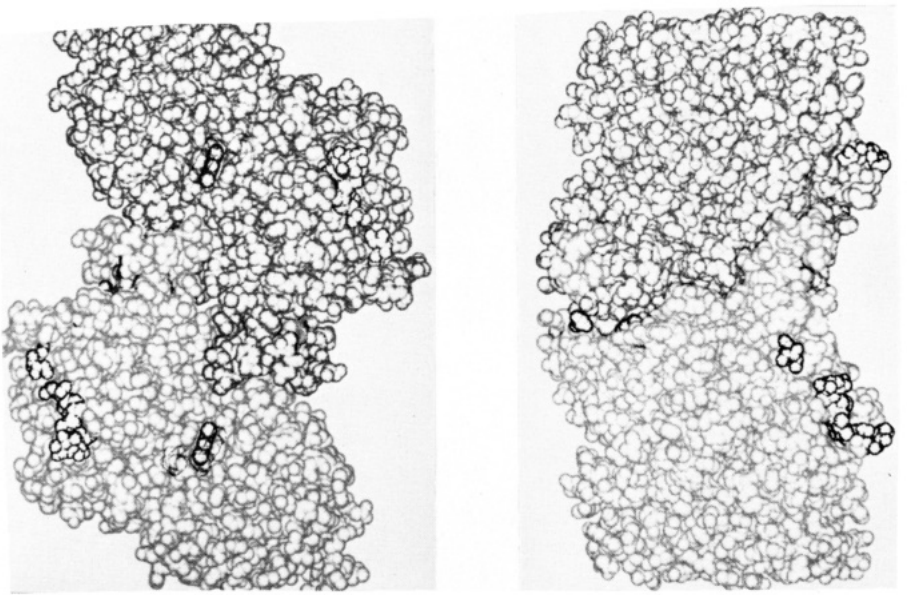

Figure 4. A space-filling representation of two views of the phosphorylase dimer. The spheres are van der Waals contact surfaces and are shaded to distinguish the individual monomers. The ligands (darkened) on the left panel are (from left to right, bottom monomer) oligosaccharide, glucose, caffeine. PLP and glucose are buried within the enzyme. The right panel shows Ser-14-P near the subunit interface. About 10 Å to the right is AMP (mostly buried) at the subunit interface. The oligosaccharide is to the right on the concave surface. The molecule measures 116 Å in the vertical dimension.

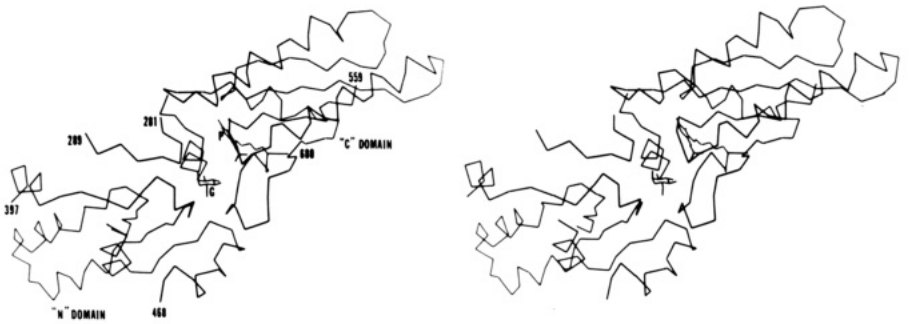

Figure 5. A view into the active site of phosphorylase. The site is formed by residues eminating from the β-sheet cores of the N- (left) and C- (right) terminal domains. Glucose is depicted at the catalytic site. The loop formed by residues 281–289 is disordered by activating ligands, thus facilitating the binding of substrates (see text).

serine and threonine residues are buried in phosphorylase whereas, among smaller globular proteins, the ratio is only one out of two.[29]

The active-site tunnel is, as in many glycolytic enzymes,[30] found at the C-terminal confluence of the β-sheet cores of the two domains. The active-site amino acids are located on six loops eminating from the secondary structural elements (Figure 5).

Subunit Contacts

The dimer is an object of dimensions 116 Å × 80 Å × 60 Å. Though it appears to have a solid exterior surface, the actual intersubunit contact area is quite small: only 6% of the total molecular surface is buried by the subunit interaction[28] (Figure 4). This arises because the tertiary structure involved consists almost entirely of external helices and loops of polypeptide chain that pack together at the subunit boundary. This is in striking contrast to the interfaces observed in a variety of nonallosteric oligomers wherein a central β sheet may extend across a subunit boundary as in insulin, concanavalin, or prealbumin, or, as in the $\alpha_1\beta_1$ contact of hemoglobin, the interface is formed of tightly packed helices. The contact surface between the phosphorylase monomers is widely distributed both in space and in primary sequence as a consequence of a convoluted and wandering main-chain linkage; it is more complex than, for example, the allosteric $\alpha_2\beta_1$ interface of hemoglobin.[31]

The majority of contacts between the monomers are van der Waals interactions, but there are also four noteworthy salt-bridge or ionic hydrogen-bonded pairs (largely shielded from solvent). These include the interactions between Arg-10 and Ser-14-P at the N terminus with Asp-32 and Arg-43 of the twofold related subunit. These contacts are sensitive to the conformational (R vs. T) state of the enzyme and play an important role in its allosteric regulation. Several other hydrogen bonds are made, but in all only 23 amino acid side chains per subunit are buried on forming the dimer. As an interesting consequence of the arrangement of external secondary structure, the subunit interface contains an interior solvent grotto with a volume equivalent to about 150 water molecules.

Though possessing a twofold symmetry axis, the dimer is a very asymmetric structure (Figure 4) with two functionally and structurally distinct surfaces[32] at op-

(29) Janin, J. *Nature (London)* **1979**, *277*, 491–492.
(30) Branden, C. *Quant. Rev. Biophys.* **1980**, *13*, 317–338.
(31) Baldwin, J. Chothia, C. *J. Mol. Biol. 129*, **1979**, 175–220.

Table I
Phosphorylase Biochemical and Physical Characteristics

property	characteristics
molecular weight (dimer)	194 800
amino acids/monomer	841
functional unit	dimer
metabolic interconversion	serine 14-phosphate
cofactor	pyridoxal phosphate

Reaction Catalyzed

$$G_N + P_i \rightleftharpoons G\text{-}1\text{-}P + G_{N-1}$$
$$K_{eq} = 0.3$$

Covalent Activation

$$Pb \underset{\text{phosphatase}}{\overset{\text{kinase}}{\rightleftharpoons}} Pa$$
(inactive) (active)

Allosteric Activation

R is active conformer

$$Pb\ (T) \underset{\text{Glucose}}{\overset{\text{AMP}}{\rightleftharpoons}} Pb\ (R)$$
$$L = 1/3000$$

T is inactive conformer

$$Pa\ (T) \underset{\text{Glucose}}{\overset{\text{AMP}}{\rightleftharpoons}} Pa\ (R)$$
$$L = 1/3$$

allosteric activators	promote R conformation	K_d, mM
AMP	intersubunit AMP/ATP site	0.002
Glycogen	storage site	1
P_i	Serine-14	
substrates P_i, G-1-P	active site	3
F-1-P, UDPG	inhibitors at active site	0.1-1

allosteric inhibitors	promote T conformation	K_d, mM
ADP, ATP	intersubunit AMP/ATP site	100
purines	I site, near active site	0.1
glucose	active site	5

posite ends of the twofold axis. One of these might be characterized as the catalytic face which mates with the 350 Å diameter glycogen particle. On this side is found the glycogen storage and activation site, the inhibitor "I" site, and the catalytic tunnel. The radius of curvature of this concave surface corresponds roughly to that of the glycogen particle itself. The opposing, regulatory face is richer in polar and charged groups exposed to the cytosol and presents a binding site for the allosteric effector AMP and recognition sites for the modifying enzymes.[32]

Activation and Conformational Changes

The structural analysis of an allosteric macromolecule requires a study of the isomeric set of structures that define its functional range. Hemoglobin has been studied in two radically different conformations, essentially the molecule with and without oxygen bound; the structural differences are discussed in detail by Baldwin and Chothia.[31] Although phosphorylase probably functions by means of a regulated transition between two major state conformations, there is certainly a spectrum of more or less stable intermediate states, so that an exacting structural analysis of more than one of these is required to understand the function and physical chemistry of the transitions. Indeed, the

(32) Fletterick, R. J.; Sprang, S.; Madsen, N. B. *Can. J. Biochem.* **1979**, *57*, 789–797.

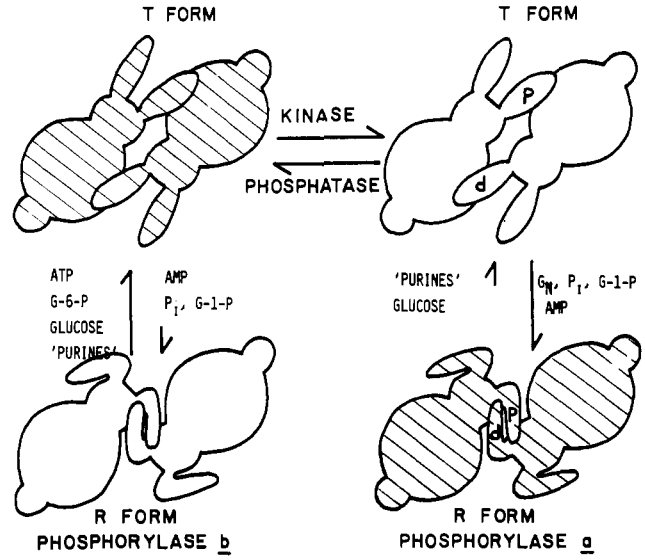

Figure 6. A schematic showing the interrelationship among the cardinal forms of phosphorylase. The striped species are the major conformers in vivo. Phosphorylase *b* is activated covalently by the kinase or by AMP and substrates. Note that the phosphatase can only function on the *inactive* conformer of phosphorylase *a*.

enzyme structures viewed in the crystal are subject to both allosteric and lattice forces and may well represent metastable conformers relative to those existing in solution. One might rationalize the functional response of phosphorylase in terms of each of the five separate sites listed in Table I contributing individually (but certainly unequally) to either the T or R conformation. Thus, phosphorylation of Ser-14 shifts the T $\rightleftharpoons$ R equilibrium toward R, but glucose returns the equilibrium back to T. Glucose and caffeine together merely enhance the ratio of T to R molecules. This model is certainly inadequate to address the relationship between structure and function, but it does provide a simple framework for understanding the allosteric response. Ligand binding in phosphorylase is cooperative (negatively or positvely) among different effector sites not only within the same subunit but also *between* the subunits as well.[17] These conformational responses are summarized at the level of our understanding of them in Figure 6, which also shows the connection between the phosphorylation–dephosphorylation event and the ligand-effected transitions.

In the following discussion we focus on the allosteric apparatus itself, the effector binding sites. At the present only a few of the structural changes that contribute to the R $\rightleftarrows$ T transition have been observed by crystallographic techniques. The full range of conformational isomers, from T to the fully activated R state, cannot be accommodated by the lattice of the crystals currently available for study. These crystals show weakened catalytic activity[33] because the structural response to the binding of allosteric effectors is sluggish.[26] The full response as elicited in solution has not been well-described by crystallographic techniques, but it has been probed in solution by ^{31}P NMR,[34,35] small-angle X-ray scattering (R. J. Fletterick, unpublished),

(33) Kasvinsky, P. J.; Madsen, N. B. *J. Biol. Chem.* **1976**, *251*, 6852–6859.
(34) Withers, S.; Sykes, B. D.; Madsen, N. B.; Kasvinsky, P. J. *Biochemistry* **1979**, *78*, 5342–5350.
(35) Withers, S.; Madsen, N. B.; Sykes, B. D. *Biochemistry* **1981**, *20*, 1748–1756.

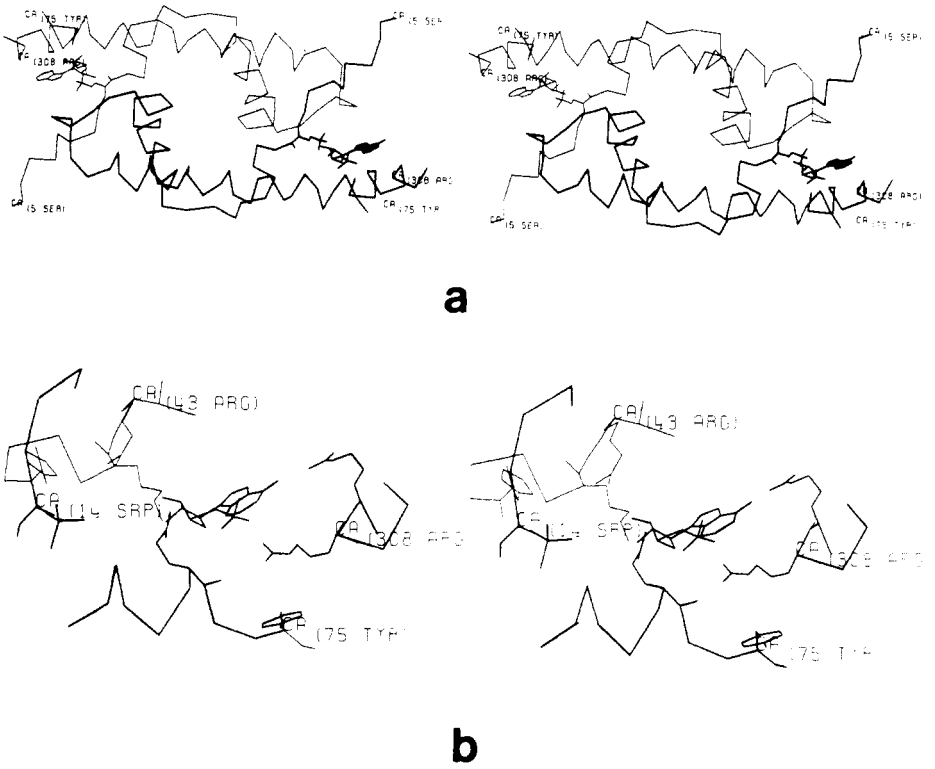

Figure 7. (a) A polypeptide chain tracing of the subunit contact region (viewed down the twofold axis of the dimer) in the neighborhood of Ser-14-P and AMP binding sites. The Ser-14-P side chain and the ligand are shown. The monomers are distinguished by light and bold lines. Note that the serine phosphate and the AMP bound to one monomer interact with an intervening loop of polypeptide chain from the twofold related subunit. Arg-10 is also probably involved in securing the N terminus to the enzyme surface through its interaction with Asp-32 on the opposing subunit. (b) A detail of the above, showing the protein side chains that interact with Ser-14-P: Arg-43′, Arg-69 (not shown), His-36, and with AMP: Lys-41′ and Gln-72 with the AMP ribose and Arg-308 and Arg-309 with the phosphate group.

and the use of cross-linking reagents or proteolysis.[36,37] Here we concentrate on the structural aspects of phosphorylase activation.

Phosphorylation of Serine-14

Protein phosphorylation is the basis of many primary regulatory mechanisms in biology.[38] The event in phosphorylase is under hormonal and nervous control and can be regarded as a temporally stable allosteric activation (Figure 1). The conformation of the covalently activated phosphorylase *a* is stable to moderate fluxes in the concentration of effector ligands. Thus, the creation of a serine phosphate at Ser-14 relieves the dependence upon AMP for activation, to which phosphorylase *b* is subject.[18] However, covalent modification is ultimately under the control of allosteric ligands, since high concentrations of negative effectors, e.g., glucose[39] and certain purines[40] (or nucleosides, as yet unidentified in vivo), strongly promote an inactive T state which renders phosphorylase *a* a good substrate for the phosphatase.[41,42] The covalent activation of phosphorylase is triggered by a complex, oligomeric, and allosterically regulated kinase which is itself activated covalently (by multiple phosphorylation) in response to nervous (Ca^{2+} fluxes) and hormonal (cAMP and epinepherine) signals.[5,18] The phosphorylation event, by mobilizing phosphorylase, announces the cell's requirement for G-1-P.

The covalent modification of Ser-14 is equivalent to the creation of a nondiffusable intramolecular allosteric effector.[43] The N-terminal 17 residues of phosphorylase form an extended polypeptide with partly helical secondary structure. In the crystal lattice formed by the *b* enzyme, and in solution as well, this chain is weakly bound at the enzyme surface and is disordered.[44] The reverse is true for phosphorylase *a*: here the Ser-14-P fastens the N terminus to the dimer surface through two specific ion-pair interactions,[43] one formed within and one between monomers (Figure 7). These contacts are important, not only in promoting an R structure, as does AMP, but also in maintaining allosteric cooperativity between the monomers. Thus, phosphorylase *b′* (with 17 amino acids removed from the N terminus) can be activated by AMP but shows no cooperativity.[45] The mechanism by which these effects are achieved is unknown, but it is clear that the structure and dynamics of the interaction at the interface is sensitive to the conformational state at the active site, some 33 Å distant. Both glucose and caffeine, effectors of the inactive T conformation, induce flexibility in the phosphorylated terminus, as measured

(36) Hajdu, J.; Dombradi, B.; Bot, G.; Friedrich, P. *Biochemistry* **1979**, *18*, 4037–4041.
(37) Dombradi, B.; Toth, B.; Bot, G.; Hajdu, J.; Friedrich, P. *Int. J. Biochem.* **1982**, *14*, 277–284.
(38) Krebs, E. G.; Beavo, J. A. *Annu. Rev. Biochem.* **1979**, *48*, 923–960.
(39) Helmreich, E.; Michaelides, M. C.; Cori, C. F. *Biochemistry* **1967**, *6*, 3695–3710.
(40) Kasvinsky, P. J.; Madsen, N. B.; Sygusch, J.; Fletterick, R. J. *J. Biol. Chem.* **1978**, *253*, 3343–3351.
(41) Madsen, N. B.; Fletterick, R. J.; Kasvinsky, P. J. *Cold Spring Harbor Conf. Cell Proliferation* **1981**, *8*, 483–495.
(42) Detwiler, T. C.; Gratecos, D.; Fischer, E. H. Biochemistry **1970**, *16*, 4818.
(43) Sprang, S.; Fletterick, R. J. *Biophys. J.* **1980**, *32*, 175–192.
(44) Weber, I. T.; Johnson, L. N.; Wilson, K. S.; Yeales, D. R. G.; Wild, D. L.; Jenkins, J. A. *Nature (London)* **1978**, *274*, 433–437.
(45) Graves, D. J.; Mann, S.; Phillip, G.; Olivera, R. J. *J. Biol. Chem.* **1968**, *243*, 6090–6099.

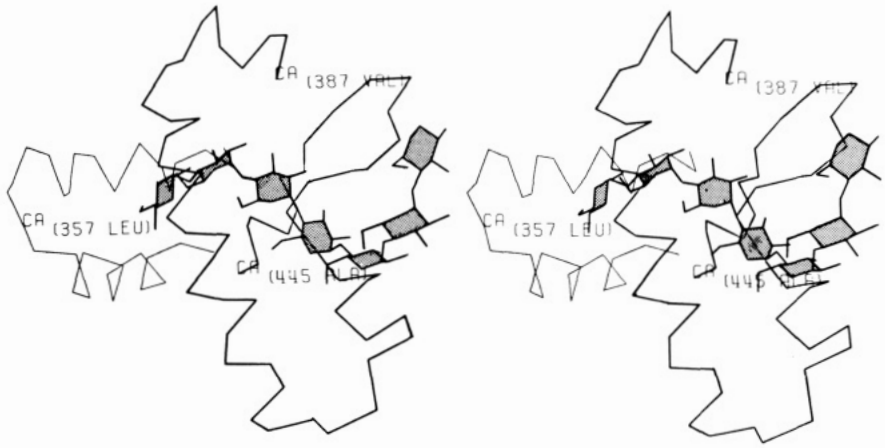

Figure 8. Binding mode of the oligosaccharide maltoheptaose (glucosyl rings are stippled) to the two-layered α-helical stack comprising the glycogen storage site. The oligosaccharide participates in hydrophobic interactions with protein residues located in the groove between the two external helices, and hydrogen bonds with residues which line this binding pocket. The oligosaccharide is bound in a helical conformation favoring an intramolecular hydrogen bond between the 2- and 3-hydroxyl oxygen atoms of adjacent residues. This pattern is broken only between the third and fourth glucosyl residues, where the hydroxyl groups form hydrogen bonds with the protein instead.

by ^{31}P NMR[34] and protease and chemical cross-linking studies,[37] and promote the attack of Ser-14 by phosphorylase phosphatase. It is likely that there are other differences in the intersubunit contact surface between phosphorylase *a* and *b*, but these are unknown at the present.

AMP/ATP Intersubunit Site

The binding site for AMP is only 15 Å distant from that of the Ser-14-P (Figure 7). Both sites are located at the subunit interface and involve residues from both monomers. AMP is bound at the vertex formed by the C terminii of two α helices (residues 50–75 and 287–310),[23] and its phosphate binding subsite is locally rich in arginine residues.[46] This tertiary structure bears no resemblance to the "nucleotide binding fold"[27] characteristic of many nucleotide binding sites. The AMP and Ser-14 sites share a loop of polypeptide chain (Lys-41–Arg-43). It could be rationalized that the two ligands stabilize similar R-like conformations and that, in consequence, activation by phosphorylation of Ser-14 is strikingly similar to activation by AMP. There is little synergism between AMP and Ser-14 phosphorylation activation of phosphorylase. Phosphorylase *a* has about 80–90% of its full activity in the absence of AMP while phosphorylase *b*, at 50 μM AMP is about 80% as active as phosphorylase *a*.[17] The two activation phenomena are independent but closely related. The cooperativity between the AMP site and the catalytic site 33 Å away has been directly observed in solution by ^{31}P NMR relaxation wherein G-1-P directly tightens the interaction of AMP with the enzyme.[35] Although the substrate and AMP sites are connected by a stretch of polypeptide chain (see below), no structural changes are noted in crystals of phosphorylase *a* when AMP is introduced. Curiously, phosphorylase *b* crystals tend to shatter under the same conditions.

Phosphorylase *b* is inhibited in vitro by ATP, which appears kinetically to be a simple competitor for the AMP site. There is no explanation for the fact that ATP bound at the AMP site does not activate phosphorylase *b*, even though the crystallographic study of Johnson et al.[46] show the two ligands to bind identically. ATP may stabilize a slightly different T conformation in solution and the phosphates of AMP and ATP may bind in a different way as they indeed appear to do in the crystal structure of phosphorylase *a* (Sprang and Fletterick, unpublished).

Activation by Glycogen

It was a surprise to find that glycogen binds at a "storage" site some 30 Å distant from the active site and 40 Å from the AMP site.[47] Kinetic analysis showed that maltoheptaose, a seven-residue oligomer of α-D-glucose, promotes the binding of substrates (G-1-P), P_i, and glycogen) at the active site and accounts for the activation and subunit cooperativity that has been observed for glycogen. This site (Figure 8) is located at a pair of helices which form the external layer of a double-layered helical stack covering the β-sheet core of the regulatory (N-terminal) domain. The polysaccharide binds in a left-handed helical conformation only modestly perturbed from strict regularity by the protein side chains that form the binding site.[48] Both hydrophobic and hydrophylic contracts are involved.[57] It is remarkable that the saccharides, and presumably glycogen, bind at least 20-fold more tightly to the storage site than the active site itself.[47] Indeed, crystallographic analysis has never revealed oligosaccharides bound at the catalytic site, even in partially activated crystals.

The structural changes induced by glycogen binding are the least well-understood of all the ligand activations studied either crystallographically or spectroscopically. When added with substrate analogues bound at the active site, maltoheptaose produces the most extensive structural changes within the crystal lattice observed for any combination of activators.[26] The mechanism by which glycogen promotes the binding of substrates is unknown, although the storage site is adjacent to two chain segments that are involved in the binding of substrates.

(46) Johnson, L. N.; Stura, E. A.; Wilson, K. S.; Sansom, M. S. P.; Weber, I. T. *J. Mol. Biol.* **1979**, *134*, 639–653.

(47) Kasvinsky, P.; Madsen, N. B.; Fletterick, R. J.; Sygusch, J. *J. Biol. Chem.* **1978**, *253*, 1290–1296.

(48) Goldsmith, E. G.; Sprang, S.; Fletterick, R. J. *J. Mol. Biol.* **1982**, *156*, 411–427.

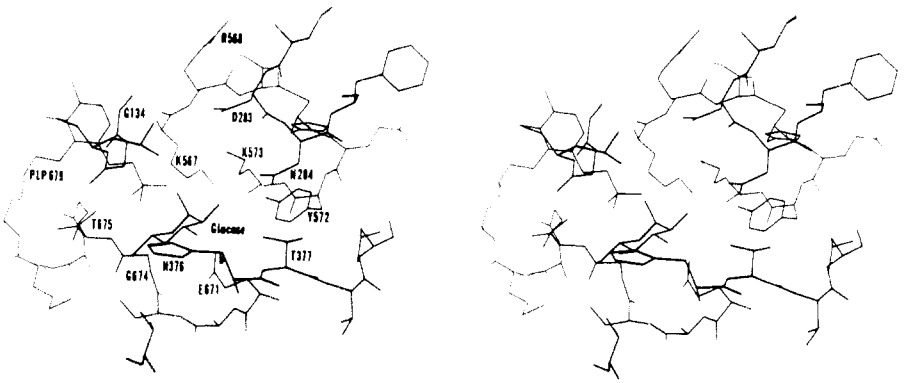

Figure 9. Residues in the immediate vicinity of the active site of the glucose-inhibited conformation of phosphorylase *a*. The view is approximately orthogonal to that of Figure 5. The coordinates were derived from a model building and refinement procedure using X-ray diffraction data at 2.1 Å resolution.[36]

The storage site is the in vivo glycogen binding locus. In fact, it is a standard purification scheme for the enzyme to spin down the glycogen from cell extracts and dissociate the phosphorylase with maltotriose.[49] All but 20% of the enzyme can be recovered this way. The presence of glycogen or oligosaccharide is also required to prevent the formation of phosphorylase tetramers,[4] the preferred but catalytically incompetent oligomer for the R state enzyme. Possibly the tetramer interface covers the catalytic surface, as would the glycogen particle.

Active-Site Inhibition and Activation

The static, T-like structures of phosphorylase *a* (Figure 9) reveal a complex active-site apparatus designed to bind both an inhibitor, glucose, and an activator/substrate, glucose 1-phosphate, at the same site with equally high specificity.[18,50] These two compounds are not simple competitive inhibitors, but are effectors of the opposing T and R conformations. The active site thus possesses a remarkable degree of discrimination which results in the use of substrate binding energy toward the stabilization of two quite different conformations. The structural basis of this active-site specificity is the division of the binding site into two distinct subsites, one interacting with the glucosyl moiety and the other, the phosphate ion or phosphate group. Glucose stabilizes a conformation in which the phosphate binding site is filled by residues of the protein itself, whereas G-1-P liberates these residues and permits cooperative conformation changes at the subunit interface and the activator and substrate binding sites on the opposing subunit.

The interactions between α-D-glucose and the T state enzyme have been well-defined.[50] This ligand is completely buried from solvent by the enzyme. All of the hydroxyl groups except the 1-position of the sugar, which is in a C-4 equatorial pyranose conformation, make hydrogen bonds with the protein side chains and main chain of Gly-134. It is noteworthy that the 1-hydroxyl, the position substituted by a phosphate moiety in G-1-P, does not interact. The "virtual" phosphate site in the T enzyme is occupied by the carboxyl group of Asp-283. This aspartic acid residue is probably an important key to the allosteric signals since it is part of a loop of chain that connects the AMP-activator site, through the helix terminating at Arg-309, to a sequence involved in subunit contacts (252–260)(Figure 2). The neighboring residue, Asn-284, is hydrogen-bonded to the 2-hydroxyl group of glucose. This loop of chain is the site of one of the major conformational changes that is definable in the crystal on activation of the enzyme.[14]

Both the substrate, G-1-P, and its analogue, glucose 1,2-cyclic phosphate (GCP, a potent inhibitor with K_i = 0.1 mM), bind to phosphorylase *a* crystals. These ligands cause local conformational rearrangements of sufficient magnitude to result in a partial loss of isomorphism with the native crystals, so that the structural changes cannot be modeled in detail. The parallel experiment with phosphorylase *b* crystals[15,16] reveals no such changes, suggesting that the catalytic site in the *b* crystals assumes at least a partially activated conformation. The substrate analogues bind similarly to both forms of the enzyme. In the *a* crystals we observe the glucosyl moiety bound in the same manner as glucose, making use of the same hydrogen-bonding groups in the enzyme, while the phosphoryl group takes up the position occupied by Asp-283 in the T state. In both *a* and *b* crystals, the phosphoryl moiety is less than 7 Å (phosphate to phosphate) from the PLP phosphate such that both lie on the same side of the glucosyl ring. A complete transition to the R state is not effected by this ligand in phosphorylase *a* as the conformation of the enzyme is constrained by the crystal lattice. Even so, the residues Asn-282–Phe-286 (Figures 5,9) are clearly observed to shift or disorder. Thus, the phosphate competes with an active-site residue for its own binding site. If the second activator, maltopentaose, is added to crystals containing GCP, conformational changes are so large as to be uninterpretable or more frequently the crystals disintegrate. It is noteworthy that AMP cannot substitute for maltopentaose in activating the enzyme in the crystals. At least in the crystal lattice, all effectors are not alike in function.

Inhibition at the (Purine) I Site

Although it is not known which physiological ligands function here, the I site is the best understood of the various effector sites since inhibitors stabilize the native structure and can be bound without disrupting the crystals. This observation is consistent with the idea that glucose and purine stabilizes the same T conformation. A variety of kinetics,[40,51] NMR,[34] cross-link-

(49) Appleman, M.; Krebs, E.; Fischer, E. *Biochemistry* **1966**, *5*, 2101–2107.
(50) Sprang, S. R.; Goldsmith, E. J.; Fletterick, R. J.; Withers, S. J.; Madsen, N. B. *Biochemistry*, in press.

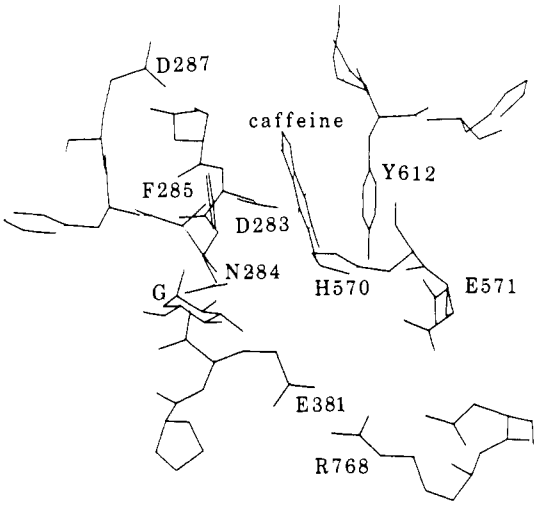

Figure 10. View in the orientation of Figure 5 of the entrance to the catalytic site. Caffeine and glucose are illustrated in their observed binding mode in the T-state crystals.

ing,[36,37] and X-ray diffraction[52] experiments suggest that purines and their analogues inhibit phosphorylase *a* synergistically with glucose by stabilizing the T conformer. The I site is analogous to the 2,3-DPG site in hemoglobin in that it exists only on the T conformer,[53] i.e., I site ligands are exclusive effectors. In contrast, AMP is a nonexclusive activator that binds to both T and R conformers. In both cases, the binding sites have different affinities and different conformations as the enzyme conformation switches between the T and R states.

The I site in phosphorylase *a* is shown to be thermodynamically and structurally (Figure 10) accountable solely by a three-ring stacking interaction involving intercalation of the fused heteroatom ring of the purine or analogue between the Tyr-612 (C-terminal domain) and Phe-285 (N-terminal domain) side chains.[53] Part of the ligand binding energy is used to stabilize the same loop (Asn-282–Phe-286) which binds glucose (at Asn-284) in the T state but which is known to move when the enzyme is activated by Pi or G-1-P at the catalytic site.

Concluding Remarks

The details of the dynamic and structural response for an allosteric macromolecule are best understood in hemoglobin.[31] The general principles that also apply to phosphorylase are as follows: (1) effector sites are near or at subunit (or domain) interfaces; (2) the nature of the effector protein binding interactions may be radically different for the different conformations; (3) the structural differences among the conformations are attenuated at large distances from the effector binding site; (4) not only is the tertiary structure altered by effector binding, the dynamics of the macromolecule (or portions thereof) change (via interactions at the subunit interface); (5) the conformation at the subunit interface is correlated with that at the allosteric effector sites by virtue of protein-chain segments that link the two.

Without knowledge of both R and T structures, it is premature to define the putative structural conformational linkages in phosphorylase, although it is evident that all of the allosteric sites and the catalytic site are interconnected either directly through an intervening chain segment or through nonbonded contacts, both within and between subunits. Studies of the activation process have been carried out with crystals of phosphorylase in the T state and require the use of two activators to obtain significant structural changes. Further, one of these must be an oligosaccharide such as maltopentaose which functions in the crystal through its interactions with the storage site on the N-terminal domain but not with the active site. The manner in which effectors modulate the conformation of phosphorylase in the solid state is highly sensitive to lattice forces and, as one might expect, the phosphorylation state of the enzyme. We have noted that AMP disorders phosphorylase *b* crystals, while G-1-P is bound without evident conformational changes. Just the opposite is true in crystalline phosphorylase *a*. The suspicion arises that, whatever the situation in solution or in vivo, the allosteric sites can be decoupled in the crystal lattice and the molecules within, in an allosteric sense, are conformationally chimeric.

The global aspects of the allosteric transition in phosphorylase are unknown at present. It is not yet apparent whether a shift in quaternary structure is implicated as in hemoglobin[31] or individual domains within the protein are rotated as in hexokinase upon binding glucose.[56] Certainly, an understanding of this event requires a detailed description of the fully R state phosphorylase structure. With recent successes in generating activated crystal forms both from bovine liver (Stern, Huang, and Thaller, unpublished) and rabbit muscle (Withers and Madsen, unpublished), such a description may be close at hand.

We acknowledge many discussions with Elizabeth Goldsmith, Steve Withers, and Neil Madsen. The work down in the authors' laboratory was supported by the NIH Grants AM06293 (SRS), AM26081 (RJF), and NSF Grant PCM80-01950 (RJF).

glycogen phosphorylase, 9035-74-9.

(51) Kasvinsky, P.; Schechosky, S.; Fletterick, R. J. *J. Biol. Chem.* **1978**, *253*, 9102–9106.
(52) Sprang, S.; Fletterick, R. J.; Stern, M. J.; Yang, D.; Madsen, N. B.; Sturtevant, J. S. *Biochemistry* **1982**, *21*, 2036–2048.
(53) Arnone, A. *Nature (London)* **1972**, *237*, 146–149.
(54) Klein, H. W.; Schlitz; Helmreich, E. J. M. *Cold Spring Harbor Conf. Cell Proliferation* **1981**, *8*, 305–320.
(55) Withers, S. G.; Madsen, N. B.; Sykes, B. D.; Takagi, M.; Shimomura, S.; Fukui, T. J. Biol. Chem. **1981**, *256*, 10759–10762.
(56) Anderson, C. M.; Zucher, F. H.; Steitz, T. A. Science (*Washington D.C.*) **1979**, *204*, 375.
(57) Goldsmith, E. J.; Fletterick, R. J. *Pure Appl. Chem.*, in press.