Laue and monochromatic diffraction studies on catalysis in phosphorylase b crystals

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Abstract

The conversion of substrate, heptenitol, to product, β -1-C-methyl, α -D-glucose-1-phosphate (heptulose-2-P), in crystals of glycogen phosphorylase b has been studied by Laue and monochromatic diffraction methods. The phosphorylation reaction in the crystal was started following liberation of phosphate from a caged phosphate compound, 3,5-dinitrophenyl phosphate (DNPP). The photolysis of DNPP, stimulated by flashes from a xenon flash lamp, was monitored in the crystal with a diode array spectrophotometer.

In the Laue diffraction experiments, data to 2.8 Å resolution were collected and the first time shot was obtained at 3 min from the start of reaction, and data collection comprised three 800-ms exposures. Careful data processing of Laue photographs for the large enzyme resulted in electron density maps of almost comparable quality to those produced by monochromatic methods. The difference maps obtained from the Laue measurements showed that very little catalysis had occurred 3 min and 1 h after release of phosphate, and a distinct peak consistent with the position expected for phosphate, in the attacking position was observed.

Data collection times with monochromatic crystallographic methods on a home source took 16 h for data to 2.3 Å resolution. Sufficient phosphate was released from the caged phosphate in the crystal from 5 flashes with a xenon flashlamp within 1 min for the reaction to go to completion within the time scale of the monochromatic data collection procedures. The heptulose-2-P product complex has been refined and the model agrees with that obtained previously with the major difference that the interchange of an aspartic acid (Asp 283) by an arginine (Arg 569) was not observed at the catalytic site. This change is part of the activation process of glycogen phosphorylase and may not have taken place in the current experiments because the caged compound binds weakly at the inhibitor site, restricting conformational change, and because activators of the enzymic reaction were not present in the crystal. In experiments with monochromatic radiation in which low phosphate concentrations were generated either by fewer photons or by diffusion of known phosphate concentrations, mixtures of substrate and product were observed. It was not possible through crystallographic refinement at 2.3 Å resolution to establish the fractional occupancies of the enzyme-substrate and enzyme-product complexes, but the results did indicate that the reaction was proceeding slowly, consistent with approximate calculations for the likely rate of the reaction in the crystal. In these experiments in which only partial reaction had occurred, there was connecting density between the phosphate and the O2 hydroxyl of the sugar substrate. The recent evidence for the influence of the O2 hydroxyl on the catalytic mechanism is discussed.

Keywords: enzyme mechanism; glycogen phosphorylase; Laue diffraction; time-resolved experiments

Laue diffraction, utilizing the white X-ray beam of bright synchrotron radiation sources and very short X-ray data collection times, has opened up the possibility of time-resolved studies on dynamic events in protein crystals. Applications have included

the description of the P21-GTP complex (Schlichting et al., 1990), the studies on the activation of caged chymotrypsin and the subsequent slow binding of an inhibitor (Stoddard et al., 1991), pH jump experiments with guanidinobenzoyl-trypsin (Singer et al., 1993), the structure of compound I intermediate of cytochrome c peroxidase (Fulop et al., 1994), and location of zinc-chelated waters in carbonic anhydrase (Lindahl et al., 1992). However, time-resolved Laue diffraction has not been widely used. The application requires methods to initiate reactions in the crystal and is effective only for those reactions that generate semi-stable intermediates that accumulate in the crystal and create a nearly homogeneous population. The applica-

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Abbreviations: GPb and GPa, glycogen phosphorylase b and a; heptulose-2-P, β -1-C-methyl, α -D-glucose-1-phosphate; DNPP, 3,5-dinitrophenyl phosphate; DTT, dithiothreitol; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; PLPP, pyridoxal 5'-diphosphate.

tion is unlikely to yield results for reactions that are accompanied by substantial conformational changes in the protein if these changes are constrained by the crystal lattice. The application requires robust protein crystals that are highly ordered and that can withstand the intense white-beam irradiation. Finally, the application requires careful data processing of the crowded Laue films generated from proteins with large unit cells. In the present work we show that Laue methods can be applied to molecules as large as glycogen phosphorylase (MW 97,444; 1,4- α -D-glucan:orthophosphate α -glucosyltransferase [EC 2.4.1.1]) to produce difference Fourier maps of almost comparable quality to those obtained by conventional methods. We have used the method, in conjunction with monochromatic methods, to follow catalysis in glycogen phosphorylase crystals. Preliminary accounts of this work have been published (Duke et al., 1991, 1992).

Glycogen phosphorylase is a classic control enzyme that catalyzes the phosphorolysis of glycogen in muscle and liver to yield glucose-1-P. The regulation of glycogen phosphorylase either by kinase-promoted phosphorylation on Ser 14 or by noncovalent binding of effectors (such as AMP, glucose-6-phosphate, glucose) may be understood to a first approximation in terms of the Monod, Wyman, and Changeux theory in which the enzyme exists in 2 (or at least 2) states: a T state with low affinity for substrates and low activity, and an R state with high affinity and high activity. X-ray structural studies have defined the conformations of T and R states for both phosphorylase b and phosphorylase a (Sprang & Fletterick, 1979; Sprang et al., 1988, 1991; Barford & Johnson, 1989; Acharya et al., 1991; Barford et al., 1991) and the binding sites for different ligands (e.g., Johnson et al., 1989, 1992, 1993; Oikonomakos et al., 1991; Johnson, 1992). The present experiments on catalysis in the crystal were performed with T-state GPb crystals in which the enzyme is known to be active but with reduced activity and lower affinity for substrate than the R form of the enzyme (Kasvinsky & Madsen, 1976).

The phosphorylase subunit (MW 97,444) has a compact almost spherical shape approximately 60 Å in diameter with 2 major projections of the chain that are involved in subunit-subunit contacts in the physiologically functional dimer. The catalytic site is situated at the center of the molecule, some 15 Å from the surface, at the base of a narrow tunnel formed by the interface of the 2 domains of the monomer and close to the essential cofactor, pyridoxal phosphate. The allosteric effector site (site N), which binds both AMP and glucose-6-P and a number of other phosphorylated ligands, is situated over 30 Å away at the subunit-subunit interface. The Ser 14 phosphorylation site in the activated R-state molecule is also in the vicinity at the subunit interface and some 12 Å from the AMP site. In the T state, access to the catalytic site is blocked by a loop of chain, residues 282–287, termed the 280s loop. The 280s loop is connected via a short β -strand to a helix, the tower helix, that together with the corresponding helix from the other subunit forms an important subunit-subunit contact. At the entrance to the catalytic site tunnel there is an inhibitor-binding site (site I) which binds purine and other aromatic compounds and which is formed by the aromatic residues Phe 285 (from the 280s loop) and Tyr 613. In the T state, an acidic residue, Asp 283 from the 280s loop, is directed into the catalytic site and is hydrogen bonded via 2 water molecules to the cofactor 5'-phosphate. On conversion to the R state, there are associated quaternary and tertiary structural

changes that link the allosteric effector and Ser-P sites via the subunit-subunit contacts to the catalytic site. In particular, the 280s loop is displaced, His 571 breaks its contact with Asp 283 and forms a new interaction with Tyr 613, and Arg 569 replaces Asp 283 at the catalytic site. The mutual interchange between an acidic group and a basic group at the catalytic site appears to be the major change that creates a high-affinity phosphate-recognition site for substrate while the displacement of the 280s loop allows access for bulky oligosaccharide and glycogen substrates.

The rate-limiting step in the reaction is the interconversion of the ternary phosphorylase-phosphate-glucosyl complex to the ternary phosphorylase-glucose-1-P-glucosyl complex (evidence reviewed by Graves & Wang, 1972) and this gives rise to the expectation that, provided data collection times are fast enough, a ternary enzyme-substrate complex might be detected in the crystal. Glycogen, the natural glucosyl substrate of phosphorylase, is too large to be used in crystal experiments. Linear oligosaccharides such as maltoheptaose are effective substrates, but their affinity for phosphorylase is 2 orders of magnitude less than for branched substrates (Hu & Gold, 1975) and no equilibrium binding of oligosaccharide at the catalytic site of phosphorylase has been observed in crystallographic experiments to date. The 7-carbon glycosyl substrate, heptenitol, has proved the most informative substrate in the diffraction studies (Johnson et al., 1990). In the presence of phosphate, phosphorylase catalyzes the conversion of heptenitol to heptulose-2-P in a reaction that exhibits similar characteristics to the action of the enzyme with its natural substrate (Klein et al., 1986). Heptulose-2-P is a potent inhibitor of phosphorylase ($K_i = 14 \mu\text{M}$) and is a dead-end product that is released only slowly from the enzyme. A combination of X-ray, NMR, and biochemical experiments (Johnson et al., 1989, 1990; Palm et al., 1990) have led to an explanation for the involvement of the essential cofactor pyridoxal phosphate in catalysis and to proposals for the catalytic mechanism. The substrate heptenitol binds to the catalytic site with all polar groups, with the exception of the ring oxygen, involved in hydrogen bonds both as hydrogen bond donors and acceptors. In the heptulose-2-P complex the phosphate position is stabilized by contacts to the main-chain atoms at the start of helix α 6, the side chain of Lys 574, and in the activated state by Arg 569, which shifts from a buried position in the T state and displaces an acidic group, Asp 283, from the catalytic site. The 5'-phosphate of the cofactor pyridoxal phosphate makes contacts to main-chain nitrogens at the start of the α 21 helix and to Lys 568. In the proposed mechanism (Fig. 1), the cofactor phosphate acts as a general acid to promote attack by the inorganic phosphate at the C7 atom. After protonation of the methylene carbon, the glucosyl-carbonium ion intermediate is stabilized by the negatively charged phosphate. There are no other suitable protein groups in the vicinity to promote stabilization. The reaction is completed by nucleophilic attack of the substrate phosphate on the carbonium ion to give heptulose-2-P. The transition-state stabilization by the dianion phosphate substrate within the confined and shielded environment of the catalytic site means that the reaction proceeds in the direction of phosphorolysis and not hydrolysis. The mechanism can be readily extended to the natural reaction (Johnson et al., 1990).

In previous time-resolved experiments with monochromatic radiation (Hajdu et al., 1987), several reactions were studied that included the phosphorolysis of heptenitol to heptulose-2-P and

Fig. 1. A simplified diagram of the proposed catalytic mechanism for the phosphorylase-catalyzed conversion of heptenitol to heptulose-2-P. The 5'-phosphate group of the pyridoxal (PXL) phosphate cofactor promotes general acid catalysis by inorganic phosphate on the C7 methylene carbon leading to the formation of a carbonium-oxonium ion intermediate. The transition-state intermediate is stabilized by the nearby presence of the negatively charged phosphate. The reaction is completed by nucleophilic attack of the phosphate at the C1 atom to form heptulose-2-P.

of maltoheptaose to glucose-1-P. Times for data collection were 45 min and 25 min, respectively. The former reaction gave a tantalizing glimpse of what could be a reactive ternary enzyme-substrate complex. In the current experiments, we have reduced the time interval for data collection to about 1 min and the earliest time for recording data to 3 min. In previous work, catalysis in the crystal was begun by diffusion of substrates into preformed crystals. Diffusion is rapid ($t_{1/2}$ for phosphate is less than 1 min) but diffusion of high concentrations of phosphate results in a transient disorder of the crystals which takes about 8–10 min to heal (Johnson & Hajdu, 1989). In the present work, we have explored the use of caged phosphate, 3,5-dinitrophenyl phosphate, in which the molecule is made biologically inert by covalent attachment of a photosensitive group and the reaction initiated by liberation of phosphate by photolysis.

Results

Spectral measurements

DNPP is photosensitive and is converted to 3,5-dinitrophenol and P_i (Fig. 2) by irradiation at 300–350 nm with a quantum yield of about 0.6. The quantum yield is comparable to that of the more frequently used caged ATP ((2-nitrophenyl)ethyl ester) compound (Corrie et al., 1992). DNPP has an absorption spectrum similar to that of 3,5-dinitrophenol (protonated form) with $\epsilon_{340} = 3,100 \text{ M}^{-1} \text{ cm}^{-1}$. Its photolysis can be monitored by measuring release of 3,5-dinitrophenolate ($pK = 6.8$; $\epsilon_{400} = 2,800 \text{ M}^{-1} \text{ cm}^{-1}$ [Parke, 1961; Kirby & Vargolis, 1967; Corrie et al., 1992]). The effective quantum yield of DNPP makes it an attractive compound for caged phosphate work, but the compound suffers from significant absorption at the illumination wavelength. Following a flash from a xenon flash lamp (wavelength 300–400 nm; energy 100 mJ per flash), a yellow layer ap-

peared on the front surface of the crystal that diffused to the far side of the crystal within less than a minute to give the crystal a uniform yellow color. Thus, the time for the liberated cage (and presumably the phosphate) to achieve a uniform concentration in the crystal was about 1 min. (A rather longer time was observed in work reported by Hadfield and Hajdu [1994] in which liberation of phosphate from DNPP was stimulated by illumination from a nitrogen laser with $\lambda = 337.1 \text{ nm}$.) Phosphorylase crystals themselves exhibit absorption at 330 nm from the pyridoxal phosphate, and this absorption also reduces the transmission of light through the crystal. (The optical density of a phosphorylase crystal 0.4 mm thick is about 1.1 at 330 nm [Hadfield & Hajdu, 1993].) The absorption of the liberated cage at 400 nm was an advantage in providing a ready diagnostic for liberation of phosphate but also provided a further barrier to transmission of light through the crystal following irradiation. Despite these difficulties, the spectroscopic measurements at 400 nm with a crystal of thickness 0.32 mm, which had been soaked in a solution of 21 mM DNPP, showed that the first flash from the xenon flash lamp liberated approximately 2.6 mM phosphate (12.3%) and thereafter each flash liberated a constant proportion of about 5–6% of the remaining DNPP (assuming the pH of the solution in the crystal was 6.7 and the pK for the phenol/phenolate equilibrium was 6.8) (Fig. 3). Similar results were obtained with crystals that had been soaked in 10 mM DNPP. The experiments showed that the amount of phosphate released was proportional to the concentration of caged phosphate and the number of 100-mJ flashes from the xenon flash lamp. The curves shown in Figure 3 were used to provide estimates for the amount of phosphate liberated in the Laue data set 1 and the experiments CHLITE, CHLITEA, DNP1, and DNP5. In later work the concentration of phosphate released was measured directly following flashing and before X-radiation (e.g., SRS1 and the Laue data set 2).

Fig. 2. Schematic diagram showing the photolysis of DNPP to 3,5-dinitrophenol and inorganic phosphate. 3,5-Dinitrophenolate absorbs at 400 nm, $\epsilon_{400} = 2,800 \text{ M}^{-1} \text{ cm}^{-1}$.

Laue data

Laue diffraction data were collected in 2 experiments. In one (Laue data set 1) the crystal was soaked in 50 mM heptenitol and 45 mM DNPP, and data were recorded before (data set DARK)

Fig. 3. Liberation of phosphate from DNPP as a function of the number of flashes. The plot shows $\ln[D/(D - P_i)]$ versus number of flashes for crystals soaked in 10 mM and 21 mM DNPP, where D is the initial concentration of DNPP and P_i the concentration of phosphate after flashing the crystal. The linear dependence of $\ln[D/(D - P_i)]$ on the number of flashes has been derived from the assumption that the amount of phosphate liberated is approximately first order and dependent on the concentration of unliberated DNPP and the number of flashes.

and after (3MIN and 1H) illumination with 5 flashes from the xenon flash lamp. In the other experiment (Laue data set 2: 19 h) the crystal was soaked in 50 mM heptenitol and 30 mM DNPP, and data were collected at various time intervals, but only those collected after 19 h were processed. A summary of the data processing statistics for the Laue data sets is given in Table 1. Considerable care was needed in the data processing in order to produce interpretable difference Fourier syntheses for this large protein. The films are crowded with spots. (Approximately 28,000 spots are recorded to a resolution of 2.8 Å on a film 125 × 125 mm placed 130 mm from the crystal.) It was important to refine crystal and film orientation parameters to obtain a precise prediction of spot position. The processing program LEAP utilizes a large area for estimation of background intensities (a box of 500 × 500 μm² from which peaks are subtracted using known spot size and position) and these improved background measurements contributed to the precision of the intensities. At 2.8 Å resolution, about 9,000 of the spots have separations less than the spot diameter (250 μm) and are monitored as spatial overlaps. Spatial overlap spots whose separations were greater than about half the width of the spots (150 μm) were deconvoluted using knowledge of the profiles (Wakatsuki, 1993, and in prep.) and these represented about 40% of the reflections flagged as spatial overlaps. Spots with separations less than 150 μm were rejected. The harmonic overlap reflections comprise almost all the low-resolution terms and their systematic higher-order harmonics (Cruickshank et al., 1987). Prior calculations had shown that omission of these terms in Fourier syntheses led to substantial ripples around the peaks and longer-range spurious peaks separated 1/2, 1/3, etc. of the unit cell from the true peak (Duke et al., 1992). It was important to rescue these terms by deconvolution of the harmonic overlaps reflections. Deconvolution of the doublet spots resulted in about 1,000–1,600 additional reflections per film pack and between

Table 1. Laue data statistics^a

Data set	Laue data set 1									Laue data set 2		
	DARK			3MIN			1H			19H		
Film pack	A1AF	A2AF	A3AF	A4AF	A5AF	A6AF	A10AF	A11AF	A12AF	E22AF	E23AF	E24AF
ϵ/μ	150	150	150	200	150	200	150	150	150	150	150	150
AFSCALE												
$I_A/I_B R_m^b$	0.078	0.087	0.089	0.083	0.081	0.085	0.083	0.086	0.099	0.085	0.090	0.089
No spots output	21,572	21,023	22,009	19,199	21,843	18,940	18,540	18,816	17,788	22,290	21,940	21,953
Overall R_m^c	0.089	0.109	0.122	0.094	0.114	0.135	0.105	0.119	0.149	0.094	0.097	0.090
UNSCRAM												
No doublets deconvoluted	1,647	1,449	1,423	1,554	1,431	1,187	1,291	1,193	952	1,573	1,368	1,319
LAUENORM												
No reflections output		14,498			12,193			11,884			14,718	
Normalization R^d		0.101			0.110			0.132			0.112	
Completeness (2.8 Å)		62%			52%			51%			63%	
Fractional change in F^e		0.129			0.144			0.156			0.123	
RMS ρ^f		144			180			175			127	

^a Laue data set 1, soak solution: 50 mM heptenitol, 45 mM DNPP. After 5 flashes the phosphate concentration was estimated to be 12–14 mM from extrapolation of spectroscopic measurements at 21 mM DNPP. Laue data set 2, soak solution: 50 mM heptenitol, 30 mM DNPP. Phosphate concentration was measured to be 0.6–1.5 mM. Three exposures, each 800 ms, were recorded at ϕ values differing by 11.5° with 6 films per film-pack to give data sets A1AF, etc.

^b I_A/I_B merging $R = \sum |I_A - sI_B| / \sum I_A$, where I_A and I_B are the A and B film intensities, respectively, and s is the calculated scale from the Victoreen coefficients.

^c Overall $R_m = \sum_h \sum_i |I - I_i| / \sum_h \sum_i I_i$, where I is the mean intensity and I_i the i th measurement of intensity for reflection h , and the summation is over all reflections and all films A through F for which the reflection is present.

^d Normalization R factor $= \sum_h \sum_i |I - I_i| / \sum_h \sum_i I_i$, where I is the mean intensity of reflection h after normalization scales to a single wavelength have been applied to the individual intensities I_i .

^e Fractional change in $F = \sum_h |F_{PL} - F_P| / \sum_h F_P$, where F_P and F_{PL} are the structure factor amplitudes for the native protein (monochromatic data) and the protein plus ligand (Laue data) and the summation is over all common reflections h .

^f The RMS difference in electron density calculated over the asymmetric unit with the maximum electron density set to 1,000 arbitrary units.

3,400–4,500 reflections per data set (Table 1). Finally, it was important to ensure that the resolution used in the data processing corresponded to the resolution of the data recorded. A theoretical method has been proposed for the estimation of the limit of resolution in Laue photographs that uses the clear gaps surrounding prominent zone lines in Laue diffraction patterns with the help of gnomonic projections (Cruickshank et al., 1991, 1992). In practice we found this method not easy to apply and the resolution limit was established by trial and error from integrations carried out at different resolutions which established the best resolution as 2.8 Å (Duke, 1993). In monochromatic experiments, phosphorylase crystals diffract to 1.9 Å resolution. The poorer resolution in the Laue photographs may be a consequence of radiation damage, the poor sensitivity of X-ray film at short wavelengths (the wavelengths at which most of the high-angle data are generated), and/or insufficient exposure to generate spots for the weak high-angle data. The Laue data, as judged from the merging R values (between 0.10 and 0.12), are satisfactory but are not as good as those recorded with monochromatic methods. The third exposure from the same volume of the crystal showed poorer intensity agreement statistics, indicating some damage. The total number of reflections output represent 62%, 52%, and 51% of the data to 2.8 Å resolution for the DARK, 3MIN, and 1H experiments, respectively. A plot of the completeness of the data for singlets and for singlets plus doublets is shown in Figure 4. Refinement of the structure observed from the 1-h Laue diffraction experiment is described later.

Laue data set 1: DARK, 3MIN, 1H

No direct spectroscopic measurements were made on the concentration of phosphate liberated in this experiment. However, the crystal was flashed under similar conditions to those described above in the spectroscopic work (Fig. 3). Extrapolation from these data to the Laue experiment in which the DNPP concentration was 45 mM gave the liberated phosphate concentration 12–14 mM.

The DARK difference map calculated from data before liberation of the phosphate showed heptenitol bound at the catalytic site of GPb, with additional density indicating a shift and firmer location of a water molecule that is hydrogen bonded to one of the 5' phosphate oxygens of pyridoxal phosphate and the O4 hydroxyl of heptenitol (Fig. 5A). There was also electron density for the binding of DNPP at the allosteric control site N, where the compound binds through its phosphate group to 2 arginine residues (discussed later). There was no definite evidence for the binding of DNPP at the inhibitor site I, which blocks the entrance to the catalytic site cleft. The 3MIN difference map calculated from data recorded 3 min after liberation of phosphate had a slightly higher noise level than the DARK map. Heptenitol and the ordered water were apparent at the catalytic site and there was additional, rather ragged density close to the position expected for the phosphate (Fig. 5B). In the 1H map calculated 1 h from the time of liberation of phosphate, the additional electron density is stronger and resolved from the water molecule (Fig. 5C). There is no connecting density between

Fig. 4. Histogram showing percentage completeness of data for the Laue data set DARK to 2.8 Å resolution for singlet reflections (cross-hatched) and for doublet reflections (shaded). The diagram shows the absence of low resolution singlet reflections.

the phosphate and the heptenitol positions even when the contour level is lowered. At site N, the electron density indicated that DNPP was bound as would be expected because photolysis had liberated only 30% of the caged phosphate. There was no evidence for binding of DNPP at site I. The Laue experiments demonstrated that liberation of phosphate from DNPP had taken place at the catalytic site but, within the time frame of the experiments and at the concentration of phosphate liberated, catalysis had not proceeded to saturate all the catalytic sites with product.

Laue data set 2: 19H

In this experiment, the phosphate liberated was recorded with the diode array spectrophotometer and found to be between 0.6 and 1.5 mM. The low phosphate concentration was a result of the poor alignment of the flash lamp bulb compared with previous experiments. The data recorded 19 h after liberation (Table 1) gave a difference map that was similar to the 1-h experiment described above. In addition to heptenitol and the ordered water, there was electron density at the position expected for the phosphate that was not connected to the heptenitol. DNPP was bound at the allosteric site N and not at the inhibitor site I. Because the difference map after 19 h indicated that the reaction had not gone to completion, the earlier time shots in this series were not processed.

Monochromatic data

Data collection statistics are shown in Table 2 and the results of the refinement of selected complexes are given in Table 3.

Data set DNPPD

The control experiment (DNPPD), in which 2.3-Å-resolution data (Table 2) were collected from a crystal soaked in 50 mM DNPP, showed the caged phosphate bound at the allosteric site N, where the major contact is between the phosphate group and

2 arginine residues, Arg 309 and Arg 310, and at the inhibitor site I, where the dinitrophenol group is sandwiched between the aromatic rings of Phe 285 and Tyr 613.

Data sets CHDARK, CHDARKA, CHLITE, and CHLITEA

Both experiments CHDARK, in which a crystal was soaked in 30 mM DNPP and 50 mM heptenitol and data collected immediately, and CHDARKA, in which a crystal was soaked in 45 mM DNPP and 50 mM heptenitol and data collected after a 24-h delay, showed similar results. DNPP was bound at sites N and I, with an indication that the binding at site I was weaker, heptenitol was bound at the catalytic site, and the water molecule that bridges the cofactor phosphate and the O4 of heptenitol was more ordered (Fig. 5D). The experiments showed that there was no apparent liberation of phosphate under conditions of X-radiation and that the DNPP molecule was stable in the crystal for long periods. Refinement of the complex, which included a manual shift of the side chain of Arg 309 to contact the phosphate of DNPP at site N as indicated in the difference map, showed the usual small conformational changes in the protein structure (e.g., shifts in positions of the side chains of Ile 136, Asp 283, Asn 284, His 377, Thr 378) that are characteristic of ligands binding at the catalytic site (Martin et al., 1990) and no further conformational changes. The starting conformation for heptenitol was a half chair (ring torsion angles C1-C2-C3-C4-C5-O5-C1-C2-C3 = -43° , 63° , -61° , 39° , -18° , 21°) as determined previously with the program COSMIC (Hadju et al., 1987). During refinement, the ring adopted a chair conformation (ring torsion angles -40° , 54° , -54° , 43° , -36° , 32°). The major shifts in atomic positions resulting from the change in ring geometry are at C1 (displacement about 0.3 Å) and the methylene carbon C7 (displacement about 0.8 Å). At the allosteric site, the phosphate of DNPP dominates the binding. The phosphate group makes 4 hydrogen bonds to arginine residues Arg 242, Arg 309, and Arg 310 and occupies a position similar to that observed for the phosphate moiety of glucose-6-P (Johnson et al., 1993). The remainder of the DNPP molecule makes few van der Waals contacts, the most significant of which are to the side chains of Arg 193 and Leu 68. The aromatic ring is between Trp 67 and Val 45' (from the other subunit), but these residues are too distant to contribute to binding (7 Å and 5 Å, respectively). The position of the aromatic ring is similar but not identical to the position of the glucose moiety observed in the glucose-6-P complex (Johnson et al., 1993) but displaced slightly toward the position occupied by the ribose of AMP bound at this site. Evidently, the allosteric effector site can accommodate a number of different groups with the binding directed by the phosphate. At site I, the binding of DNPP is weak as indicated by the electron density and the large *B* factors (Table 3). The aromatic ring is sandwiched between residues Phe 285 and Tyr 613. The phosphate and one of the nitro groups are exposed to solvent. The other nitro group points into the protein toward residues His 571 and the main-chain atoms of Asp 283 and Asn 284. The polar contacts to heptenitol are summarized in Table 4. Each hydroxyl group participates in a hydrogen bond to protein groups.

In the experiment CHLITE, a crystal was soaked under similar conditions to those of CHDARK (50 mM heptenitol and 30 mM DNPP) and then subjected to 6 flashes over a period of 30 s from the xenon flash lamp. It is estimated that approxi-

Fig. 5. Difference Fourier maps in the vicinity of the catalytic site of phosphorylase showing selected residues and the pyridoxal phosphate. (*Continues on facing page.*)

Table 2. Data collection statistics for monochromatic data sets^a

Data set	DNPPD	CHDARK	CHLITE	CHDARKA	CHLITEA	DNP1	DNP5	SRS1	SRS2	LOWPI
Soak solution	50 mM DNPP	50 mM Heptenitol, 30 mM DNPP	50 mM Heptenitol, 45 mM DNPP	50 mM Heptenitol, 45 mM DNPP	50 mM Heptenitol, 45 mM DNPP	50 mM Heptenitol, 45 mM DNPP	50 mM Heptenitol, 45 mM DNPP	50 mM Heptenitol, 30 mM DNPP, 2.5 mM AMP	50 mM Heptenitol, 3 mM P _i	50 mM Heptenitol, 3 mM P _i
No. of flashes	0	0	6	0	10	1	5	12	12	0
Estimated phosphate (mM)	0	0	9–10	0	21–22	3–5	12–14	3	—	3
Delay before data collection (h)	0	0	24	24	48	24	0	22	27	0
No. of observations	156,213	81,689	83,582	80,222	79,813	81,710	84,702	79,217	90,989	82,559
No. of unique reflections	39,287	34,609	35,296	34,874	34,057	34,773	35,915	34,373	41,969	34,739
Merging R_m	0.086	0.080	0.075	0.070	0.088	0.071	0.086	0.072	0.082	0.060
Completeness (2.3 Å)	85%	79%	80%	80%	78%	79%	81%	78%	87%	79%
Fractional change in F	0.131	0.117	0.134	0.125	0.192	0.168	0.174	0.120	0.135	0.096
RMS ρ	135	78	79	75	107	105	100	81	90	63

^a Symbols are defined in Table 1 except fractional change in F here refers to fractional change in amplitudes for monochromatic data sets for both native and ligand-complexed proteins. Phosphate concentration estimated from extrapolations of spectroscopic measurements made at 21 mM DNPP for data sets CHLITE, CHLITEA, DNP1, and DNP5. Phosphate concentrations measured directly for data sets SRS1 and LOWPI. For the data set SRS2, no spectroscopic measurements were made and the lamp alignment was different from the calibration conditions. Hence it was not possible to extrapolate.

mately 9–10 mM phosphate was liberated under these conditions, although direct measurements were not made. Data were collected after 24 h from the time of the first flash and the resulting difference map showed that the reaction had taken place in the crystal. In the experiment CHLITEA (50 mM heptenitol and 45 mM DNPP), in which 10 flashes were used to liberate an estimated 21–22 mM phosphate and data collected after a 48-h delay, the reaction had evidently gone to completion. Heptulose-2-P was observed bound at the catalytic site (Fig. 5E) and DNPP at site N and site I. The difference map showed no further conformational changes to those observed in the CHDARK experiment. This was confirmed by refinement of data set CHLITE and CHLITEA with XPLOR in which a simulated annealing cycle was included. The results of the refine-

ments were identical to within the precision of the data. Although the difference map for CHLITE indicated slightly lower connecting density between C1 and O1 than in CHLITEA, the refinement of the complex with the same protocols used for CHLITEA gave essentially the same result with B factors for the heptulose and phosphate components of 12 Å² and 33 Å², which are almost identical for those obtained from the refinement of the CHLITEA data (17 Å² and 34 Å², respectively). In both refinements there was no evidence for shifts in the side-chain positions of Arg 569 to contact the phosphate of heptulose-2-P nor for the displacement of the 280s-loop residues Asp 283 and Asn 284. Thus, in the present structure, the phosphate of the heptulose-2-P makes a contact through the OP2 oxygen to the 5'-phosphate of the pyridoxal phosphate and a

Fig. 5. Continued. A–C: Difference Fourier maps obtained from Laue diffraction data to 2.8 Å resolution with the coordinates of the refined structures from DNP1 superimposed. **A:** DARK (before flashing): heptenitol (Hpt 0) is observed bound to the catalytic site and a water molecule (top right) has become more ordered and shifted closer to the 5'-phosphate group of pyridoxal phosphate (PLP 999). **B:** 3MIN after 5 flashes from the xenon flash lamp to liberate phosphate from 45 mM DNPP. Some additional density corresponding to the phosphate position is observed. The map is noisier than the DARK map and the putative phosphate density is not resolved from the water molecule density. **C:** 1H after flashing. The phosphate peak is stronger and better resolved than in the 3MIN map. Contour levels are at 430 arbitrary units where the maps have been normalized so that the maximum density is 1,000 arbitrary units. **D–H:** Difference Fourier maps obtained from monochromatic data to 2.3 Å resolution. **D:** CHDARKA showing heptenitol bound at the catalytic site and no liberation of phosphate from DNPP before flashing. **E:** CHLITEA, in which data were measured after 10 flashes from the flash lamp to liberate phosphate from 45 mM DNPP followed by a delay of 48 h. The coordinates of the refined structure of the product heptulose-2-P obtained are superimposed. Maps D and E are contoured at a contour of 335 arbitrary units where the maximum electron density is 1,000 arbitrary units. **F:** DNP1, in which data were measured after 1 flash from the xenon flash lamp to liberate phosphate from 45 mM DNPP followed by a delay of 24 h. Some reaction has taken place but the reaction has not gone to completion. There is continuity of electron density from the phosphate peak to the O2 hydroxyl. **G:** DNP5, in which the crystal was subjected to 5 flashes to liberate phosphate from 45 mM DNPP followed by immediate data collection. The reaction has produced heptulose-2-P. **H:** LOWPI, in which the crystal was soaked in 3 mM phosphate for 10 min followed by immediate data collection. Very little reaction has taken place, but at a lower contour level (250 arbitrary units) there is connectivity between the phosphate peak and the O2 hydroxyl as in DNP1 (Fig. 5F). Maps F–H are contoured at a normalized contour of 300 arbitrary units.

Table 3. Refinement parameters^a

Data set	CHDARK	CHLITE	DNP1-1	DNP1-2	DNP1-3	DNP1-4
Ligand model	Heptenitol, DNPP	Heptulose-2-P, DNPP	100% heptenitol, 100% phosphate, DNPP	100% heptenitol, DNPP	80% heptenitol, 20% heptulose-2-P, DNPP	50% heptenitol, 50% heptulose-2-P, DNPP
No. of atoms	7,377	7,372	7,394	7,394	7,394	7,394
No. of reflections	34,505	35,088	34,561	34,561	34,561	34,561
Final <i>R</i>	0.202	0.205	0.215	0.218	0.216	0.215
RMS diff. bond lengths (Å)	0.019	0.018	0.020	0.020	0.019	0.018
RMS diff. bond angles (°)	3.6	3.4	3.7	3.7	3.6	3.6
Average <i>B</i> factors (Å ²)						
Heptenitol	17		22	21	23	12
Phosphate			68			
Heptulose-2-P						
Heptulose		12			14	29
2-Phosphate		33			14	35
Wat 896 OH3	19	14	23	26	20	19
DNPP(N)	57	42	53	48	43	44
DNPP(I)	78	70	69	63	58	58
Protein	29	30	35	35	27	37

^a The resolution range for all refinements was 100–2.3 Å.**Table 4.** Distances (Å) from polar groups to heptenitol, phosphate, and heptulose-2-P in the refined complexes

Ligand atom	GPb atom	Heptenitol CHDARK	Heptenitol and phosphate DNP1	Heptulose-2-P CHLITEA	Heptulose-2-P ^a
O2	Asn 284 ND2	3.1	3.2	3.3	
	Tyr 573 OH	3.2	2.8	2.9	3.2
	Glu 672 OE1	2.9	2.8	3.0	3.3
	Wat 890 OH1	3.0	3.0		
	H2P 998 OP4			2.8	2.8
	PHO 1 OP4		2.3		
O3	Glu 672 OE1	2.8	2.8	2.9	2.9
	Ala 674 N	3.0	3.1	3.2	
	Gly 675 N	3.1	3.1	3.2	3.2
O4	Gly 675 N	2.7	2.7	2.9	2.8
	Wat 896 OH3	2.7	3.0	2.8	2.8
O6	His 377 ND1	2.6	2.7	2.9	2.7
	Asn 484 OD1	—	3.1	2.7	2.9
O1 or OP1	Wat 896 OH3		2.8		
	Arg 569 NH2				3.3
OP2	PLP 999 OP2		2.4	2.7	2.3
	Wat 878 OH1		2.8		
	Wat 896 OH3		2.8	2.7	2.8
	Lys 574 NZ		2.8		
OP3	Asn 284 ND2		2.9		
	Arg 569 NH2			2.3	
	Tyr 573 OH		3.0	3.0	2.8
	Lys 574 NZ		2.4	2.6	2.9
	Hept 1 O2		2.3	2.8	2.8
OP4	Gly 135 N		2.7	2.7	3.2
	Arg 569 NH2				2.3
	Asp 283 OD1		3.1	3.0	
	Wat 878 OH1		2.9	2.8	

^a From Johnson et al. (1990).

further contact through the OP4 oxygen to the side chain of Asp 283. These contacts suggest that this site would have a lower affinity for phosphate than a site in which Arg 569 has displaced the Asp 283 as observed previously (Johnson et al., 1990). It is possible that the conformational change is inhibited by binding of the caged phosphate (or the liberated cage) at inhibitor site I. Although this binding appears to be weak, the ligand would help restrain the 280s loop in the closed conformation. The average *B* factors for heptulose-2-P and the water at the catalytic site are similar to those obtained for the protein atoms, but those for DNPP bound at the allosteric effector site N and at the inhibitor site I are significantly higher (Table 3). The contacts to the heptulose-2-P are illustrated in Figure 6B and are summarized in Table 4. The contacts are similar to those previously observed (Johnson et al., 1990) with the exception of those to Asp 283, Asn 284, and Arg 569, as discussed above. A short contact between the heptulose-2-P (OP2) and the cofactor phosphate (OP2) observed previously (2.3 Å) has improved to a more acceptable hydrogen bond distance (2.7 Å). The ring torsion angles of the glucopyranose ring were characteristic of the chair conformation. The bond angle C1-O1-P = 147.4° has increased from that observed previously (135°). The torsion angle O5-C1-O1-P = 208° is similar to that observed previously (224°). The average *B* factor for the phosphate moiety (33 Å²) is greater than that for the heptulose (12 Å²). A similar difference was observed previously in the refinement of the heptulose-2-P complex (Johnson et al., 1990) in which the values were 20 Å² and 7 Å², respectively, for the phosphate and sugar atoms with data to 2.9 Å resolution only. In the refinement of the glucose-1-P complex at 2.4 Å resolution, the *B* factors were 46 Å² and 36 Å², respectively, for the phosphate and glucose moieties (Martin et al., 1990). The glucose-1-P is bound less tightly than

heptulose-2-P, but both complexes exhibit higher mobility (or possibly less occupancy for heptulose-2-P) for the phosphate moiety compared to the sugar.

Data sets DNP1 and DNP5

Further investigations of the release of phosphate and the time scale of the reaction were made with the series DNP1 and DNP5, in which crystals were soaked in concentrations of 50 mM heptenitol, 45 mM DNPP, and subjected either to 1 flash and a 24-h delay before data collection (DNP1) or to 5 flashes followed by immediate data collection (DNP5). It is estimated that approximately 3.5–5.5 mM phosphate was liberated from 1 flash and 12–14 mM phosphate was liberated from the 5 flashes. The difference electron density map for DNP1 showed that the reaction had not gone to completion (Fig. 5F). The peaks at the catalytic site that cover the heptenitol and the phosphate positions are resolved. There is no connecting density via the C1 atom, but there is connecting density between the phosphate peak and the O2 hydroxyl of heptenitol. In DNP5 the electron density at the catalytic site showed formation of heptulose-2-P (Fig. 5G). In both DNP1 and DNP5 there was density for the unliberated caged phosphate at site N. At site I the density was weaker but gave an indication that this site was occupied. In DNP5 there was no density for the phosphate moiety at site I, indicating the possibility that only the liberated dinitrophenol had bound.

Least-squares refinement was carried out with data set DNP1 to establish if the ligands bound represented an active ternary complex of heptenitol and phosphate with the phosphate in the attacking position or a mixture of substrate heptenitol and product heptulose-2-P. In the refinement with the ternary enzyme-

Fig. 6. **A:** The refined structures of heptulose-2-P (solid bonds) and heptenitol and phosphate (open bonds) and selected residues at the catalytic site. The isolated sphere to the center right of the figure is the water that becomes more ordered and hydrogen bonds between the O4 hydroxyl of the sugar ring to the 5'-phosphate of pyridoxal phosphate. The O2 hydroxyl is hydrogen bonded to the phosphate oxygen and to Glu 672. The major difference between the covalent heptulose-2-P and the noncovalent heptenitol phosphate structures is in the position of the C1 atom of the glucopyranose ring and the orientation of the phosphate oxygens. **B:** The refined structure of the heptulose-2-P complex showing hydrogen bonds to residues at the catalytic site.

substrate complex (DNP1-1; Table 3), the phosphate (100% occupancy) was modeled as the dianion and van der Waals interactions were turned off in order that the phosphate should be free to adopt either a covalent or noncovalent position. Heptenitol and DNPP were included in their previously refined positions. This structure refined to an overall R value of 0.215 (refinement DNP1-1; Table 3). The resulting $2F_o - F_c$ Fourier synthesis indicated satisfactory positions for all atoms. There was no connecting density between the phosphate and the heptenitol. The phosphate refined to a position similar to that of heptulose-2-P. The polar contacts are summarized in Table 4. There are some close polar contacts between the phosphate oxygens and Lys 574, the O2 hydroxyl of heptenitol, and the co-factor phosphate. The van der Waals contacts were satisfactory with the exception of a close contact between OP1 and the C1 atom of heptenitol (2.5 Å), which might be expected if the phosphate were in the attacking position. The average B factor for the phosphate was 68 Å² compared with an average B factor for heptenitol of 22 Å², indicating that the phosphate group had higher mobility or was bound with lower occupancy than heptenitol in the crystal. In a second refinement (DNP1-2; Table 3), the phosphate was omitted from the coordinate file. The resulting R value was 0.218, which was slightly worse than in the previous refinement. The $2F_o - F_c$ map showed a small peak at the position of the phosphate group and provided definite evidence for additional matter at that site. In a third refinement (DNP1-3; Table 3), the notion of mixed substrate/product was explored. Heptenitol was included with 80% occupancy and heptulose-2-P with 20% occupancy. The resulting R value was 0.216 and the $2F_o - F_c$ map showed heptenitol and only very weak density for the phosphate. A difference map was calculated from these data in which the structure factors for the protein plus ligand were those calculated from the 80% heptenitol/20% heptulose-2-P complex and the native structure factors were those measured for the native protein. This difference map showed some density for the phosphate, in addition to the strong density for the heptenitol and connecting density via the O2 hydroxyl of the heptenitol, and the appearance was similar to the experimental map. In a fourth refinement (DNP1-4; Table 3), both heptenitol and heptulose-2-P were included with 50% occupancy. The resulting $2F_o - F_c$ map showed a stronger phosphate peak than in the third refinement and no connection between the phosphate and heptenitol. Comparison of the 3 refinements (DNP1-1, DNP1-3, and DNP1-4; Table 3) showed, as expected, that there was a strong correlation between B factor and occupancy.

The refinements indicated that phosphate was bound but it was not possible to discriminate between the 2 models (ternary substrate complex or mixture of substrate plus product). In searching for other criteria to discriminate the models, we note that, on the one hand, it was possible to simulate the appearance of the difference maps with an interpretation based on a mixture of substrate and product whereas, on the other hand, in the free phosphate model, the phosphate did not refine to a covalent position despite lack of van der Waals restraints, supporting the notion that its position is consistent with noncovalently bound phosphate. The position of the phosphorous atoms for the noncovalent and covalently bound phosphate are identical. The major differences between the noncovalent and the covalent structures lie in the slightly different orientations of the phosphate oxygens and in the geometry around the C1 atom of

the heptenitol/heptulose-2-P (Fig. 6). In heptenitol, C1 is trigonal and is shifted approximately 0.6 Å away from the phosphate compared to the tetrahedral C1 position in heptulose-2-P.

Data sets SRS1 and SRS2 (Table 2)

Spectroscopic measurements indicated that about 3 mM phosphate had been released in experiment SRS1 in which the lamp was in poor alignment. The resulting difference Fourier synthesis showed that, despite the long time delay between flashing and the start of data collection (22 h), catalysis had not proceeded to completion. The appearance of the map was similar to that observed for DNP1, with the phosphate peak resolved and connecting density to the O2 hydroxyl of heptenitol. The results of the refinement of heptenitol with or without phosphate were similar to those obtained for the data set DNP1 (Table 3). The difference map for data set SRS2, which was flashed under similar conditions to SRS1 (but for which there were unfortunately no spectroscopic measurements), showed that after a time delay of 27 h, heptulose-2-P was bound at the catalytic site. Although 2.5 mM AMP had been included in the crystal soak solutions, no AMP was observed to be bound at the allosteric effector site N. Only DNPP (which is present at much higher concentrations) was bound.

Data set LOWPI (Table 2)

The results with the caged phosphate indicated that the reaction was proceeding slowly with the small amounts of phosphate liberated. It was possible that the binding of the unreleased cage at the allosteric effector site N and at the inhibitor site I had inhibited the enzyme. Hence, an experiment was performed (LOWPI) in which a known concentration of phosphate, 3 mM (to mimic the concentration released in the caged phosphate experiments DNP1 and SRS1), was diffused into the crystal together with 50 mM heptenitol. Monochromatic data were collected immediately after the 10-min soak was completed (Table 2). The difference Fourier synthesis showed similar features to those observed for DNP1 and SRS1. Heptenitol was bound at the catalytic site and the phosphate peak was evident (Fig. 5H). At a contour level comparable to that used to display the DNP1 difference map, there was no connectivity between the phosphate, but at a lower contour level, connectivity from the O2 hydroxyl of heptenitol to the phosphate peak was apparent. There was no density for phosphate at the allosteric effector site N, and it appears that, at 3 mM concentration, phosphate is not able to saturate this site.

Refinement with Laue data set 1

The structure obtained from the Laue data set 1 1H experiment was refined with XPLOR. The starting structure was that obtained from the refinement with the monochromatic data set DNP1 in which heptenitol but not phosphate was included at the catalytic site and DNPP at the allosteric site. The Laue data set is only 51% complete and a cautious approach to refinement was adopted. All atoms were fixed except those within 6 Å of the heptenitol at the catalytic site. After 30 cycles of positional refinement of the 184 atoms, the R value was 0.216 for 10,171 reflections between 8 and 2.8 Å resolution. Examination of a $2F_o - F_c$ electron density map showed the small peak for the phosphate well resolved from the heptenitol with no electron density connecting the 2 molecules. The density for side chains

Arg 569 and Asp 283 was fragmented and, at low contour level, there was indication for alternative conformations of these residues. There was also a definite indication for a rotation in the position of the DNPP at the allosteric site. These residues were rebuilt and refinement continued for a further 30 cycles of positional refinement. The resulting R value was similar to that observed before (0.215), and the RMS deviations in bond lengths and bond angles were 0.02 Å and 3.6°, respectively. The $2F_o - F_c$ map indicated reasonable density for Arg 569 in its new position close to the phosphate peak, but the density for Asp 283 and the 280s loop was fragmented, suggesting disorder or multiple conformations. Although the refinement was satisfactory in that it supported the identity of the phosphate peak discrete from the heptenitol, refinement with limited data sets at moderate resolution requires careful evaluation with trial calculations to test the validity of the proposed conformational shifts. Such trial calculations are in progress and will be reported elsewhere.

Discussion

The binding studies with DNPP and heptenitol with no illumination from the flash lamp showed that the caged compound was stable in the crystal under the conditions of irradiation. The refined structure from the data set CHDARK defined the heptenitol complex and the interactions of the 7-carbon compound with the protein. The heptenitol refined to the chair conformation with standard bond lengths and bond angles despite a starting structure of the half-chair conformation. A chair geometry has also been observed for another ligand with trigonal geometry at C1, the hydroximogluconolactone when complexed with GPb (Papageorgiou et al., 1991). It appears that the catalytic site has no special preference for a half-chair geometry. This is consistent with the observation from stereoelectronic effects that cleavage of an α -glycosidic bond does not necessarily require distortion from the chair to half-chair geometry prior to bond cleavage (unlike cleavage of a β -glycosidic bond), although such geometry is essential for stabilization of the carbonium-oxonium ion intermediate once formed. D-Gluconolactone, a classical half-chair transition-state analogue for several glycosidase reactions, is not a tight inhibitor of GPb by itself (Gold et al., 1971).

In several experiments with monochromatic X-rays (CHLITE, CHLITEA, DNP5, SRS2), the reaction has gone to completion following release of phosphate and long incubation times. Crystallographic refinement showed satisfactory geometry for the heptulose-2-P at the catalytic site and reasonable interactions with the protein molecule, including a direct hydrogen bond from the product phosphate to the cofactor 5'-phosphate. It was surprising that the conformational change observed in the previous heptenitol-2-P complex (Johnson et al., 1990), in which Arg 569 displaced Asp 283 at the catalytic site to form the phosphate recognition site, did not take place. A possible reason may be provided by the binding of the DNPP (or the released cage) at the inhibitor site. This binding, although weak, would assist in restraining the 280s loop in the T-state conformation and thus in preventing the conformational change. In the previous experiments, a higher concentration of phosphate (50 mM) was used and an oligosaccharide activator was also present in the crystal and these may have assisted in the promotion of the conformational change. In the present complex, there is an unexpected

hydrogen bond from the product phosphate to the side chain of Asp 283.

The monochromatic data sets in which there appears to have been partial reaction (e.g., DNP1, SRS1, and LOWPI), and the Laue experiments in which there appears to have been no reaction or very small reaction, have been the most difficult to interpret largely because of the limited resolution of the data, the similarity in structure between the substrate and product complexes, and because we have no additional measurements, apart from the X-ray data, of the progress of the reaction in the crystal. Although a fine-time resolution of just over 1 min for data collection times with the Laue method has been achieved, the problem of spatial heterogeneity is more difficult to solve. In the crystal, some molecules may have bound substrate, some may have bound product, and others may have no ligand bound. Where there is a convenient signal to indicate the different states of the reaction, as for example in the work on aspartate aminotransferase (Malashkevich et al., 1993), then it is possible to use this evidence to guide refinement of complexes with fractional values for the contributions of the appropriate intermediates. In the absence of this knowledge, the refinements of the DNP1 data set showed that X-ray measurements at 2.3 Å resolution are not sufficient to distinguish between different interpretations for a protein the size of glycogen phosphorylase, although such interpretations may well be possible for smaller proteins and where there is a greater distinction between the structures of the enzyme-substrate complex and the enzyme-product complex. Hence, we must use other methods of inference and comparison to establish the likely nature of the complexes in the crystal.

The phosphorylase-catalyzed conversion of heptenitol to heptulose-2-P results in a product that is tightly bound ($K_i = 14 \mu\text{M}$), and product saturation of the catalytic site can be readily achieved once a sufficient number of substrate molecules have been turned over. The substrate, heptenitol, has a $K_m = 15 \text{ mM}$ (Klein et al., 1986). Concentrations of heptenitol in the crystal were high (50 mM) and thus it is expected that the catalytic site will be saturated at the start of the reaction but that heptenitol will be replaced by heptulose-2-P as the reaction proceeds. Estimation of the likely saturation by the substrate phosphate is problematic. The K_m for phosphate of GPb in the absence of AMP is about 80 mM (Buc, 1967), and, in the reverse reaction, the K_m for glucose-1-P for GPb in the presence of IMP (similar to the crystallization conditions) is about 37 mM (Black & Wang, 1968). However, experiments with activated phosphorylase have shown that there is a synergy in substrate binding in which the enzyme-glycosyl binary complex has an approximately 10-fold lower dissociation constant for phosphate than the free enzyme and likewise the enzyme-phosphoryl complex has a 10-fold lower dissociation constant for the glucosyl substrate than the free enzyme (evidence summarized by Graves & Wang, 1972). We have observed such synergy in phosphate binding to a binary complex in experiments with the transition-state analogue norjirimycin tetrazole (E.P. Mitchell, S.G. Withers, A. Vasella, P. Ermert, & L.N. Johnson, in prep.). An increase in affinity between 2- and 10-fold for phosphate was observed. Likewise, although gluconolactone by itself is not a particularly potent inhibitor (K_i for the free enzyme is 7.5 mM), in the enzyme-glycogen-phosphate complex, the K_i is 0.025 mM (Gold et al., 1971). Hence, we may expect that, in the presence of heptenitol, there may well be synergistic binding of phos-

phate, and the affinity of GPb for phosphate is likely to be greater than that indicated from dissociation constants for the free enzyme.

The concentration of active sites in the tetragonal GPb crystals is 7 mM based on a calculation from the known molecular weight of the asymmetric unit in the phosphorylase crystal lattice. For full saturation in the crystal there must be at least an equivalent number of substrate or product molecules to saturate the site. In experiments in which a crystal has been soaked in a volume of liquid that exceeds the crystal size by many times, there are sufficient substrate molecules and binding saturation will be governed by the dissociation constant. But if, as in the present experiments, the phosphate is liberated from the caged phosphate within the crystal, then at least 7 mM must be liberated to satisfy the active sites and preferably a higher concentration to overcome the high dissociation constant. The caged phosphate DNPP has a good quantum yield, but its high absorption, together with the absorption of the GPb at the wavelength of illumination, has presented some challenges in attempts to liberate high concentrations of phosphate in a short period of time.

It is expected that the reaction will proceed slowly in the T-state crystals. The work of Kasvinsky and Madsen (1976) showed that reaction rates in the crystal were reduced by about 11–50-fold compared to those in solution. In the present experiments no activator molecules were present, although phosphate, at high concentrations, can activate T-state GPb. It is difficult to give a precise estimate of the effect on rates, but we note that rates for phosphate activation are at least 30 times less than those for AMP activation (Engers et al., 1970), and activation by oligosaccharide binding at the glycogen storage site can contribute a further factor of 10 (Philip et al., 1982). Finally, binding of DNPP at the inhibitor site may further slow the reaction. These approximations suggest that, under the crystal experimental conditions, rates could be at least 9,000 times slower than those measured for AMP-activated phosphorylase in solution. The turnover for the heptenitol arsenolysis (which is not complicated by product inhibition) is 18 min^{-1} at saturating substrate concentrations in the presence of AMP and oligosaccharide (Klein et al., 1986). Hence, rates in the crystal might be less than 0.12 per h. It is assumed that the reaction proceeds by the same mechanism despite the lower rates, but there is no direct evidence.

In the experiments where the reaction has gone to completion (CHLITE [phosphate concentration approximately 11 mM; delay time 24 h, data collection time 16 h]; CHLITEA [phosphate concentration approximately 22 mM, delay time 48 h; data collection time 16 h]; and DNP5 [phosphate concentration approximately 12–14 mM, delay time 0, data collection 16 h]), the results are consistent with the above calculations for the rate of reaction, the concentration of phosphate liberated, and the expected concentration of heptulose-2-P generated in the crystal. For example, in 16 h the enzyme molecule would have had the opportunity to turn over substrate twice and even 1 turnover would yield sufficient heptulose-2-P to saturate the catalytic site. The experiments confirm that release of phosphate from the caged compound can provide sufficient phosphate to allow the reaction to proceed within 16 h.

In 3 further experiments there appears to have been partial reaction. In DNP1 (phosphate concentration approximately 3.5–5.5 mM, delay time 24 h, data collection time 16 h), the time

scale of the experiment should have been sufficient to allow the reaction to go to completion, but the phosphate concentration was low, 5 times lower than in the corresponding experiment DNP5. A similar result was obtained with SRS1 (3 mM phosphate liberated, delay time 22 h, data collection 16 h). With the lower concentration of phosphate, the reaction proceeds more slowly and the amount of heptulose-2-P formed is limited by the phosphate molecules present in the crystal. At 3–5 mM phosphate there would be insufficient heptulose-2-P molecules to saturate all the active sites in the crystal (concentration 7 mM) even if all substrate molecules had been turned over. (We estimate that, because crystals adhere to the capillary tube with a small volume of liquid, the volume of reaction solution may be up to approximately 2 times greater than the volume of the crystal.) The possibility of limiting phosphate concentration was tested in the experiment LOWP1, in which a crystal was soaked in a solution of 3 mM phosphate for 10 min followed by data collection over 16 h. The appearance of the difference map indicated less reaction than in the DNP1 experiment. In this experiment, the enzyme in the crystal had access to sufficient phosphate at low concentration during the 10-min soak and, had the reaction proceeded to completion in that time, then all active sites would have been saturated with product. Prior to data collection, the bulk of the solution had been removed and thus the only phosphate present was that in the interstitial spacings of the crystal, as in the experiments in which phosphate was liberated from caged phosphate. The observation that reaction had not gone to completion in this experiment supports the notion that the reaction is proceeding slowly and raises the expectation that a ternary enzyme–substrate complex might be observable in shorter time intervals.

The Laue experiments with data recorded over a time interval of 76 s at 3 min and 1 h from the liberation of about 14 mM phosphate from caged phosphate gave difference maps in which there is a resolved phosphate peak with no connecting density to the heptenitol. The peak in the 1-h experiment is slightly stronger and better characterized than the peak in the 3-min experiment, and its position was substantiated in the $2F_o - F_c$ electron density map after the limited refinement in which phosphate was not included. In the Laue experiment recorded with very much lower phosphate (0.6–1.5 mM) and 19 h after the start of reaction, a similar small resolved peak is also observed for phosphate. Does this peak represent phosphate in the attacking position or does it represent a small amount of product, so small that the connecting density by the glycosidic oxygen does not show up? This question is difficult to answer. The refinement of the Laue data set 1 showed that the phosphate peak persisted even though the phosphate was not included in the refinement with no connectivity to the heptenitol, providing support for the notion that it represented phosphate in the attacking position. Moreover, in this structure, the restricted refinement indicated that Arg 569 had moved into the active conformation at the catalytic site and had displaced Asp 283.

Two lines of evidence suggest that the phosphate peak could represent the phosphate in the attacking position despite the low affinity for phosphate in the T state. Firstly, in the crystals of both R-state GPa and GPb grown from ammonium sulfate, a sulfate ion occupies a position that is very similar to the proposed phosphate attacking position (Barford & Johnson, 1989; Barford et al., 1991). Unfortunately, the R-state crystals exhibit some disorder and are not suitable for time-resolved Laue dif-

fraction. However, in monochromatic experiments, heptulose-2-P has been formed in the crystals and observed with difference maps (S. Wakatsuki, unpubl. obs.). The phosphate position of the heptulose-2-P is very similar to that observed in the T state. These observations suggest that in the R-state crystals where the phosphate-recognition site is fully formed, the position occupied by the phosphate is similar to that occupied in the T-state crystals. Secondly, the phosphate position in the norjirimycin phosphate complex with T-state GPb (where there is no reaction) is very similar to the phosphate position seen in the heptulose-2-P complex (separation 0.4 Å) and to the center of the peak seen in the Laue experiments (E.P. Mitchell, S.G. Withers, P. Ermert, A. Vasella, & L.N. Johnson, in prep.). This supports the notion that the phosphate binding sites in the covalent and noncovalent complex are similar. In the norjirimycin tetrazole experiment there is synergy between binding of the carbohydrate and the phosphate. No binding of phosphate at the catalytic site in the absence of other ligands, even at 100-mM concentrations, has been observed in crystallographic experiments, but in the presence of the tetrazole, phosphate is bound at 10-mM concentrations. It is possible that a similar synergy arises for the heptenitol phosphate binding from the interactions of the phosphate with the trigonal C1 and methylene carbons. No synergistic binding of phosphate is observed with glucose.

The proximity of the acidic group Asp 283 to the phosphate provides an explanation of why the reaction proceeds slowly. Phosphorylase allosteric behavior is characterized both by an enhanced affinity toward its substrate and an enhanced catalytic efficiency on the T to R transition (Black & Wang, 1968). Some effectors, such as IMP, enhance activity without giving rise to an increased affinity for substrate glucose-1-P, whereas AMP activation results in an increase in affinity and activity. The presence of the aspartate is likely to disrupt the proton relay system whereby the cofactor phosphate assists the inorganic phosphate in a general acid attack on the methylene carbon of heptenitol (Fig. 1). Instead the proton may be directed to the aspartate and not to the methylene carbon. Thus, full activation may require displacement of Asp 283 while increase in affinity for phosphate requires replacement of this group by Arg 569.

Over the years, crystallographic binding studies on glycogen phosphorylase have shown several different phosphate-recognition sites at the catalytic site (summarized by Leonidas et al., 1992, and Sprang et al., 1992). In complexes of R-state glycogen phosphorylase with the modified cofactor, PLPP, the β -phosphate was observed in a position displaced from the glucosyl binding site toward the main-chain nitrogen of Arg 569. A second position for the β -phosphate directed toward the substrate was observed in the work of Sprang et al. (1992). These authors have speculated that the first of the β -phosphate positions might represent a phosphate site in a prereaction ternary complex and that the second site might represent the site in the reactive complex. The second site is certainly closer to the position observed in the present studies, but is of course constrained by the covalent link to the α -phosphate of the modified cofactor PLPP. In complexes with glucose-1-P both in the T state and in the R state, the phosphate occupies a position that is not in contact with the 5'-phosphate of the pyridoxal phosphate (Johnson et al., 1990, 1992). This position also corresponds to the position observed for orthophosphate binding to T-state GPa (Withers et al., 1982). As previously discussed, this site is unlikely to be a productive site for the reaction pathway

(Johnson et al., 1990; Sprang et al., 1992). Only under the constraints of the additional methyl group in the β configuration in heptulose-2-P is the phosphate directed down toward the cofactor phosphate or in the compound glucose-1,2-cyclic-phosphate (Jenkins et al., 1981; Withers et al., 1982). In the natural reaction with oligosaccharide substrate, we proposed (Johnson et al., 1990) that the oligosaccharide played a role in directing the phosphate toward its productive site. The present studies on the ternary complex with a monosaccharide substrate show the phosphate in the presumed reactive site. From site-directed mutagenesis studies with *Escherichia coli* phosphorylase, in which the effects of mutations on both the forward and reverse reactions of phosphorylase were studied, Becker et al. (1993) have proposed that there might be 2 binding modes for the terminal glucosyl residue, an S mode observed for synthesis and a P mode observed for degradation. The crystallographic studies have identified only 1 binding mode for monosaccharides bound at the catalytic site of glycogen phosphorylase. This binding mode exhibits highly specific interactions. However, the 1 outstanding problem in the complete elucidation of the phosphorylase catalytic mechanism is the failure to observe oligosaccharide substrates bound in either the T-state or the R-state crystal forms of glycogen phosphorylase. Studies with the *E. coli* enzyme are in progress to address this problem.

The strong interaction between the phosphate oxygen and the O2 hydroxyl of the heptenitol that is observed in the experiments in which there has been partial catalysis has implications for mechanism. The importance of the internal hydrogen bond between the phosphate and the O2 hydroxyl in stabilization of the conformation of heptulose-2-P (which has an unusual torsion angle about the C1-O1 bond) has already been noted (Johnson et al., 1990). Glucose-1,2-cyclic phosphate is a reasonable inhibitor of glycogen phosphorylase ($K_i \approx 0.3$ mM) (Hu & Gold, 1978; Kokesh et al., 1977; Jenkins et al., 1981; Withers et al., 1982). In this compound the phosphate is covalently attached to the 2-oxygen and the phosphate is constrained to adopt a similar conformation as in heptulose-2-P. Thus, the O2 hydroxyl assists the binding and correct orientation of the phosphate substrate both with regard to the C1 position of heptenitol and to the 5'-phosphate of the cofactor. More recent evidence suggests that the O2 hydroxyl may also play a dynamic role in catalysis. Street et al. (1989) have shown that 2-deoxy, 2-fluoro-glucose-1-P is a very poor substrate for phosphorylase, with a V_{max} reduced by 3×10^5 but with little change in K_m compared to the natural substrate glucose-1-P. It was concluded that replacement of the hydroxyl group by the more electronegative fluorine resulted in destabilization of the charged carbonium-oxonium ion transition state and thus a decrease in turnover rate. The electronic contribution of the groups at the 2 position was further supported in experiments on the nonenzymic acid-catalyzed hydrolysis of deoxy sugars. Rates with the 2-deoxy glucose-1-P proceeded 3×10^3 -fold faster than the unmodified glucopyranosyl phosphate (Withers et al., 1989). In experiments with *E. coli* maltodextrin phosphorylase, which exhibits conservation of amino acids in the vicinity of the catalytic site to the rabbit muscle enzyme, a 2-fold increase in rate and a 30-fold increase in K_m was observed for the enzyme-catalyzed reaction using 2-deoxy derivative substrates (Becker et al., 1993). In all sugar substrates studied in the crystal, the O2 hydroxyl is involved in hydrogen bonds to Tyr 573 and Glu 672 and these interactions appear to be important for binding of sugar substrates (Fig. 6B).

However, site-directed mutagenesis experiments (Schinzel & Palm, 1990; Schinzel & Drueckes, 1991; Becker et al., 1993) with the *E. coli* phosphorylase have shown that mutations corresponding to E672D or Y573F (using the rabbit muscle numbering) do not substantially change the apparent affinity for substrate, but there is a decrease in k_{cat} of 8-fold for the Y573F mutant and 560-fold for the E672D mutant when the reaction is followed in the direction of degradation. (Similar values for K_m and similar changes in k_{cat} between wild type and mutants are also observed when the reaction is followed in the direction of oligosaccharide synthesis.) These results show that Glu 672 plays a significant role in catalysis, probably through at least 2 effects. Firstly, Glu 672 contributes to the correct electrostatic environment within the catalytic site through its network of hydrogen bonds to Lys 574 and Tyr 573 and slightly longer contacts with the cofactor 5'-phosphate group (4.3 Å) and the attacking phosphate (3.4 Å) and, secondly, Glu 672 may act through its interaction with the 2-OH to reduce the unfavorable electrostatic contribution of the electronegative oxygen to stabilization of the transition state and thereby allow for an increase in rate.

In summary, the work has shown that Laue diffraction data can give rise to difference maps of almost comparable quality to those obtained by conventional methods for a large protein molecule. The method developed for utilization of DNPP as a caged phosphate compound showed that sufficient phosphate could be released in reasonable times (e.g., 30 s) to stimulate catalysis. The results with the Laue diffraction data show that very little reaction has occurred within the first 3 min and 1 h of reaction and that the position of the phosphate peak is consistent with the position expected for phosphate in the attacking position. However, the monochromatic data sets in which the reaction has proceeded further have shown the difficulties in distinguishing between a ternary enzyme-substrate complex and a product-enzyme complex where there is no other diagnostic available to provide estimates of the different states of the enzyme in the crystal. The results from the partial reaction have served to emphasize the importance of the O2 hydroxyl in the reaction in assisting in the orientation and stabilization of the phosphate and in its influence on the stabilization of the transition state.

Materials and methods

Materials

DNPP was synthesized and characterized as described in Duke et al. (1991) and was a generous gift from Dr. D.R. Trentham (National Institute for Medical Research, Mill Hill, UK). Its photochemical properties have been reported (Kirby & Vargolis, 1967; Corrie et al., 1992). Heptenitol was synthesized by the method of RajanBabu and Reddy (1986) and was a generous gift from Dr. G.W.J. Fleet (Dyson Perrins Laboratory, Oxford, UK). All other chemicals were from Sigma.

Crystals of T-state rabbit muscle GPb were grown from solutions containing 25–30 mg/mL GPb, 10 mM BES, 10 mM magnesium acetate, 2 mM DTT, 2 mM IMP, pH 6.7, as described previously (Johnson et al., 1974; Oikonomakos et al., 1985). The crystals were a generous gift from Dr N.G. Oikonomakos (National Hellenic Research Foundation, Athens). The crystals are tetragonal spacegroup $P4_32_12$, unit cell dimen-

sions $a = b = 128.5$ Å, $c = 116.3$ Å, with 1 phosphorylase subunit MW 97,444 per asymmetric unit.

Spectroscopic measurements

Crystals were transferred to thin-walled quartz capillary tubes and soaked in solutions of substrate and DNPP in 10 mM BES, 10 mM magnesium acetate at pH 6.7 (standard buffer) for 0.5 h. The bulk of the solution was then removed so that the crystal adhered to the wall of the tube and the capillary sealed in the usual way for X-ray measurements. The release of cage was effected by a series of 1-ms flashes from a high-intensity xenon flash lamp (Hi-Tech Scientific, Salisbury, UK; Rapp & Guth, 1988) into a spot approximately 2 mm in diameter focused at approximately 20 mm from the lamp. The wavelength range for illumination of the crystal was 300–400 nm with a UG11 filter and the energy per pulse was estimated to be 100 mJ. The lamp required a recovery period of 5 s between flashes. Spectra were recorded with an Oriel diode array spectrophotometer adapted with fiber optic light paths for measurements on crystals (Hadfield & Hadju, 1993). In an experiment designed to measure the amount of phosphate liberated per flash, a crystal of T-state GPb (dimensions $2.2 \times 0.32 \times 0.2$ mm) was soaked in a solution of 21 mM DNPP in standard buffer for 0.5 h, mounted in a quartz capillary tube on a goniometer, and aligned with the crosshairs of the microscope in the spectrophotometer. Before and after flashing the crystal was rotated 90° so that the same face of the crystal faced the flashlamp and then the spectrophotometer beam. Spectra were recorded every 20 s after each flash for a series of 38 flashes. A similar experiment was performed with a crystal soaked in 10 mM DNPP.

Laue data collection

Laue diffraction photographs were recorded at station 9.7 (Helliwell et al., 1986) at the Synchrotron Radiation Source (SRS), Daresbury, UK, with X-rays in the wavelength range 0.2–2.5 Å. Three exposures with the spindle axis rotated 11.5° between exposures were recorded on CEA 25 reflex film with 6 films, with no interleaving foil per filmpack. The c axis of the crystal was inclined approximately 5.2° to the spindle axis. The crystal-to-film distance was 130 mm, the collimator size 200 µm; the circulating current of the synchrotron was about 200 mA and exposures were 800 ms. After each set of 3 exposures, the crystal was translated to expose a new portion of the crystal. It has been suggested (Clifton et al., 1991) that 2 exposures taken at different spindle settings should be sufficient to record about 80% of the data from a tetragonal crystal. However, because the crystal showed a change in position after flashing, exposures at 3 different spindle settings were used to generate the data set. The only alignment protocol was to ensure that the beam was not directed down a principal axis normal to a crystal face. The angular difference of 11.5° between exposures was selected from trial calculations. Analysis of the data showed that almost 90% of the data were recorded on the first 2 photos. After 3 exposures there was a burn mark running through the crystal where the beam had passed and this provided guidance for how far the crystal needed to be translated to expose a fresh portion. With a crystal of length 1.6 mm (parallel to the crystallographic c axis), about 4 data sets could be recorded per crystal.

Data set 1

A crystal of T-state GPb (dimensions approximately $1.6 \times 0.4 \times 0.4$ mm) was soaked in a solution containing 50 mM heptenitol, 45 mM DNPP in standard buffer for 0.5 h at 20 °C. The first Laue photographs were recorded before flashing (DARK) with the crystal-to-film distance set at 130 mm. The crystal was then removed from the hutch and flashed 5 times with the xenon flash lamp; the total time for the 5 flashes was 30 s and the crystal was not rotated between flashes. The crystal was returned to the hutch and 3 Laue photos recorded at 3 min (2 min 29 s, 3 min 8 s, 3 min 47 s); 15 min (14 min 39 s, 15 min 16 s, 15 min 54 s); and 1 h (58 min 52 s, 59 min 30 s, 1 h 8 s) where zero time was taken as the time for the first flash. The space in the hutch on station 9.7 is restricted and it was not possible to flash the crystal in situ on the camera. A rate-limiting step in the experiment was the time taken to replace the crystal after flashing (about 2 min). A further rate-limiting step was the time taken to rotate the crystal 11.5° and to advance the carousel between photos (about 35 s). The Laue photos recorded at 3 min showed no streaking of spots in contrast to photos recorded when phosphate had been diffused into the crystals (Johnson & Hajdu, 1989).

Data set 2: 19H

In this experiment, measurements from the diode array spectrophotometer were made after flashing the crystal to record the concentration of phosphate released. A crystal (dimensions $1.0 \times 0.6 \times 3.8$ mm) was soaked in a solution of 50 mM heptenitol, 30 mM DNPP, and 2.5 mM AMP in standard buffer for 0.5 h and mounted for X-ray measurements. AMP was added to the reaction mixture to facilitate activation of the enzyme. A set of 3 Laue photos were recorded from the crystal before flashing as described above except the crystal-to-film distance was 105 mm. The crystal was removed from the hutch and subjected to 12 flashes from the xenon flash lamp in a series of 3 sets of 4 flashes. The crystal was rotated 90° between each flash and after every fourth flash there was a 2.5-min delay. Prior experiments had indicated that this protocol for flashing resulted in liberation of greater quantities of phosphate than a protocol in which only 1 face of the crystal was exposed to the beam. However, it was found later that the bulb on the flash lamp was slightly out of alignment during these experiments and less phosphate was liberated than anticipated. The measurements from the diode array spectrophotometer indicated concentrations of phosphate between 0.6 and 1.5 mM phosphate. Laue data sets were recorded at 30 min, 1 h, 2 h, 4 h, 6 h, 9.75 h, and 19 h. Exposure times for the 19-h photos were 1 s with a beam current of 133 mA.

Laue data processing

Films were scanned with an Optronics P-100 microdensitometer using a 50- μ m spot and raster. Comparison of Laue diffraction patterns obtained for the DARK sets and for the subsequent sets after flashing showed there had been movement of the crystal. This appears to be a consequence of flashing the crystal and not the removal of the crystal from the camera because movement of the crystal was also observed in experiments on station 9.5, where there is room in the hutch to flash the crystal in situ on

the X-ray camera. It is likely that the heat from the light causes some of the mother liquor around the crystal to evaporate, which then results in a small shift of the crystal itself. The series of Laue photos recorded after flashing indicated that there was no further change in orientation of the crystal on irradiating with X-rays. Spot diameters on the film were measured to be between 200 and 300 μ m with the exception of a few strong nodal spots that had larger diameters. Crystal orientation parameters were determined with the SERC Collaborative Computing Project No. 4 (CCP4) suite of programs for Protein Crystallography (distributed by the Daresbury Laboratory, Warrington, UK) (Helliwell et al., 1989). A series of trial integrations indicated that data beyond 2.8 Å resolution could not be reliably measured and gave poor intensity statistics. Spots were therefore predicted with the program GENLAUE using a spot width (δ) of 250 μ m to 2.8 Å resolution. At this stage all spots were predicted, including those that were spatially overlapped (criterion for spot overlap parameter $\epsilon = 0$).

Integrations of intensities were performed with LEAP (Laue Evaluation and Analysis Programme, S. Wakatsuki, 1993, and in prep.). LEAP provides graphical display of fitted profiles, allows a measure of background intensities from a wide area of film (a box of dimensions 500×500 μ m centered on the spot), and provides deconvolution of spatially overlapped spots from knowledge of the spot profiles. Three cycles of film-parameter refinement were performed and the resulting spot predictions carefully assessed with the aid of the graphics display. Intensities were integrated and spots that had previously been flagged as spatially overlapped if their separation was less than the spot width 250 μ m were deconvoluted and integrated. Spots with separation less than a cutoff (ϵ) of 150 μ m were rejected. Spots with separation less than ϵ were rejected. For 3 of the film packs it was necessary to increase this value of ϵ slightly in order to obtain integration. The B to F films of the 6-film pack were scaled to the A film with the CCP4 program AFSCALE, a program that determines the wavelength-dependent film-absorption scale factors. These scale factors, which are represented by Victoreen coefficients, were used to deconvolute the doublet harmonic overlap reflections with the program UNSCRAM. Higher-order overlaps were judged to be too difficult to deconvolute with precision (Helliwell et al., 1989). Intensities were normalized to a single wavelength with the program LAUENORM in the wavelength range 0.5–0.9 Å and 0.95–2 Å. The program derives appropriate scale factors from a comparison of the intensities of symmetry-related reflections that have been recorded at different wavelengths. The lower cut-off of 0.5 Å was chosen to optimize the merging *R* agreement. Reflections generated at wavelengths below 0.5 Å were found to be weak and less precisely measured than those at longer wavelengths. The break in wavelength between 0.9 and 0.95 Å was made to avoid the discontinuity in scaling arising from the bromine-absorption edge of the film. Reflections for which $I < \sigma(I)$ were rejected. The unique reflections were scaled to the intensities for the native T-state GPb recorded on the synchrotron with monochromatic radiation and difference Fourier syntheses calculated.

Monochromatic data

Data were collected to 2.3 Å resolution on a Siemens-Nicolet Xentronics IPC area detector with graphite monochromated CuK α radiation from an Ru-200 Rigaku rotating anode X-ray

generator (60 kV, 60 mA). The crystal-to-detector distance was 16 cm and the detector angle was adjusted to 22°. Data frames of 0.2° were collected with exposure times between 90 and 120 s, for a total angular range of at least 90°. The total data collection time was 16 h. The data were processed using XENGEN (Howard et al., 1987) to produce a set of unique intensities, which were scaled to a native set of intensities recorded under similar conditions on the area detector. Difference Fourier maps were produced with the CCP4 software.

Several sets of monochromatic data were recorded to check the stability of DNPP under X-ray conditions and the binding sites in the crystal, to establish that the reaction with phosphate liberated from the caged phosphate could proceed in the crystal, to monitor the progress of the reaction with minimal number of flashes, and finally, to check the progress of the reaction in crystals that had been flashed under identical conditions to those used in the Laue experiments.

Data set DNPPD

A crystal was soaked in 50 mM DNPP for 0.5 h, mounted for X-ray measurements, and data collection begun immediately.

Data sets CHDARK, CHLITE and CHDARKA, CHLITEA

Crystals were soaked in solutions containing 50 mM heptenitol, 30 mM DNPP in standard buffer for 0.5 h. One crystal was subjected to 6 flashes with a 30-s delay between each flash and 180° rotation of the crystal after every 2 flashes. Data were recorded after a delay of 24 h (CHLITE). A second crystal was not flashed and data collection started immediately (CHDARK). Two further crystals were soaked in solutions containing 50 mM heptenitol, 45 mM DNPP in standard buffer. One crystal was subjected to 10 flashes over a period of 5 min and data collection begun after a 48-h delay (CHLITEA), whereas the other crystal was not flashed and data collection begun 24 h after the end of the soak (CHDARKA).

Data sets DNP1 and DNP5

Crystals were soaked in solutions containing 50 mM heptenitol, 45 mM DNPP in standard buffer for 0.5 h. One crystal was subjected to a single flash and data collected after a period of 24 h (DNP1). A second crystal was subjected to 5 flashes without rotation of the crystal and data collection begun immediately after flashing (DNP5).

Data sets SRS1 and SRS2

Crystals were soaked in solutions containing 50 mM heptenitol, 30 mM DNPP, 2.5 mM AMP in standard buffer for 0.5 h and mounted for X-ray analysis. The crystals were flashed 12 times and the crystals rotated 90° between each flash with a 2.5-min pause between flashes 4 and 5 and 8 and 9 to allow the buildup of DNP to disperse. This protocol was carried out at the SRS and was identical to that used in the Laue experiment data set 2 (19H). Crystals were returned to the home laboratory and monochromatic data collected commencing 22 h (SRS1) and 27 h (SRS2) after the start of the reaction. Spectroscopic measurements indicated that approximately 3 mM phosphate had been liberated for crystal SRS1 (dimensions 2.2 × 0.4 × 0.4 mm). As in the 19H Laue experiment, the lamp was out of alignment and less phosphate was liberated than anticipated. The crystal

SRS2 had dimensions 3.0 × 0.4 × 0.4 mm, but due to malfunction of the spectrometer, no spectral measurements were made.

Data set LOWPI

A crystal was soaked in 50 mM heptenitol, 3 mM phosphate for 10 min and data collection begun immediately after the end of the soak.

Refinement of the complexes obtained with data sets CHDARK, CHLITE, DNP1, and SRS1 were carried out using XPLOR (Brünger, 1988; Brünger et al., 1989a, 1989b) on a DEC 3000 model 400 workstation. Individual atomic *B*-factor refinement was carried out in the final cycles with target standard deviations of 1.5 and 2 Å² for bonded atoms and atoms linked by 1 other atom, respectively. The starting structure for heptenitol was the half-chair conformation that had been generated by the program COSMIC as described in Hadju et al. (1987). The heptulose-2-P structure was taken from the refined structure observed in the phosphorylase-heptulose-2-P complex (Johnson et al., 1990; PDB reference 6GPB). DNPP was generated from the structure of an aromatic ring with phosphate and nitro groups added with standard geometry. The starting protein structure was the native T-state GPb structure refined at 1.9 Å resolution (PDB reference 1GPB). Waters from the GPb structure (except those that had been displaced by the ligand) were included in the refinement. Standard XPLOR topology and parameter files were used. The parameter files for sugars were based on those derived by Ha et al. (1988), which have values for the dihedral force constants of 1.021 kcal/mol (X-C-C-X), 0.443 kcal/mol (X-C-O-X), and 0.928 kcal/mol (X-C-OE-X), and those of Weiss et al. (1990) who introduced a strong improper torsion angle force constant (500 kcal/mol/rad²) to restrain the chirality at the epimeric centers. Refinement consisted of 200 cycles of positional refinement followed by 60 cycles of *B*-factor refinement. Simulated annealing was carried out on one of the complexes (CHLITE) by heating to the nominal temperature of 3,000 K followed by a slow cool procedure in which the temperature was reduced by 25 K with 50 cycles of simulated annealing refinement with a time step of 0.005 ps at each temperature until the temperature of the bath (300 K) was reached. Final $2F_o - F_c$ maps in which the terms were weighted by Rayment weights (Rayment, 1983) were examined for satisfactory fit of atoms to the electron density. Estimates of the precision of coordinates taken from previous studies indicate errors on the order of 0.2 Å. Limited refinement of the structure was carried out with the Laue data set 1 in which atomic positions were fixed except for those atoms within 6 Å of the catalytic site.

Display of density maps and refined structures was performed using the molecular graphics program FRODO (Jones, 1978, 1985) implemented on an Evans and Sutherland PS390 graphics terminal. Potential hydrogen bonds were assigned if the distance between 2 electronegative atoms was less than 3.3 Å and if the angles formed between these 2 atoms and the preceding atom was greater than 90°. Van der Waals interactions were assigned where the separation between non-hydrogen atoms was less than 4 Å.

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