Kinetic properties of tetrameric glycogen phosphorylase *b* in solution and in the crystalline state

D.D. LEONIDAS, N.G. OIKONOMAKOS, A.C. PAPAGEORGIOU,
AND T.G. SOTIROUDIS

Institute of Biological Research and Biotechnology, National Hellenic Research Foundation,
48 Vas. Constantinou Avenue, Athens 11635, Greece

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Abstract

R-state monoclinic *P2*₁ crystals of phosphorylase have been shown to be catalytically active in the presence of an oligosaccharide primer and glucose-1-phosphate in 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM dithiothreitol, the medium in which the crystals are grown or equilibrated for crystallographic studies (Barford, D. & Johnson, L.N., 1989, *Nature* 360, 609–616; Barford, D., Hu, S.-H., & Johnson, L.N., 1991, *J. Mol. Biol.* 218, 233–260). Kinetic data suggest that the activity of crystalline tetrameric phosphorylase is similar to that determined in solution for the enzyme tetramer. However, large differences were found in the maximal velocities for both oligosaccharide or glucose-1-phosphate substrates between the soluble dimeric and crystalline tetrameric enzyme.

Keywords: crystal activity; dimer activity; glycogen phosphorylase; tetramer activity

Glycogen phosphorylase catalyzes the phosphorolytic cleavage of the α -1,4-glycosidic linkage occurring at the nonreducing ends of glycogen or polysaccharide chains resulting in the formation of glucose-1-P. The enzyme, in resting muscle, exists in the inactive dephosphorylated *b* form (GP*b*) but can be activated by the cooperative binding of AMP and inhibited by ATP and glucose-6-P (Graves & Wang, 1972). The main route for physiological activation is by phosphorylase kinase-catalyzed conversion to the phosphorylated *a* form (GP*a*) with the covalent attachment of a phosphate group to a single Ser (Ser-14). Conversion of GP*b* to GP*a* results in an enzyme that is active in the absence of AMP. GP*a* exhibits an increased affinity for AMP but a decreased affinity for ATP and glucose-6-P (Madsen, 1986; Johnson et al., 1989; Oikonomakos et al., 1992). Both GP*b* and GP*a* exhibit at least two conformational states: T (less active;

low affinity for substrate) and R (active; high affinity for substrate) in the terminology of Monod et al. (1965). In vitro GP*a* exists in a tetrameric state even at relatively low concentrations, whereas GP*b* at fairly high concentrations, when activated by AMP, reversibly associates to form tetramers (Dombradi, 1981; Munoz et al., 1983). In solution GP*a* tetramers have only 12–33% (at 20–35 °C) of the full phosphorylase activity but may be dissociated by glycogen (or oligosaccharides) and T-state inhibitors (e.g., glucose and caffeine) to give fully active or fully inactive dimers, respectively (Wang et al., 1965a,b; Metzger et al., 1967; Helmreich et al., 1967; Graves et al., 1968a; Huang & Graves, 1970; Withers et al., 1979).

Studies on structure/function relation and catalysis of phosphorylase have gained impetus in recent years from high-resolution structures now known for T-state GP*b* (Weber et al., 1978; Sansom et al., 1985; Acharya et al., 1991) and T-state GP*a* (Sprang & Fletterick, 1979; Sprang et al., 1988), respectively. A comparison of the two structures (Sprang et al., 1988) has shown the conformational changes that result from phosphorylation of Ser-14 and create a high-affinity AMP allosteric site. In the T-state, crystal structure lattice forces prevent communication of these changes to the catalytic site, which is over 30 Å

Reprint requests to: N.G. Oikonomakos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, Athens 11635, Greece.

Abbreviations: GP*b* and GP*a*, glycogen phosphorylase *b* and *a*, 1,4- α -D-glucan:orthophosphate α -glucosyltransferase (EC 2.4.1.1); glucose-1-P, α -D-glucose-1-phosphate; glucose-6-P, glucose-6-phosphate.

from the Ser-14 and AMP allosteric site. T-state GPb tetragonal crystals have been obtained in the presence of the weak activator IMP (Johnson et al., 1974; Oikonomakos et al., 1985) and T-state GPa tetragonal crystals have been obtained in the presence of the physiological inhibitor glucose (Fletterick et al., 1976). Both crystalline enzymes have shown a reduced specific activity when compared to soluble enzymes (Kasvinsky & Madsen, 1976). An understanding of the mechanism for the allosteric response has come from the recent crystal structure determination of R-state GPb (Barford & Johnson, 1989) and R-state GPa (Barford et al., 1991), which showed how large quaternary structure changes between T- and R-state phosphorylases resulted in tertiary structural changes at ligand-binding sites (Johnson & Barford, 1990). R-state GPb and GPa crystals have been obtained in the presence of ammonium sulfate (Fasold et al., 1972; Madsen et al., 1972; Barford & Johnson, 1989; Barford et al., 1991). The crystals are monoclinic, $P2_1$ with unit cell dimensions $a = 119 \text{ \AA}$, $b = 190 \text{ \AA}$, $c = 88.2 \text{ \AA}$, $\beta = 109.35^\circ$ with one tetramer MW 390,000 per asymmetric unit (Barford et al., 1991). It is well-known that sulfate anions are able to activate GPb to a considerable extent (Engers & Madsen, 1968) and that this activation could be further stimulated by AMP (Sotiroudis et al., 1979). A study of the effect of ammonium sulfate on the solution properties of GPb (Leonidas et al., 1990) showed that ammonium sulfate-activated GPb displays characteristics of GPa such as an enhanced affinity for AMP, a reversal of the enzyme inhibition by glucose-6-P and enzyme tetramerization. Under the conditions of crystallization, the tetrameric enzyme also exhibits a high affinity for AMP (apparent $K_d = 4 \mu\text{M}$) (Leonidas et al., 1990). The X-ray analysis of GPb activated by ammonium sulfate at a resolution of $2.9 \text{ \AA}$ revealed that sulfate mimics the Ser-14 phosphate by binding to the Ser-14 phosphate site and that the sulfate ion and N-terminal residues cooperate in forming a structure similar to the R-state GPa (Barford & Johnson, 1989; Barford et al., 1991). In fact, kinetic studies in solution have recently demonstrated that sulfate is unable to activate GPb' (Leonidas et al., 1991), a proteolytic species that lacks the N-terminal 16 residues (Graves et al., 1968b). Recently, Sprang et al. (1991) have determined the structure of an R-state tetrameric GPb reconstituted with pyridoxal 5'-diphosphate cocrystallized in place of the natural cofactor with AMP from polyethylene glycol. The overall structure is similar to the ammonium sulfate-activated GPb structure (Barford & Johnson, 1989) and gives a new insight on the mechanism by which AMP activates GPb.

Apparently, the significance of the R-state crystal structure will depend on whether the R-state crystals exhibit appreciable activity. It would be interesting therefore to know the structure/function relationship between the protein molecule in solution and the protein molecule in the crystalline state. The purpose of this study was to

determine whether the GPb tetramer stabilized by ammonium sulfate in solution is active and see how crystallization of this molecular species affects the catalytic properties of the enzyme within the crystal lattice. With this in view, a comparison of the enzymic activity of a protein molecule in the crystal with that of the tetramer and dimer in solution was undertaken.

Results

Properties of tetrameric GPb in solution

Activation by glycogen or oligosaccharide

Figure 1 shows that addition of glycogen to GPb (1.0 mg/mL) preincubated with 0.9 M ammonium sulfate and 1 mM AMP, prior to the measurement of enzymic activity, resulted in an increase of enzymic activity. This result is in agreement with previous findings on the activation and/or dissociation of GPa tetramers or AMP-activated GPb tetramers by glycogen and other polysaccharides (Wang et al., 1965a, 1970; Metzger et al., 1967; Graves et al., 1968a; Huang & Graves, 1970). The effect of maltohexaose (or maltoheptaose) on GPb activation, in the presence of 0.9 M ammonium sulfate and 1 mM AMP, was also studied. It was found (data not shown) that activation was dependent upon both enzyme and oligosaccharide concentration. However, although different extents of activation were observed at different concentrations of oligosaccharide for a given enzyme concentration, the

Fig. 1. Effect of glycogen on the activity of GPb. GPb (1.2 mg/mL) was preincubated at 30°C for 15 min in the assay buffer (10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol, pH 6.8) containing 1.06 M ammonium sulfate and 1.2 mM AMP. Glycogen was then added to give a final concentration of 1.05%, and aliquots were removed at various intervals indicated for measurement of activity at 30°C with glucose-1-P. The assay mixtures contained as final concentrations 1.0 mg/mL enzyme, 0.9 M ammonium sulfate, 1 mM AMP, 1% glycogen, and 20 mM glucose-1-P. Assay times varied from 20 to 60 s.

rates for activation were found to be much higher ($t_{1/2}$ about 1–2 min) than those obtained by glycogen (an approximate value of $t_{1/2} = 28$ min can be calculated from Fig. 1). Furthermore, progress curves of phosphate release for aliquots withdrawn 0.5–2 min after the addition of oligosaccharide and assayed at times varying from 10 to 60 s showed upward curvatures, indicating that even during the assay enzyme activation was not negligible. When GPb was preincubated with 0.9 M ammonium sulfate without AMP, activation by glycogen or oligosaccharide proceeded too fast to follow by the rapid assay method employed. Thus, under these conditions, the specific activity at the various times of preincubation remained constant throughout; enzyme activation by glycogen or oligosaccharide proceeded too fast even at 16 °C or 25 °C at various enzyme concentrations (0.01–3.50 mg/mL) tested (data not shown). Presumably, the assays in which activation by glycogen or oligosaccharide was too fast to measure had the GPb in the dimeric state.

Ultracentrifugation

To relate catalytic activity of GPb to its quaternary structure, ultracentrifugation experiments were carried out under conditions similar to those of the assay conditions (30 °C). Table 1 shows the sedimentation constants ($s_{20,w}$) determined for GPb (4 mg/mL) in the presence of ammonium sulfate and various ligands. The values of 7.9–8.8S and 12.4–14.1S obtained in this study closely correspond to those of a dimer and tetramer, respectively (Wang et al., 1965b; Cohen et al., 1971; Withers et al., 1979, 1982). The following results were obtained: (1) In the absence of AMP, GPb sedimented 35% as a dimer ($s_{20,w} = 8.7$ S) and 65% as a tetramer ($s_{20,w} = 14.0$ S). (2) The addition of glucose-1-P (40 mM) caused no significant change in the sedimentation pattern of the enzyme. (3) In the presence of 1 mM AMP, GPb sedimented 75% as a tetramer ($s_{20,w} = 13.1$), whereas a 25% fast-moving component was formed with an $s_{20,w}$ of 18.8S, probably representing higher aggregates. (4) T-state inhibitors such as glucose (50 mM) and caffeine (2 mM) induced complete dissociation of the tetrameric species. However, further addition of 1 mM AMP transformed the dimeric species into tetrameric, completely in the case of glucose (50 mM) but partially in the case of caffeine (2 mM).

Kinetics

Figure 2 shows a plot of specific activity of GPb in the presence of ammonium sulfate with 1 mM AMP as a function of protein concentration. The plot obtained is similar to that obtained previously for GPa (Graves et al., 1968; Huang & Graves, 1970), indicating a dissociation-association system. Because the specific activity, obtained at high protein concentrations, versus the reciprocal of enzyme concentration plot could not be extrapolated to zero activity an inactive tetramer seems unlikely (Fig. 3, inset). In the presence of ammonium sulfate, 1 mM AMP

Table 1. Sedimentation properties of GPb in the presence of ammonium sulfate^a

Addition	$s_{20,w}$, S	% of total
None	8.7	35
	14.0	65
AMP (1 mM)	13.1	75
	18.8	25
Glucose (50 mM)	8.2	100
Glucose (50 mM) + AMP (1 mM)	13.4	100
Caffeine (2 mM)	8.8	100
Caffeine (2 mM) + AMP (1 mM)	8.4	60
	13.8	40
Glucose-1-P (40 mM)	7.9	40
	12.4	60
Glucose-1-P (20 mM) + AMP (1 mM) ^b	13.5	80
	19.8	20

^a All sedimentation velocity measurements were performed with 4 mg/mL GPb in 9 mM β -glycerophosphate (pH 6.8), 0.5 mM EDTA, 1 mM dithiothreitol buffer in the presence of 0.8 M ammonium sulfate (pH 6.8) at 30 ± 1 °C at a rotor speed of 50,000 rpm. The movement of protein boundaries was followed by Schlieren optics. Pictures were taken at various times (15–35 min) after the rotor had reached full speed. The percentage of the components with different sedimentation coefficients was estimated by measuring the area under the sedimentation peak above the baseline (MSE Technical Publication No. 73, supplement) by cutting out the required area and weighing it. Error in the estimation of areas of poorly resolved components (or minor peaks) was as high as 20%.

^b With 4.5 mg/mL enzyme.

was able to induce substantial tetramerization even at very low enzyme concentrations. Huang and Graves (1970) have developed a mathematical approach for evaluating equilibrium constants and kinetic parameters from initial rate measurements for an enzyme undergoing a one-step dissociation-association reaction. Following this approach (see legend of Fig. 3), specific activity values of 1.70 and 55.6 μ mol/mg/min can be calculated for the tetrameric and dimeric GPb, respectively, and a dissociation constant (K_d) value of 0.0012 mg/mL for the equation $E_T \rightleftharpoons 2E_D$, where E_T and E_D represent the tetrameric and dimeric species, in 20 mM glucose-1-P, 1 mM AMP, 1% glycogen, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM dithiothreitol buffer (pH 6.8) containing 0.9 M ammonium sulfate. The theoretical line drawn in Figure 2, by using the calculated values for the association of GPb dimers to tetramers, fits very well the experimental points up to the concentration of 0.8 mg/mL. However, fitting at higher protein concentrations was not satisfactory. Similar deviations from theoretical lines were observed previously for GPa (Graves et al., 1968a; Huang & Graves, 1970). These observations may suggest that formation to some extent of higher aggregates, presumably inactive, at high enzyme concentrations cannot be excluded, in agree-

Fig. 2. Specific activity of GPb as a function of protein concentration. Activities were measured at 30 °C and pH 6.8 in the direction of glycogen synthesis by preincubating the enzyme with 20 mM glucose-1-P, 1 mM AMP, 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol buffer before initiating the reaction with 1% glycogen. Assay volumes were 100 μ L and times varied from 10 to 20 s. Control specific activities (not shown) were determined by preincubating GPb at various concentrations with glycogen at 30 °C for 15 min prior to the addition of the glucose-1-P/ammonium sulfate/AMP mixture (1-min assays). The mean value from six different enzyme concentrations was 56.6 ± 0.4 μ mol/mg/min. (—) Theoretical line calculated as described in the text and Figure 3.

ment with the ultracentrifugal results (Table 1). This also may be connected with the tendency of AMP-GPb to crystallize in the presence of ammonium sulfate with an octameric aggregation state (D.D. Leonidas & N.G. Oikonomakos, unpubl.). The corresponding values for the specific activity of GPa tetramer and K_d , in a buffer consisting of 0.5% NaCl, 40 mM β -glycerophosphate, and 1 mM dithiothreitol, were 3.3 μ mol/mg/min and 0.0353 mg/mL, respectively, at pH 6.8 and 30 °C (Huang & Graves, 1970). The thermodynamic parameters obtained for the dissociation of GPa tetramers at the temperature range 20–35 °C were 60 kcal/mol and 170 eu for standard enthalpy and entropy changes, respectively, with a standard free energy change varying between 10.0 and 7.56 kcal/mol (Huang & Graves, 1970). The value of the standard free energy change for the dissociation of GPb tetramers to dimers (calculated from the apparent K_d) is 11 kcal/mol.

Kinetics of crystalline GPb and GPa

It was found that suspensions of phosphorylase microcrystals in 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol buffer, pH 6.8 (assay buffer), were catalytically active. The microcrystals were found to be stable at both room temperature and 30 °C and not to crack during the assay experiment. Therefore, they did not have to be stabilized by cross-

Fig. 3. Analysis of specific activity data of Figure 2. Determination of specific activities and dissociation constant, K_d , for the equilibrium (tetramer) = 2(dimer), assuming active tetramer. v , v_D , and v_T represent observed specific activity, specific activity of dimer, and specific activity of tetramer, respectively. A value of $v_T = 1.7$ μ mol/mg/min, calculated by plotting specific activity observed at high concentrations (1.0–3.5 mg/mL) versus the reciprocal enzyme concentration from extrapolation to $1/E_0 = 0$ (inset), was used for calculation of $1/(v - v_T)$ and $E_0(v - v_T)$, where E_0 is total enzyme concentration. From the values of x and y intercepts, $v_D = 55.6$ μ mol/mg/min and $K_d = 0.0012$ mg/mL can be calculated according to Huang and Graves (1970).

linking as in the case of the tetragonal microcrystals (Kasvinsky & Madsen, 1976). Figure 4 indicates that crystal size influenced the specific activities, which were higher with smaller crystals than with larger crystals. The smaller crystals for both GPb and GPa were obtained within 1 or 2 h (see Materials and methods). The average sizes of these crystals were $20 \times 30 \times 2$ μ m³ for GPb and $20 \times 5 \times 5$ μ m³ for GPa, respectively. With crystals of the above size the assays were linear with time (Fig. 5), indicating an absence of diffusion limitation. Although crystal thickness was higher than the critical thickness defined by Makinen and Fink (1977), our results are in good agreement with those of Kasvinsky and Madsen (1976) who found no substrate diffusion effects for T-state tetragonal crystals of minimum dimension less than 10 μ m.

In order to rule out the possibility that substantial dissolution of microcrystals may occur during the assay, a number of control experiments were carried out. Thus, filtration, through minisart filters (0.22 μ m), of suspensions of microcrystals that had been preincubated for 60 min in the assay buffer either in the absence or in the presence of 80 mM glucose-1-P or 30 mM maltohexaose gave filtrates that showed no trace of activity (determined under conditions for dimeric enzyme with 1% glycogen, 20 mM glucose-1-P, 1 mM AMP, and 0.9 M ammonium sulfate). However, preincubation of microcrystals with 1% glycogen gave filtrates that showed a specific activity of about 0.1 μ mol/mg/min, suggesting that some

Fig. 4. Determination of specific activity for microcrystalline suspensions of GPb by extrapolation to zero time. GPb monoclinic microcrystals were prepared at 16 °C and pH 7.5 by incubating a solution of AMP-free GPb in the presence of 1.1 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 3 mM dithiothreitol, and 0.02% NaN₃ as described in the Materials and methods. Initial enzyme concentration was 10 mg/mL. Crystallization progress was stopped at the indicated times and microcrystals were washed with 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM dithiothreitol buffer, pH 6.8, several times until no activity of soluble enzyme could be detected. Activity was measured at 30 °C in the direction of oligosaccharide synthesis in the above buffer with 20 mM glucose-1-P and 15 mM maltohexaose. Assay time varied between 15 and 50 min. Crystalline enzyme concentration varied from 100 μ g/mL to 500 μ g/mL. Crystal sizes varied between approximately $20 \times 30 \times 2 \mu\text{m}^3$ (reached within the first 6 h) to $50 \times 80 \times 20 \mu\text{m}^3$ (reached within 72 h). The reciprocal of specific activity is plotted against crystallization time. A specific activity value of 0.32 $\mu\text{mol}/\text{mg}/\text{min}$ can be calculated by extrapolation to $t = 0$.

Fig. 5. Assay of crystalline GPa as a function of time at pH 6.8 and 30 °C with 20 mM glucose-1-P and 30 mM maltoheptaose in 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol, and 0.9 M ammonium sulfate. Experimental points fit closely a curve drawn according to the first-order reaction equation $k = \ln[x_e/(x_e - x)]/t$, where k is the first-order reaction constant, x_e is the amount of phosphate released at equilibrium, and x is the amount released at a given time t (Engers et al., 1970). The concentration of microcrystals was 71.7 $\mu\text{g}/\text{mL}$.

crystal dissolution may occur. Assuming a specific activity for the dimeric GPb of 55.4 $\mu\text{mol}/\text{mg}/\text{min}$, the solubility of the crystals in the presence of 1% glycogen (for 60 min) can be calculated to be less than 0.2%.

The effects of oligosaccharide and glycogen substrates as well as the activator AMP on the activity of phosphorylase in the crystal are summarized in Table 2 and compared with those in solution. GPb microcrystals gave a specific activity of 0.31 $\mu\text{mol}/\text{mg}/\text{min}$ in the presence of 15 mM maltohexaose and practically no activity in the presence of 1% glycogen. This result may be taken as an indication that glycogen is incapable of entering the crystal lattice. In the presence of 1 mM AMP, no increase in the catalytic activity was obtained with maltohexaose (15 mM) as substrate, but a significant increase in the activity (as compared to that in the absence of AMP) with glycogen (1%) as substrate. In the presence of both 15 mM maltohexaose and 1% glycogen added together, the activity was found to be additive. Because in solution phosphorylase activities with 1% glycogen or 1% glycogen plus 15 mM maltohexaose are approximately the same (Table 2) and given that glycogen cannot enter the crystal lattice but it can cause some crystal dissolution, this result indicates that more than 70% of the observed activity in this particular experiment can be attributed to the crystalline enzyme.

On the basis of these observations, experiments were carried out on the dependence of crystalline GPb and GPa activities with respect to either glucose-1-P or oligosaccharide in the absence of AMP to further characterize the kinetic properties of the enzyme in the crystal. The results are summarized in Table 3.

Effect of ammonium sulfate on oligosaccharide activation of soluble dimeric GPb

In order to compare the kinetics for crystalline and soluble dimeric GPb under identical conditions with oligosaccharides as variable substrates, the effect of ammonium sulfate on maltohexaose or maltoheptaose activation of dimeric GPb was studied at different glucose-1-P concentrations and constant concentration of AMP (1 mM). The effect of ammonium sulfate on the kinetic properties of dimeric GPb with glycogen as substrate has been reported previously (Leonidas et al., 1990). It has been shown that ammonium sulfate induces a dramatic stimulation of AMP-dependent activity of GPb (Leonidas et al., 1990). In contrast to GPb, GPa is inhibited rather than activated by ammonium sulfate (Leonidas et al., 1990, 1991). The kinetic parameters of GPb in the absence or presence of ammonium sulfate (0.9 M) are shown in Table 4. It appears that ammonium sulfate (0.9 M) causes a significant increase of catalytic activity of GPb as reflected in increased maximal velocity values for either maltohexaose or maltoheptaose. The relatively small effect of ammonium sulfate on glucose-1-P affinity most

Table 2. Activities of phosphorylase in the crystal and in solution in the presence of either oligosaccharide or glycogen substrates^a

Enzyme	Substrate	Activity ($\mu\text{mol}/\text{mg}/\text{min}$)	
		Crystal	Solution
GPa	Maltoheptaose	0.16 ± 0.01	5.6 ± 0.1
GPa	Glycogen	<0.02	44.7 ± 0.3
GPb	Maltohexaose	0.31 ± 0.02	0.16 ± 0.02
GPb	Glycogen	<0.02	4.9 ± 0.1
GPb + AMP	Maltohexaose	0.31 ± 0.02	20.0 ± 0.3
GPb + AMP	Glycogen	0.10 ± 0.02	55.4 ± 0.7
GPb + AMP	Maltohexaose + glycogen	0.43 ± 0.03	52.6 ± 0.6

^a Crystalline (100–200 $\mu\text{g}/\text{mL}$) and soluble enzymes (50 $\mu\text{g}/\text{mL}$) were assayed at 30 °C as described in the Materials and methods with either oligosaccharide (15 mM) or glycogen (1%) at a constant concentration of glucose-1-P (20 mM) without or with AMP (1 mM) in 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol buffer, pH 6.8.

likely arises from competitive inhibition by sulfate, which also binds to the catalytic site (Barford & Johnson, 1989).

It should be emphasized that all the experiments for dimeric GPb referred to in Tables 2 and 4 were carried out by using soluble enzyme derived from crystals that had been previously dissolved as described in the Materials and methods.

Discussion

Demonstration of enzymic activity in the crystalline state, comparable to that observed in solution, represents the most rigorous criterion for the definition of enzyme con-

formation in the crystal (Johnson, 1985; Hajdu et al., 1988). Enzyme activity data in the crystalline state are therefore of importance in relating the three-dimensional structure in the crystalline state to biological function as based on the X-ray diffraction evidence. This correlation has been proven in many cases (Doscher & Richards, 1963; Kallos, 1964; Bello & Nowoswiat, 1965; Quiocchio & Richards, 1966; Sluyterman & De Graaf, 1969; Rossi & Bernhard, 1970, 1971; Makinen & Fink, 1977; Vas et al., 1979; Mozzarelli et al., 1982).

Kinetic studies on the activity of glutaraldehyde cross-linked T-state tetragonal crystals of GPb and GPa (performed under the conditions of crystallization) have shown that the catalytic rate decreases markedly (Kasvinsky & Madsen, 1976). Comparison of V_{max} and K_m values of the crystalline enzyme with those obtained in solution reveals that crystallization predominantly affects V_{max} , the reduction ranging from 10- to 50-fold. Thus, on going from solution to crystal the K_m values for the substrates glucose-1-P and maltoheptaose do not change as compared to those of the soluble enzyme but V_{max} values decrease considerably (Kasvinsky & Madsen, 1976). Loss of catalytic efficiency was attributed to changes in the catalytic site caused by crystallization, i.e., to changes in the structure of the transition state as a result of lattice forces in the crystal (Kasvinsky & Madsen, 1976). In tetragonal crystals no binding of oligosaccharides at the catalytic site has been observed. Oligosaccharides (even at a concentration of 0.7 M) bind only to the glycogen storage site and not to the catalytic site (Sansom et al., 1984). Low affinity of the crystal for oligosaccharide is associated with the lack of access to the catalytic site from the solvent (Hajdu et al., 1987; Barford et al., 1988). However, formation of products was observed in crystallographic experiments in which mixtures of substrates were diffused rapidly into crystals and data collected within 1 h using the bright Synchrotron Radiation Source in Daresbury (UK) (Hajdu et al., 1987), indicating that conformational changes can take place in the T-state crystalline GPb that allow both substrates of the reaction to visit the catalytic site (Sansom et al., 1984; Johnson et al., 1989).

The kinetic experiments, presented here, with $P2_1$ monoclinic microcrystals of GPb and GPa produced by crystallization in the presence of ammonium sulfate indicate that both crystalline enzymes, when assayed in the direction of glycogen synthesis and under the conditions of crystallization possess intrinsic activity. The rate of 0.31 $\mu\text{mol}/\text{mg}/\text{min}$ for 20 mM glucose-1-P obtained in the presence of GPa establishes the catalytic activity of this form of the enzyme. It is remarkable that GPb crystals display activity with oligosaccharide without AMP. Addition of AMP produces no significant effect. Crystallographic studies have shown that the global tertiary and quaternary structures of the R-state GPb-sulfate-AMP complex (produced after soaking native R-state crystals in a solution containing 100 mM AMP and 1.0 M

Table 3. Kinetic parameters of GPb and GPa in the crystalline state^a

Enzyme	Variable substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{mg}/\text{min}$)	Fixed substrate
GPb	Glucose-1-P ^b	23 ± 2	0.70 ± 0.05	Maltohexaose
GPb	Maltohexaose ^c	85 ± 15	1.83 ± 0.27	Glucose-1-P
GPa	Glucose-1-P ^d	31 ± 5	0.45 ± 0.04	Maltoheptaose
GPa	Maltoheptaose ^e	54 ± 9	0.83 ± 0.10	Glucose-1-P

^a Crystalline enzymes (100–200 $\mu\text{g}/\text{mL}$) were assayed at pH 6.8 and 30 °C in 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol buffer as described in the Materials and methods at either various oligosaccharide concentrations at a fixed concentration of glucose-1-P (20 mM) or various glucose-1-P concentrations at a fixed concentration of oligosaccharide (15 mM) in the absence of AMP.

^b Concentration range was 5–60 mM.

^c Concentration range was 10–30 mM.

^d Concentration range was 10–77 mM.

^e Concentration range was 8–30 mM.

Table 4. The effect of ammonium sulfate on the kinetic parameters of soluble dimeric GPb^a

Variable substrate	In the absence of ammonium sulfate		In the presence of 0.9 M ammonium sulfate	
	K_m (mM)	V_{max} ($\mu\text{mol}/\text{mg}/\text{min}$)	K_m (mM)	V_{max} ($\mu\text{mol}/\text{mg}/\text{min}$)
Glucose-1-P ^b	7.8 $\pm$ 1.6	18.4 $\pm$ 1.1	12.0 $\pm$ 1.0	34.0 $\pm$ 1.3
Maltohexaose ^c	36.5 $\pm$ 6.0	44.9 $\pm$ 5.2	39.5 $\pm$ 9.7	74.6 $\pm$ 14.5
Glucose-1-P ^d	9.2 $\pm$ 0.4	9.4 $\pm$ 0.2	18.9 $\pm$ 0.8	33.0 $\pm$ 0.8
Maltoheptaose ^e	42.6 $\pm$ 7.0	25.0 $\pm$ 3.2	43.9 $\pm$ 9.5	62.9 $\pm$ 9.7

^a Soluble enzyme (50 $\mu\text{g}/\text{mL}$) was assayed at pH 6.8 and 30 °C at several concentrations of glucose-1-P and oligosaccharide and constant concentration of AMP (1 mM) in 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol in the absence or presence of 0.9 M ammonium sulfate. Enzyme and oligosaccharide were preincubated for 15 min at 30 °C before initiating the reaction with a glucose-1-P/AMP mixture (with or without ammonium sulfate).

^b Concentration varied from 6 to 30 mM at a constant concentration of maltohexaose (15 mM).

^c Concentration varied from 5 to 20 mM at a constant concentration of glucose-1-P (20 mM).

^d Concentration varied from 6 to 30 mM at a constant concentration of maltoheptaose (15 mM).

^e Concentration varied from 3 to 30 mM at a constant concentration of glucose-1-P (20 mM).

ammonium sulfate) are almost identical to those of unliganded R-state GPb (Barford et al., 1991). The kinetics of the enzyme crystals, studied over the same concentration of glucose-1-P (20 mM), do not differ markedly from those of the enzyme tetramer in solution (studied with 1% glycogen in the presence of AMP). Catalysis proceeds in the crystalline state with practically the same rates as in solution (Fig. 3; Table 3), indicating that the packing of the molecules in the crystalline state does not significantly alter the structure. Thus, tetramers in the crystalline state and in solution exhibit similar V_{max} values (1.83 $\mu\text{mol}/\text{mg}/\text{min}$ for crystalline GPb as determined by varying maltohexaose and 1.70 $\mu\text{mol}/\text{mg}/\text{min}$ for soluble GPb, determined at saturating concentration of glycogen). The K_m values of glucose-1-P and oligosaccharide for their complexes with phosphorylase-oligosaccharide and phosphorylase-glucose-1-P in the crystal were found to vary between 23–31 mM and 54–85 mM, respectively (Table 3). These values are comparable with those obtained for the dimeric GPb (in the presence of AMP) (Table 4). However, enzyme activity is reduced by a factor of about 40–50 on going from soluble dimeric GPb to crystalline tetrameric GPb, strongly indicating different conformations in the two physical states. Therefore, there remains the question of why tetramers are less active than dimers. Is it because the association of dimers to tetramers partially blocks access to the catalytic site (Barford & Johnson, 1992)? Is it because the glycogen storage site or a site remote from the catalytic site that helps the oligosaccharide bind (especially branched oligosaccharides) and which possibly contributes to catalysis is blocked in the tetrameric state and thus cannot activate? In the absence of structural data concerning oligosaccharide binding, a detailed description of this phenomenon must also await structure determination of the fully active dimeric AMP-activated GPb.

In conclusion, the results reported here indicate that the crystalline enzyme is just as catalytically active as the corresponding tetrameric enzyme in solution and that the kinds of conformational changes that may take place both in solution and in the crystal during the catalytic cycle are also very similar in the two physical states. These new kinetic data on the R-state phosphorylase will be helpful in relating the structure in the crystalline state to the catalytic mechanism based on the X-ray crystallographic evidence.

Materials and methods

Enzyme preparations

GPb was isolated from rabbit skeletal muscle according to Fischer and Krebs (1962) by using 2-mercaptoethanol instead of L-cysteine and recrystallized at least four times. GPa was prepared from GPb by the action of phosphorylase kinase (Cohen, 1973) and recrystallized three times. Traces of bound nucleotides were removed from both enzymes as described by Melpidou and Oikonomakos (1983).

Determination of enzyme activity in solution

Phosphorylase activity in the direction of glycogen synthesis was measured at 30 °C and pH 6.8 in a buffer consisting of 0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM dithiothreitol. With oligosaccharide as the variable substrate care was taken to ensure that the average increase in oligosaccharide chain length did not exceed two glucose units (Kasvinsky et al., 1978). The activity of the dimeric GPb was measured by preincubating the enzyme (5–50 $\mu\text{g}/\text{mL}$) with glycogen or oligosaccharide for 15 min at 30 °C before initiating the

reaction with the substrate mixture consisting of glucose-1-P, AMP, and ammonium sulfate. When tetrameric GPb was assayed, the enzyme (10–3,500 $\mu\text{g/mL}$) was pre-incubated with glucose-1-P, AMP, and ammonium sulfate for 15 min at 30 °C before initiating the reaction with glycogen. To minimize dissociation of tetrameric enzyme by glycogen during assay, aliquots were withdrawn at times varied from 10–20 s for the range 3,500–50 $\mu\text{g/mL}$ to 30–60 s for the range 30–10 $\mu\text{g/mL}$, respectively, and transferred in 0.2% sodium dodecylsulfate to stop the reaction. Inorganic phosphate released in the phosphorylase reaction was determined according to Fiske and Subbarow (1925).

Crystallization of GPb and GPa

Solutions of AMP-free GPb and GPa at a concentration of 10–13 mg/mL were prepared in 1.1 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 3 mM dithiothreitol, and 0.02% NaN_3 , pH 7.5, put in centrifuge tubes, and left overnight at 16 °C. Some precipitation occurred, and after centrifugation the enzyme solutions were transferred to 10- or 20-mL glass test tubes. Enzyme concentration in the supernatant was usually varied between 8 and 9 mg/mL. Microcrystals started to grow from 1 to 2 h. The crystals appeared to be reasonably uniform on optical examination under the microscope. However the size varied between different tubes left for different times. No amorphous material could be detected in the crystallization tubes. The largest crystals appeared in 2 weeks. At that time more than 90% of the protein was crystallized. Two different habits were grown: one approximately thin square plates, for GPb, and one prismatic (needle-shaped) crystals, for GPa, showing elongation along the *a* axis. X-ray crystallographic data, collected by using the Synchrotron Radiation Source at Daresbury Laboratory (Daresbury, UK), from big crystals showed that both characteristic habits were identical to the monoclinic forms of GPb and GPa (Barford & Johnson, 1989; Barford et al., 1991).

Determination of enzyme activity in the crystal

Phosphorylase microcrystals were prepared for assay in the following way: the mother liquor from the suspension of crystals was drawn off as completely as possible with a Pasteur pipette and replaced with ammonium sulfate buffer (0.9 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 6.8). The crystals were allowed to settle for 10 min (or in some cases forced to settle by centrifugation at very low speeds), and the supernatant was withdrawn. The crystals were gently washed with ammonium sulfate, and this procedure was repeated several times until no significant activity of soluble enzyme could be detected. The crystals were assayed essentially as described for the soluble tet-

rameric species, except that assay times varied between 10 and 50 min and that in most of the kinetic experiments glycogen was substituted by oligosaccharide. The reaction mixtures were incubated with mild shaking to ensure that crystals remained in suspension and were uniformly distributed throughout the solution. The same group of crystals, i.e., the smaller ones obtained within 1 or 2 h, was used for all the measurements concerning the determination of the kinetic parameters of substrates.

Ultracentrifugation

Ultracentrifugation experiments were carried out on a Beckman Model L5-50 analytical ultracentrifuge at a rotor speed of 50,000 rpm and 30 ± 1 °C. All sedimentation velocity measurements were performed with 4 mg/mL GPb in 0.8 M ammonium sulfate, 10 mM β -glycerophosphate, 0.5 mM EDTA, 1 mM dithiothreitol, pH 6.8, in the absence or presence of substrates and effectors as indicated. The movement of protein boundaries was followed by Schlieren optics. Pictures were taken at various times 20 min after the rotor had reached full speed. Sedimentation coefficients calculated by the sedimentation velocity method were corrected for viscosity and density of the buffer to water at 20 °C (Oikonomakos et al., 1985).

Protein concentration

Protein concentration of soluble enzyme was determined from absorbance measurements at 280 nm using an absorbance index $A_1^{1\%} = 13.2$ (Kastenschmidt et al., 1968). Protein concentration of the crystalline enzyme was either similarly determined spectrophotometrically by measuring the protein concentration of suspensions of crystals diluted 1:40 with 10 mM β -glycerophosphate buffer (pH 6.8) containing 0.5 mM EDTA and 1 mM dithiothreitol or by the procedure of Lowry et al. (1951) as modified by Hartree (1972) using GPb as standard. Under these conditions the crystals dissolve readily, whereas the dissolved enzyme showed full activity (54–56 $\mu\text{mol/mg/min}$ with 20 mM glucose-1-P, 1% glycogen, 1 mM AMP, and 0.9 M ammonium sulfate).

Kinetic analyses

Initial rates were calculated from the pseudo-first-order reaction constants (Engers et al., 1970) by using a nonlinear-regression data-analysis program (Leatherbarrow, 1987, 1990) by assuming that each experimental point (μmol of phosphate) is subject to about the same proportion of random error (Cornish-Bowden & Wharton, 1988). The program calculates the reaction constants and the standard errors of these values. For the statistical evaluation of the kinetic parameters, obtained by applying the Michaelis–Menten equation, initial rates were treated by nonlinear explicit-weighting regression analysis (Leather-

barrow, 1987), i.e., by providing an explicit value for the standard deviation of each rate. In this case, the program calculates K_m and V_{max} and the standard errors for these values.

Oyster glycogen (Serva) was freed of AMP according to Helmreich and Cori (1964). AMP, glucose-1-P (dipotassium salt), oligosaccharides (maltohexaose and maltoheptaose), ammonium sulfate, and dithiothreitol were products of Sigma Chemical Co. β -Glycerophosphate and EDTA were purchased from BDH Chemicals.

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References

- Acharya, K.R., Stuart, D.I., Varvill, K.M., & Johnson, L.N. (1991). *Glycogen Phosphorylase b: Description of the Protein Structure*. World Scientific, London, Singapore.
- Barford, D., Hu, S.-H., & Johnson, L.N. (1991). Structural mechanism for glycogen phosphorylase control by phosphorylation. *J. Mol. Biol.* 218, 233–260.
- Barford, D. & Johnson, L.N. (1989). The allosteric transition of glycogen phosphorylase. *Nature* 340, 609–616.
- Barford, D. & Johnson, L.N. (1992). The molecular mechanism for the tetrameric association of glycogen phosphorylase promoted by protein phosphorylation. *Protein Sci.* 1, 472–493.
- Barford, D., Schwabe, J.W.R., Oikonomakos, N.G., Acharya, K.R., Hajdu, J., Papageorgiou, A.C., Martin, J.L., Knott, J.C.A., Vasella, A., & Johnson, L.N. (1988). Channels at the catalytic site of glycogen phosphorylase: Binding and kinetic studies with the β -glycosidase inhibitor D-glucanohydroxymethyl-1,5-lactone N-phenylurethane. *Biochemistry* 27, 6733–6741.
- Bello, J. & Nowoswiat, E.F. (1965). The activity of crystalline ribonuclease A. *Biochim. Biophys. Acta* 105, 325–332.
- Cohen, P. (1973). The subunit structure of rabbit-skeletal-muscle phosphorylase kinase, and the molecular basis of its activation reactions. *Eur. J. Biochem.* 34, 1–14.
- Cohen, P., Duewer, T., & Fischer, E.H. (1971). Phosphorylase from dogfish skeletal muscle. Purification and a comparison of its physical properties to those of rabbit muscle phosphorylase. *Biochemistry* 10, 2683–2694.
- Cornish-Bowden, A. & Wharton, C.W. (1988). *Enzyme Kinetics*, pp. 1–18. IRL Press, Oxford, Washington, D.C.
- Dombradi, V. (1981). Structural aspects of the catalytic and regulatory function of glycogen phosphorylase. *Int. J. Biochem.* 13, 125–139.
- Doscher, M.S. & Richards, F.M. (1963). The activity of an enzyme in the crystalline state: Ribonuclease S. *J. Biol. Chem.* 238, 2399–2406.
- Engers, H.D. & Madsen, N.B. (1968). The effects of anions on the activity of phosphorylase b. *Biochem. Biophys. Res. Commun.* 33, 49–54.
- Engers, H.D., Shechosky, S., & Madsen, N.B. (1970). Kinetic mechanism of phosphorylase a. I. Initial velocity studies. *Can. J. Biochem.* 48, 746–754.
- Fasold, H., Ortanderl, F., Huber, R., Bartels, K., & Scwager, P. (1972). Crystallization and crystallographic data of rabbit muscle phosphorylase a and b. *FEBS Lett.* 21, 229–232.
- Fischer, E.H. & Krebs, E.G. (1962). Muscle phosphorylase b. *Methods Enzymol.* 5, 369–373.
- Fiske, C.H. & Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375–400.
- Fletterick, R.J., Sygusch, J., Murray, N., Madsen, N.B., & Johnson, L.N. (1976). Low-resolution structure of the glycogen phosphorylase a monomer and comparison with phosphorylase b. *J. Mol. Biol.* 103, 1–13.
- Graves, D.J., Huang, C.Y., & Mann, S.A. (1968a). Catalytic activity and subunit assembly of rabbit muscle glycogen phosphorylase. In *Control of Glycogen Metabolism* (Whelan, W.J., Ed.), pp. 35–47. Proceedings of the Fourth Meeting of FEBS, Oslo, 1967. Academic Press, London, New York.
- Graves, D.J., Mann, S.A.S., Philip, G., & Oliveira, R.J. (1968b). A probe into the catalytic activity and subunit assembly of glycogen phosphorylase. Desensitization of allosteric control by limited tryptic digestion. *J. Biol. Chem.* 243, 6090–6098.
- Graves, D.J. & Wang, J.H. (1972). α -Glucan phosphorylases—Chemical and physical basis of catalysis and regulation. In *The Enzymes*, 3rd Ed. (Boyer, P.D., Ed.), Vol. 7, pp. 435–482. Academic Press, New York.
- Hajdu, J., Acharya, K.R., Stuart, D.I., Barford, D., & Johnson, L.N. (1988). Catalysis in enzyme crystals. *Trends Biochem. Sci.* 13, 104–109.
- Hajdu, J., Acharya, K.R., Stuart, D.I., McLaughlin, P.J., Barford, D., Oikonomakos, N.G., Klein, H., & Johnson, L.N. (1987). Catalysis in the crystal: Synchrotron radiation studies with glycogen phosphorylase b. *EMBO J.* 6, 539–546.
- Hartree, E.F. (1972). Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48, 422–427.
- Helmreich, E. & Cori, C.F. (1964). The role of adenylic acid in the activation of phosphorylase. *Proc. Natl. Acad. Sci. USA* 51, 131–138.
- Helmreich, E., Michaelides, M.C., & Cori, C.F. (1967). Effect of substrates and substrate analogs on the binding of 5-adenylic acid to muscle phosphorylase a. *Biochemistry* 6, 3695–3710.
- Huang, C.Y. & Graves, D.J. (1970). Correlation between subunit interactions and enzymatic activity of phosphorylase a. Method for determining equilibrium constants from initial rate measurements. *Biochemistry* 9, 660–671.
- Johnson, L.N. (1985). Protein crystallography. In *Modern Physical Methods in Biochemistry* (Neuberger & Van Deenen, Eds.), Part A, pp. 347–415. Elsevier Science Publishers B.V., Amsterdam.
- Johnson, L.N. & Barford, D. (1990). Glycogen phosphorylase. The structural basis of the allosteric response and comparison with other allosteric proteins. *J. Biol. Chem.* 265, 2409–2412.
- Johnson, L.N., Hajdu, J., Acharya, K.R., Stuart, D.I., McLaughlin, P.J., Oikonomakos, N.G., & Barford, D. (1989). Glycogen phosphorylase b. In *Allosteric Enzymes* (Herve, G., Ed.), pp. 81–127. CRC Press, Boca Raton, Florida.
- Johnson, L.N., Madsen, N.B., Mosley, J., & Wilson, K.S. (1974). The crystal structure of phosphorylase b at 6 Å resolution. *J. Mol. Biol.* 90, 703–717.
- Kallos, J. (1964). Catalytic activity of chymotrypsin in crystalline state. *Biochim. Biophys. Acta* 89, 364–366.
- Kastenschmidt, L.L., Kastenschmidt, J., & Helmreich, E. (1968). Subunit interactions and their relationship to the allosteric properties of rabbit skeletal muscle phosphorylase b. *Biochemistry* 7, 3590–3608.
- Kasvinsky, P.J. & Madsen, N.B. (1976). Activity of glycogen phosphorylase in the crystalline state. *J. Biol. Chem.* 251, 6852–6859.
- Kasvinsky, P.J., Madsen, N.B., Fletterick, R.J., & Sygusch, J. (1978). X-ray crystallographic and kinetic studies of oligosaccharide binding to phosphorylase. *J. Biol. Chem.* 253, 1290–1296.
- Leatherbarrow, R.J. (1987). *Enzfitter: A Non-Linear Regression Data Analysis Program for the IBM-PC*. Elsevier Biosoft, Cambridge, UK.
- Leatherbarrow, R.J. (1990). Using linear and non-linear regression to fit biochemical data. *Trends Biochem. Sci.* 15, 455–458.
- Leonidas, D.D., Oikonomakos, N.G., & Papageorgiou, A.C. (1991). Sulphate activates phosphorylase b by binding to the Ser (P) site. *Biochim. Biophys. Acta* 1076, 305–307.
- Leonidas, D.D., Oikonomakos, N.G., Papageorgiou, A.C., Xenakis, A., Cazianis, C.T., & Bem, F. (1990). The ammonium sulphate activation of phosphorylase b. *FEBS Lett.* 261, 23–27.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Madsen, N.B. (1986). Glycogen phosphorylase. In *The Enzymes*, 3rd Ed. (Boyer, P.D. & Krebs, E.G., Eds.), Vol. 17, pp. 366–394. Academic Press, New York.

- Madsen, N.B., Honikel, K.O., & James, M.N.G. (1972). Studies on glycogen phosphorylase in solution and in the crystalline state. In *Metabolic Interconversion of Enzymes* (Wieland, O., Helmreich, E., & Holzer, H., Eds.), pp. 55–71. Springer, Berlin.
- Makinen, M.W. & Fink, A.L. (1977). Reactivity and cryoenzymology of enzymes in crystalline state. *Annu. Rev. Biophys. Bioeng.* 6, 301–342.
- Melpidou, A.E. & Oikonomakos, N.G. (1983). Effect of glucose-6-P on the catalytic and structural properties of glycogen phosphorylase *a*. *FEBS Lett.* 154, 105–110.
- Metzger, B., Helmreich, E., & Glaser, L. (1967). The mechanism of activation of skeletal muscle phosphorylase *a* by glycogen. *Proc. Natl. Acad. Sci. USA* 57, 994–1001.
- Monod, J., Wyman, J., & Changeux, J.-P. (1965). On the nature of allosteric transitions: A plausible model. *J. Mol. Biol.* 12, 88–118.
- Mozzarelli, A., Berni, R., Rossi, G.L., Vas, M., Bartha, F., & Keleti, T. (1982). Protein isomerization in the NAD⁺-dependent activation of β -(2-furyl)acryloyl-glyceraldehyde-3-phosphate dehydrogenase in the crystal. *J. Biol. Chem.* 257, 6739–6744.
- Munoz, F., Valles, M.A., Pocovi, M., Gorostidi, G.E., & Garcia, F. (1983). Rate and equilibrium constants of the tetramerization process of phosphorylase *b*. *Biophys. Chem.* 18, 249–256.
- Oikonomakos, N.G., Acharya, K.R., & Johnson, L.N. (1992). Rabbit muscle glycogen phosphorylase *b*. The structural basis of activation and catalysis. In *Post-Translational Modification of Proteins* (Harding, J.J. & Crabbe, M.J.C., Eds.), pp. 81–151. CRC Press, Boca Raton, Florida.
- Oikonomakos, N.G., Melpidou, A.E., & Johnson, L.N. (1985). Crystallization of pig skeletal phosphorylase *b*. *Biochim. Biophys. Acta* 832, 248–256.
- Quiocchio, F.A. & Richards, F.M. (1966). Enzymic behavior of carboxypeptidase-A in solid state. *Biochemistry* 5, 4062–4076.
- Rossi, G.L. & Bernhard, S.A. (1970). Are the structure and function of an enzyme the same in aqueous solution and in the wet crystal? *J. Mol. Biol.* 49, 85–91.
- Rossi, G.L. & Bernhard, S.A. (1971). On the relationship between the conformation and the catalysed reactivity of acyl-chymotrypsin. *J. Mol. Biol.* 55, 215–230.
- Sansom, M.S.P., Babu, Y.S., Hajdu, J., Stuart, D.I., Stura, E.A., & Johnson, L.N. (1984). The role of pyridoxal phosphate in glycogen phosphorylase *b*. Structure, environment and relationship to catalytic mechanism. In *Chemical and Biological Aspects of Vitamin B₆ Catalysis* (Evangelopoulos, A.E., Ed.), Part A, pp. 127–146. Alan R. Liss, New York.
- Sansom, M.S.P., Stuart, D.I., Acharya, K.R., Hajdu, J., McLaughlin, P.J., & Johnson, L.N. (1985). Glycogen phosphorylase *b*—The molecular anatomy of a large regulatory enzyme. *J. Mol. Struct.* 123, 3–25.
- Sluyterman, L.A.A. & De Graaf, M.J.M. (1969). The fluorescence of papain. *Biochim. Biophys. Acta* 171, 277–284.
- Sotiroidis, T.G., Oikonomakos, N.G., & Evangelopoulos, A.E. (1979). Effect of sulphated polysaccharides and sulphate anions on the AMP-dependent activity of phosphorylase *b*. *Biochem. Biophys. Res. Commun.* 90, 234–239.
- Sprang, S.R., Acharya, K.R., Goldsmith, E.J., Stuart, D.I., Varvill, K.M., Fletterick, R.J., Madsen, N.B., & Johnson, L.N. (1988). Structural changes in glycogen phosphorylase induced by phosphorylation. *Nature* 336, 215–221.
- Sprang, S.R. & Fletterick, R.J. (1979). The structure of glycogen phosphorylase *a* at 2.5 Å resolution. *J. Mol. Biol.* 131, 523–551.
- Sprang, S.R., Withers, S.G., Goldsmith, E.J., Fletterick, R.J., & Madsen, N.B. (1991). Structural basis for the activation of glycogen phosphorylase *b* by adenosine monophosphate. *Science* 254, 1367–1371.
- Vas, M., Berni, R., Mozzarelli, A., Tegoni, M., & Rossi, G.L. (1979). Kinetic studies of crystalline enzymes by single crystal microspectrophotometry. Analysis of a single catalytic turnover in a D-glyceraldehyde-3-phosphate dehydrogenase crystal. *J. Biol. Chem.* 254, 8480–8486.
- Wang, J.H., Kwok, S.C., Wirch, E., & Suzuki, I. (1970). Distinct AMP binding sites in glycogen phosphorylase *b* as revealed by calorimetric studies. *Biochem. Biophys. Res. Commun.* 40, 1340–1347.
- Wang, J.H., Shonka, M.L., & Graves, D.J. (1965a). Influence of carbohydrates on phosphorylase structure and activity. I. Activation by preincubation with glycogen. *Biochemistry* 11, 2296–2301.
- Wang, J.H., Shonka, M.L., & Graves, D.J. (1965b). The effect of glucose on the sedimentation and catalytic activity of glycogen phosphorylase. *Biochem. Biophys. Res. Commun.* 18, 131–135.
- Weber, I.T., Johnson, L.N., Wilson, K.S., Yeates, D.G.R., Wild, D.L., & Jenkins, J.A. (1978). Crystallographic studies on the activity of glycogen phosphorylase *b*. *Nature* 274, 433–437.
- Withers, S.G., Madsen, N.B., & Sykes, B.D. (1982). Covalently activated glycogen phosphorylase: A phosphorus-31 nuclear magnetic resonance and ultracentrifugation analysis. *Biochemistry* 21, 6716–6722.
- Withers, S.G., Sykes, B.D., Madsen, N.B., & Kasvinsky, P.J. (1979). Identical structural changes induced in glycogen phosphorylase by two non-exclusive allosteric inhibitors. *Biochemistry* 18, 5342–5348.