# Anion Activation of 3-Phosphoglycerate Kinase Requires Domain Closure†

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ABSTRACT: 3-Phosphoglycerate kinase is a typical two-domain "hinge-bending" enzyme, which is known to be regulated by multivalent anions. Here a relationship between this regulation and the hinge-bending domain closure is proposed on the basis of enzyme kinetic analysis and molecular modeling. Activation of the pig muscle enzyme at low concentrations and inhibition at high concentrations of various anionic analogues of the substrate 3-phosphoglycerate or of the nonsubstrate metal-free ATP are described by a two-site model assuming separate sites for activation and inhibition, respectively. Kinetic experiments with various pairs of analogues suggest the presence of a common site for activation by all effectors, separate from the catalytic site for 3-phosphoglycerate; and a common site for inhibition, except for metal-free ATP, identical with the catalytic site of 3-phosphoglycerate. An additional inhibiting site for all of the anions investigated, including metal-free ATP, is also proposed. A similar two-site model can describe activation of the enzyme by a large excess of each substrate; here the ligand binds to the catalytic site as a substrate and to the regulatory site as an activator. Activation is exerted not only by the physiological substrate, 3-phophoglycerate, but also by a synthetic weak substrate. The activity in the reaction with 3-phosphoglycerate and MgATP is greatly enhanced by the simultaneous presence of the weak substrate. This finding clearly proves the existence of a regulatory site, separate from the catalytic site. This regulatory site, however, may only exist in the catalytically competent closed conformation of the enzyme, as indicated by molecular modeling. Docking of the regulator anions into the known X-ray structures of the enzyme revealed the appearance of an anion binding site between the two domains, including the invariant residues of Lys-215 (C-domain) and of Arg-65 among other residues of the basic cluster (N-domain), as a consequence of the large-scale substrate-induced conformational change that leads to domain closure.

3-Phosphoglycerate kinase (PGK,[1] EC 2.7.2.3) is a monomeric enzyme with two domains, one of them binding 3-phosphoglycerate (3-PG) and the other MgATP, as shown by X-ray crystallographic (*1*−*7*) and by NMR studies (*8*−*11*) of the enzyme from various sources. PGK displays a high degree of evolutionary conservation not only in its three-dimensional structure but also in the primary sequence; especially, the side chains in the active site region are conserved up to 98% (*12*).

PGK exhibits a rather interesting regulatory behaviors: activation by low concentrations of multivalent anions, including the nonsubstrate $Mg^{2+}$-free ATP, and inhibition at high concentrations (*13*−*15*). In addition, a high concentration of either substrate, 3-PG or MgATP, also causes activation (*13*, *16*, *17*). These properties were observed long before structural information became available, but such information has not yet led to an understanding of the mechanism of activation at the molecular level.

While inhibition by the nonsubstrate anions can reasonably be attributed to direct competition with the anionic substrates, to explain activation, the existence of a separate anion binding site, outside the catalytic center, has been assumed (*14*). This regulatory site has been suggested to be either close to the terminal phosphate of the bound substrate, MgATP (*18*), or in the "basic patch" region on the inner surface of the N-terminal domain (*19*−*22*). Scopes (*18*) assumed that occupancy of this site by substrates or anions activated the enzyme by accelerating the release of the product, 1,3-bisphosphoglycerate. Release of this tightly bound product from the enzyme active site has indeed been shown to be rate-limiting by recent kinetic studies of transient states (*23*, *24*).

Confirmation of the nature of the rate-limiting step, however, has not been accompanied by evidence of the existence and the location of a separate regulatory site. Site-directed mutagenesis apparently underlined the importance of the basic patch residues (*25*, *26*) but has not led to definitive conclusions about the activating site (*10*, *27*, *28*). As for the existence of any possible secondary substrate site, ligand binding studies could only detect a single site for 3-PG (*18*), whereas for the nucleotide substrates, the data are equivocal. Numerous publications made claims about the existence of a secondary nucleotide site, although sometimes without presenting completely satisfactory binding curves (*18*−*20*, *29*−*33*), while others report about only a single nucleotide site (*8*, *34*−*38*). In any case, high-resolution X-ray structural data cannot yet provide evidence about the

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[1] Abbreviations: PGK, 3-phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3-PG, 3-phospho-D-glycerate; AMP-PNP, 5′-adenylylimidodiphosphate.

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location of a separate regulatory site for substrates or activators, since the data have only indicated a single site for each substrate (*3*−*7*).

Further, the basic patch in the N-domain was shown to be the site for 3-PG (*3*) and was confirmed recently (*7*), although an alternative binding mode of this substrate has been also reported from a lower-resolution data set (*6*). In fact, from X-ray and NMR structural studies, three possible sites have been found for anions (one of them being in the basic patch); however, all of them are inside and not outside the catalytic center, and anion binding here would inhibit the enzyme by preventing substrate binding (*11*, *21*, *27*, *39*). There is therefore no firm basis for establishing the location of a separate activating site or the mechanism of activation by anions.

In this work, we have tried to assess the nature of any activating site and to look for its spatial location. For this, we have conducted kinetic experiments on pig muscle 3-phosphoglycerate kinase with systematically varied analogues of 3-PG of different sizes, bearing one, two, or three negatively charged groups. Their possible modes of binding to the enzyme were also determined by computer modeling on the basis of the available X-ray structural data.

## MATERIALS AND METHODS

*Enzymes and Chemicals*. PGK (EC 2.7.2.3) was isolated from pig muscle (*3*) and stored as a microcrystalline suspension in the presence of 2 mM dithiothreitol. Its activity was approximately 600 kat/mol using 3-phosphoglycerate and MgATP as substrates. GAPDH (EC 1.2.1.12) (350 kat/mol) was prepared from pig muscle (*40*).

Na salts of 3-phosphoglycerate (3-PG) and ATP were Boehringer products. The substrate, MgATP, was formed by addition of $MgCl_2$ (Sigma) to ATP. Due to metal ion contaminations in the usual commercial preparation, about 2% of the total ATP is chelated even in the absence of $MgCl_2$, as determined by enzymatic assay. NADH was obtained from Reanal. Na salts of dl-glycerol 3-phosphate (Sigma), glycerol 2-phosphate (Fluka), and 2-phosphoglycerate (Boehringer) were used, whereas cyclohexylammonium salts of 2-phosphoglycolate (Boehringer) and 2,3-bisphosphoglycerate (Calbiochem) were used. The Na salt of pentane 1,5-bisphosphonate, produced by chemical synthesis according to Li and Byers (*41*), was a kind gift from A. Merli (University of Parma, Parma, Italy). The arsonomethyl analogue of 3-PG, dl-2-hydroxy-4-arsonobutyrate, synthesized according to Adams et al. (*42*) and crystallized as a bis(cyclohexylammonium) salt, was a kind gift from H. B. F. Dixon (University of Cambridge, Cambridge, U.K.). That the amount of cyclohexylammonium cation, which was present in some of the experiments, did not exert a significant effect on the enzyme activity was checked. All other chemicals were reagent grade commercial preparations.

*Preparation of Enzyme Solutions*. Crystals of PGK were dissolved in 20 mM Tris/HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol, and the mixture was dialyzed against the same buffer to remove $(NH_4)_2SO_4$. The final dialysis buffer contained 2 mM dithiothreitol. GAPDH solutions were also desalted in the same way.

For the determination of protein concentrations, $A_{280}$ values of 1 mg/mL solutions of PGK and GAPDH were taken to be 0.69 (*43*) and 1.0 (*44*), respectively, for a 1 cm path length. The molecular mass of PGK was taken to be 44.5 kDa, different from the previously used value of 48 kDa (*38*). The corrected value was calculated on the basis of the known amino acid composition of the horse muscle PGK (*1*), taking into account those few residues that are replaced in pig muscle PGK as deduced from X-ray structural data (*3*).

*Enzyme Kinetic Studies*. The activity of PGK was measured, with 3-PG and MgATP as substrates, by following the oxidation of NADH spectrophotometrically at 340 nm when it reduced the product in the presence of GAPDH, as described by Tompa et al. (*45*).

Activation and inhibition studies were carried out with only a limited amount of $Mg^{2+}$ when the presence of the metal-free form of ATP was required together with a constant concentration of MgATP. Here the metal ion contamination of the commercial ATP was also taken into account. The dissociation constant of MgATP was taken to be 0.1 mM, obtained by averaging the data in the literature (*46*−*48*). Most of the experiments were carried out under these conditions, but in a few cases, excess (5 or 10 mM) $MgCl_2$ was also present.

Since sulfate competes with ATP for binding $Mg^{2+}$, high concentrations of sulfate might lower the concentration of MgATP, and thereby lower the activity. Even at the lowest concentration of $Mg^{2+}$-free ATP, however, i.e., at 1 mM $MgCl_2$ and 0.6 mM ATP and the highest sulfate concentration (i.e., 200 mM), a fall of only 8% in the activity can be attributed to this effect, in contrast with the observed fall of 85%. The dissociation constant of $MgSO_4$ was taken to be 10.23 mM (*49*), and p*K* values for ATP were taken to be 3.8 and 6.8 (*50*). Calculations were performed with the SED software (Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm, Sweden).

In the kinetic experiments, GAPDH, the coupling enzyme, was used in higher concentrations than usual, to counterbalance its inhibition by ATP, sulfate, phosphate, or the other effectors.

All the experiments were carried out at 20 °C in 20 mM Tris/HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol. This buffer ensured the low ionic strength required for studies of the effect of low concentrations of anions.

Quantitative evaluation of the data was carried out by nonlinear regression analysis using the Graphit software (R. J. Leathbarrow, 1989−1990; Erithacus Software Ltd., supplied by Sigma).

*Computer Modeling of Ligand Binding.* For molecular graphics and modeling, the Insight II 95.0 software (Biosym/MSI, San Diego, CA) was used. X-ray coordinates of pig muscle PGK complexed with 3-PG and the nucleotide analogue 5′-adenylylimidodiphosphate, MnAMP-PNP (*3*), and of *Trypanosoma brucei* PGK complexed with 3-PG and MgADP (*7*) were used as examples of the open and closed conformations of the enzyme, respectively. The coordinates of the *T. brucei* enzyme were kindly provided by B. Bernstein (University of Washington, Seattle, WA). A trigonal phospho group, which represents the group transferred in the catalyzed reaction, has been modeled into the active center of *T. brucei* PGK, in a way similar to that of Bernstein et al. (*7*).

The molecular structure of each anionic ligand used in the kinetic experiments was constructed with the above

software. The charge distribution of ligands and substrates (structures were taken from the X-ray coordinates) was calculated using the AM1 method in the AMPAC/MOPAC (QCPE, Bloomington, IN) software. Hydrogens were added to the protein assuming the condition of pH 7.5, and a Coulomb potential-docking grid was calculated for the above-mentioned PGK complexes. This docking grid showed the possible anion binding areas on the surface of the protein. Further investigations were restricted only to the area which has undergone the largest change (both steric and charge distribution) upon domain closure, i.e., to the area between the two domains. The most favorable position for anion binding within this area was preselected by calculation of the interaction energy (van der Waals and electrostatic) using the Docking module of Insight II between a model phosphate ion (in its several possible positions near the positively charged residues within the investigated area) and the protein in the closed conformation. Evolutionary conservation of the residues that make up the sites was also considered an important criterion.

From this starting position, energy minimization was performed with the Discover 95.0 software (Biosym/MSI) using the consistent valence force field for the complex consisting of *T. brucei* PGK, its substrates, the transferred phosphate, and each anionic effector, fixing all but the effector, i.e., allowing changes in conformation and spatial position only for the effector anion. Two hundred iterations with the steepest-descents method and then 10 000 iterations with the conjugate gradients method were calculated, resulting in the most probable binding mode for each effector. Calculations were repeated with different dielectric constant values, but this had only a small effect on the position of the anion. The interaction energy of the substrate 3-PG (given by the X-ray coordinates) in its catalytic site was also calculated with the Docking module and found to be about 1.5−2-fold larger than the binding energy of the various anions at the effector site in the closed protein conformation. The energy minimization procedure was also carried out for the complex consisting of the pig PGK structure, the substrates, and each anionic effector and resulted in an about 2−3-fold smaller binding energy at the part of the effector site that exists in the open conformation, compared to that of the complete effector site in the closed structure.

*Equations Used for the Calculations.* A two-site model was used to describe the simultaneous activation and inhibition caused by anions. The equation was built up according to the following considerations. The equation that describes the binding curve for a ligand (L) to a single site can be written as

$$[E]_t Y = C\frac{[L]}{K + [L]} \tag{1}$$

where $[E]_t$ is the total enzyme concentration, $Y$ is the saturation function, i.e., $[E]_t Y$ is the concentration of the ligated species, $K$ is the dissociation constant, and $C$ is called capacity and represents the maximal concentrations of the sites that can be filled by L. [L] is the molar concentration of the free (unbound) ligand, which is approximately the total [L] when catalytic concentrations of enzyme are present, i.e., $C \ll [L]$. If an enzyme (E) rapidly equilibrates with a ligand L, which activates it, the left-hand side of eq 1 stands for the concentration of the active EL complex. The activity ($v$) is proportional to this, and for the same reason, $C$ can be considered a measure of the limiting value of activity ($v_L$) at an infinite concentration of L. Thus, eq 1 can be replaced by

$$v = v_L\frac{[L]}{K + [L]} \tag{2}$$

Equation 2 is valid if the enzyme has no activity in the absence of the activator L. If the enzyme possesses activity even in the absence of the activator, the changes in activity ($v$) as a function of [L] can be described as follows:

$$v = v_u + (v_u a - v_u)\frac{[L]}{K + [L]} = v_u + v_u(a - 1)\frac{[L]}{K + [L]} \tag{3}$$

where $v_u$ stands for the activity of the unligated form of the enzyme (at a fixed substrate concentration), whereas $a$ is the factor by which activity is increased; i.e., at an infinite [L], the activity $v = v_u a$. Accordingly, by definition, the relation of $a > 1$ should always be valid. The physical meaning of the coefficient $v_u(a - 1)$ is the net activity increase at saturation of the activating site, still assuming that L binds only to this activating site.

If the ligand L, in addition to binding to the activator site, also binds to another site and thereby completely inhibits the enzyme, the expression given by eq 3 would represent the total activity that can be inhibited, i.e., the "capacity" for inhibition. If we assume that the dissociation constant of one is not affected by binding to the other, the activity, $v$, as a function of [L] can be expressed as follows:

$$v = v_u + v_u(a - 1)\frac{[L]}{K_{d(act)} + [L]} - \left[v_u + v_u(a - 1)\frac{[L]}{K_{d(act)} + [L]}\right]\frac{[L]}{K_{d(inh)} + [L]} \tag{4}$$

where $K_{d(act)}$ and $K_{d(inh)}$ are the dissociation constants for the sites of activation and inhibition, respectively. The third term with the negative sign stands for the inhibition. Equation 4 may also be presented in the following simplified form:

$$\frac{v}{v_u} = \frac{K_{d(act)}K_{d(inh)} + aK_{d(inh)}[L]}{(K_{d(act)} + [L])(K_{d(inh)} + [L])} \tag{4a}$$

Fitting this equation to the activity values ($v$), determined experimentally as a function of [L] (e.g., Figures 1 and 2), we can obtain the parameters $K_{d(act)}$ and $K_{d(inh)}$, and the factor $a$, characteristic for the investigated effector (L). Equation 4 applies in the presence of a single species of anions. In the presence of any competing anions (including the substrates), however, it still applies, except $K_{d(act)}$ and $K_{d(inh)}$ are replaced by apparent values.

A similar model can be used for the activation of the enzyme by high concentrations of substrate (i) if the catalytic and activating sites are independent and (ii) if both sites equilibrate rapidly with the substrate. Here, naturally, the enzyme has no activity without ligation with substrate. By

analogy to eq 3, in the following equation, there are only positive terms:

$$v = v_S\frac{[S]}{K_{S(cat)} + [S]} + v_S(a - 1)\frac{[S]}{K_{S(cat)} + [S]}\frac{[S]}{K_{S(act)} + [S]} \quad (5)$$

The first term represents a simple hyperbolic saturation of the catalytic site with the substrate (S); i.e., by analogy to $v_u$ in eq 3, it expresses the activity in the absence of activation. Thus, $v_S$ stands for the hypothetical activity at saturation of the catalytic site by the substrate, if activation were not taking place. In a case similar to that in eq 3, the second term is a representative of saturation of a regulatory site, which is here a nonhyperbolic function. $K_{S(cat)}$ and $K_{S(act)}$ are the dissociation constants for the catalytic and the regulating (activating) sites, respectively. The coefficient $a$, as above, is the factor by which activity increased by binding of the substrate to the regulatory site; i.e., $av_S$ is the activity of the enzyme when both catalytic and activating sites are filled by the substrate. Fitting eq 5 to the activity values ($v$), measured at various [S] values (e.g., Figure 4), we can obtain parameters $K_{S(cat)}$ and $K_{S(act)}$ and the activation factor $a$.

We now derive eq 11, in effect a modified form of eq 5, to obtain an approximate description of the more complex data of activation obtained with simultaneous presence of two different substrates (S and S′) on which the enzyme acts with extremely different activities ($v_{S'} \ll v_S$). Here both substrates essentially compete for the same catalytic and regulatory sites, and the activity would be expressed quantitatively as the sum of the contributions of the following enzyme−substrate complexes (the concentrations of each form represent fractional concentrations, and the total enzyme concentration, $[E]_t$, was taken to be unity):

$$v = v_S[ES_{cat}] + v_Sa_S[ES_{cat}S_{act}] + v_Sa_{S'}[ES_{cat}S'_{act}] + v_{S'}[ES'_{cat}] + v_{S'}a_{S'}[ES'_{cat}S'_{act}] + v_{S'}a_S[ES'_{cat}S_{act}] \quad (6)$$

where the subscripts cat and act represent catalytic and regulatory (activating) sites, respectively, to both of which the substrates S and S′ can alternatively bind. $ES_{act}$ and $ES'_{act}$ complexes are not included in eq 6 as these forms do not have activity since no substrate is bound to their catalytic sites. $v_S$ and $v_{S'}$ represent the activity when the catalytic site is saturated by the substrate S or S′, respectively. $a_S$ and $a_{S'}$ are factors by which the activity is increased when substrates S and S′, respectively, fill the regulatory site. We assume that the values of these factors depend only on the nature of the ligand that fills the regulatory site and are independent of the nature of the ligand that fills the catalytic site. Following the same terminology, parameters $K_{S(cat)}$, $K_{S(act)}$, $K_{S'(cat)}$, and $K_{S'(act)}$ are dissociation constants of S and S′ binding to the catalytic and regulatory sites, respectively.

We assume that, since $v_{S'} \ll v_S$ holds, we can neglect the last three terms of eq 6; i.e., we treat S′ purely as an inhibitor at the catalytic site and an activator at the regulatory site. Further, if we choose the experimental condition where $[S] \ll K_{S(act)}$ by keeping the concentration of the good substrate (S) at a constant low level, saturation of the activating site by S will be negligible and S will bind almost exclusively to the catalytic site. This will only be possible if $K_{S(cat)} \ll K_{S(act)}$, i.e., if the good substrate binds to the catalytic site much more tightly than to the regulatory site. Thus, the complex $ES_{cat}S_{act}$ will be present only in negligible amount, and eq 6 finally can be simplified to

$$v = v_S[ES_{cat}] + v_Sa_{S'}[ES_{cat}S'_{act}] \quad (7)$$

With these assumptions, S is the only substrate and S′ is the only activator. Under the experimental conditions defined by the constant low concentration of the good substrate S and a varying one of the poor substrate S′, although S will not compete effectively for the regulatory site, the two substrates can compete for the catalytic site. This competition leads to replacement of the good substrate (S) by the weak substrate (S′), which lowers the rate of the catalyzed reaction. Hence, S′ is both inhibitory and activating. To determine the activity, we consider first the catalytic site. The two species that have S bound in the catalytic site are $ES_{cat}$ and $ES_{cat}S'_{act}$, so the sum of their fractional concentrations is simply the degree to which the catalytic site binds S, given by the usual treatments of competitive inhibition as

$$[ES_{cat}] + [ES_{cat}S'_{cat}] = \frac{[S]K_{S'(cat)}}{[S]K_{S'(cat)} + K_{S(cat)}K_{S'(cat)} + [S']K_{S(cat)}} \quad (8)$$

The right-hand side of eq 8 can be considered the capacity for filling of the regulatory site by the poor substrate (S′), and therefore

$$[ES_{cat}S'_{act}] = \frac{[S]K_{S'(cat)}}{[S]K_{S'(cat)} + K_{S(cat)}K_{S'(cat)} + [S']K_{S(cat)}}\frac{[S']}{K_{S'(act)} + [S']} \quad (9)$$

Subtracting eq 9 from eq 8 gives

$$[ES_{cat}] = \frac{[S]K_{S'(cat)}}{[S]K_{S'(cat)} + K_{S(cat)}K_{S'(cat)}[S']K_{S(cat)}} \times \left(1 - \frac{[S']}{K_{S'(act)} + [S']}\right) \quad (10)$$

Then, substituting the right-hand side expressions of eqs 9 and 10 into eq 7 will give the following expression:

$$v = v_S\frac{[S]K_{S'(cat)}}{[S]K_{S'(cat)} + K_{S(cat)}K_{S'(cat)}[S']K_{S(cat)}} + v_S(a_{S'} - 1) \times \frac{[S]K_{S'(cat)}}{[S]K_{S'(cat)} + K_{S(cat)}K_{S'(cat)}[S']K_{S(cat)}}\frac{[S']}{K_{S'(act)} + [S']} \quad (11)$$

This equation describes, with good approximation, the activation caused by the weak substrate (S′) on the reaction with the good substrate (S); it gives the increase of activity as a function of [S′] if [S] is kept at a constant value.

When [S′] = 0, eq 11 will be greatly simplified:

$$v_0 = v_S\frac{[S]}{K_{S(cat)} + [S]} \quad (12)$$

As the value of $v_0$, i.e., the velocity at [S′] = 0 and at a

Table 1: Summary of the Kinetic Parameters for Activation by Excess Substrate as Well as for Activation and Inhibition by Various Anions[a]

| substrate or effector | [$MgCl_2$] (mM) | [3-PG] (mM) | $v_S$ or $v_u$ [$\Delta A$ (min)] | $a$ | $K_{S(act)}$ or $K_{d(act)}$ (mM) | $K_{S(cat)}$ or $K_{d(inh)}$ (mM) | $K'_{d(inh)}$ (mM) | $K_I$ (mM) |
|---|---|---|---|---|---|---|---|---|
| 3-phosphoglycerate | 1 | various | 0.16 ± 0.014 | 21.7 ± 3.6 | 9.5 ± 3.2 | 0.019 ± 0.008 | − | − |
| | 10 | various | 0.46 ± 0.048 | 6.8 ± 0.9 | 12.6 ± 4.5 | 0.027 ± 0.015 | − | − |
| 2-hydroxy-4-arsonobutyrate | 1 | no | 0.0003 ± 0.0001 | nd | nd | nd | | |
| | | 0.05 | nd[c] | 15.1 ± 4 | 70 ± 30 | 6.5 ± 2.5 | − | − |
| | | 0.2 | nd | 13.5 ± 3.5 | 50 ± 25 | 6.1 ± 2.3 | | |
| glycerol 3-phosphate | 1 | 0.2 | 0.5 ± 0.04 | 5.96 ± 1.8 | 15.0 ± 6.6 | 1.4 ± 0.4 | 0.84 | |
| | | 2 | 1.73 ± 0.2 | 5.76 ± 2.1 | 19.8 ± 5.2 | 4.8 ± 1.1 | 0.62 | 0.7[b] |
| 2-phosphoglycolate | 1 | 0.2 | 0.48 ± 0.04 | 6.75 ± 1.5 | 5.4 ± 0.3 | 6.4 ± 2.8 | 3.84 | 4.0[b] |
| 2,3-bisphosphoglycerate | 1 | 0.2 | 0.505 ± 0.03 | 6.72 ± 1.2 | 3.86 ± 0.7 | 2.43 ± 0.5 | 1.46 | |
| | | 2 | 1.71 ± 0.17 | 6.28 ± 1.3 | 4.2 ± 0.8 | 4.3 ± 0.9 | 0.56 | 0.35 ± 0.20 |
| glycerol 2-phosphate | 1 | 0.2 | 0.49 ± 0.06 | 6.67 ± 1.6 | 13.8 ± 3.5 | 11.6 ± 3.9 | 6.9 | |
| | | 2 | 1.63 ± 0.2 | 6.62 ± 1.8 | 26.8 ± 6.7 | 38.2 ± 9.2 | 5.0 | 9.6 ± 2.0 |
| | 5 | 0.2 | 0.34 ± 0.02 | 5.2 ± 0.8 | 16.2 ± 5.8 | 17.9 ± 5.2 | 10.7 | |
| pentane 1,5-bisphosphonate | 1 | 0.2 | 0.45 ± 0.05 | 7.4 ± 1.3 | 15.8 ± 3.8 | 27.4 ± 5.4 | 16.4 | 26 ± 10 |
| sulfate | 1 | 0.2 | 0.45 ± 0.06 | 7.77 ± 1.3 | 8.9 ± 2.1 | 17.4 ± 3.2 | 10.4 | 14 ± 3.0 |
| phosphate | 1 | 0.2 | 0.47 ± 0.05 | 7.47 ± 1.4 | 10.3 ± 2.6 | 10.6 ± 2.5 | 6.3 | 8.3 ± 1.6 |
| ATP | 1 | 0.2 | 0.42 ± 0.05 | 6.27 ± 0.9 | 1.6 ± 0.3 | 2.04 ± 0.4 | 1.22 | nd |

[a] The parameters were determined by fitting the experimental points on Figures 1 and 2 according to eq 4 as well as the points on Figures 4 and 5 according to eqs 5 and 11, respectively. The experimental data, obtained under other conditions (e.g., 10 mM $MgCl_2$ and 2 mM 3-PG) and not shown in the figures, are also fitted in the same way. The parameters $a$, $K_{S(act)}$, $K_{S(cat)}$, $K_{d(act)}$, and $K_{d(inh)}$ are defined by the related equations (see Materials and Methods). $K_I$ competitive inhibitory constants were determined by separate experiments, similar to the one shown in Figure 4 for glycerol 2-phosphate. $K_{d(act)}$ and $K_{d(inh)}$ are apparent constants; their values depend on [3-PG] present in the experiment. From $K_{d(inh)}$ values, the real dissociation constants $K'_{d(inh)}$ were calculated according to the equation $K'_{d(inh)} = K_{d(3-PG)}K_{d(inh)}/[[3\text{-PG}] + K_{d(3-PG)}]$. The value of $K_{d(3-PG)}$ was taken to be equal to $K_{m(3-PG)}$ (0.3 mM from ref *37*). [b] Ref *45*. [c] nd, not determined.

constant [S], is determined experimentally, the value of $v_S$ can be calculated from eq 12 as $K_{S(cat)}$ is known from a separate experiment. Thus, fitting eq 11 to the experimentally determined activity ($v$) as a function of [S′] (e.g., Figure 5), we can determine the parameters $K_{S'(cat)}$, $K_{S'(act)}$, and $a_{S'}$, characteristic for the activating substrate (S′).

For computer fitting, the Graphit software was used.

## RESULTS

*Activation and Inhibition by Various Anions, Including Substrate Analogues.* The effects of different analogues (for structural formulas, see Table 1) of the substrate, 3-PG, on the activity of PGK were investigated at relatively low, constant concentrations of substrates 3-PG and MgATP (Figure 1). All of the analogues, namely, 2-phosphoglycolate (Figure 1A), 2,3-bisphosphoglycerate (Figure 1B), glycerol 2-phosphate (Figure 1C), and pentane 1,5-bisphosphonate (Figure 1D), exhibit activation at low concentrations and inhibition at high concentrations. Activating and inhibiting effects of 2-phosphoglycerate, another short analogue of 3-PG, are also observed, but these were not evaluated quantitatively (not shown). As a comparison, activation and inhibition by the metal-free ATP (Figure 1G), as well as by simple anions, such as phosphate (Figure 1F) and sulfate (Figure 1E), were also determined under identical conditions. An activation of the enzyme by about 3-fold by the metal-free ATP is markedly greater than that observed by Khamis and Larsson-Raznikiewicz (*14*). The extent of the observed activation varies in the same range (about 1.5−2-fold) and is apparently independent of the size of the molecule and the number of charged groups. A different behavior was, however, observed for glycerol 3-phosphate, an analogue closely similar to 3-PG. It apparently does not activate, but only inhibits the enzyme, under these conditions (Figure 2A).

Both activation and inhibition could also be detected at much higher concentrations of 3-PG or MgATP, or even in the presence of a moderate excess of $MgCl_2$ (cf. Table 1). At higher 3-PG concentrations, the extent of activation is

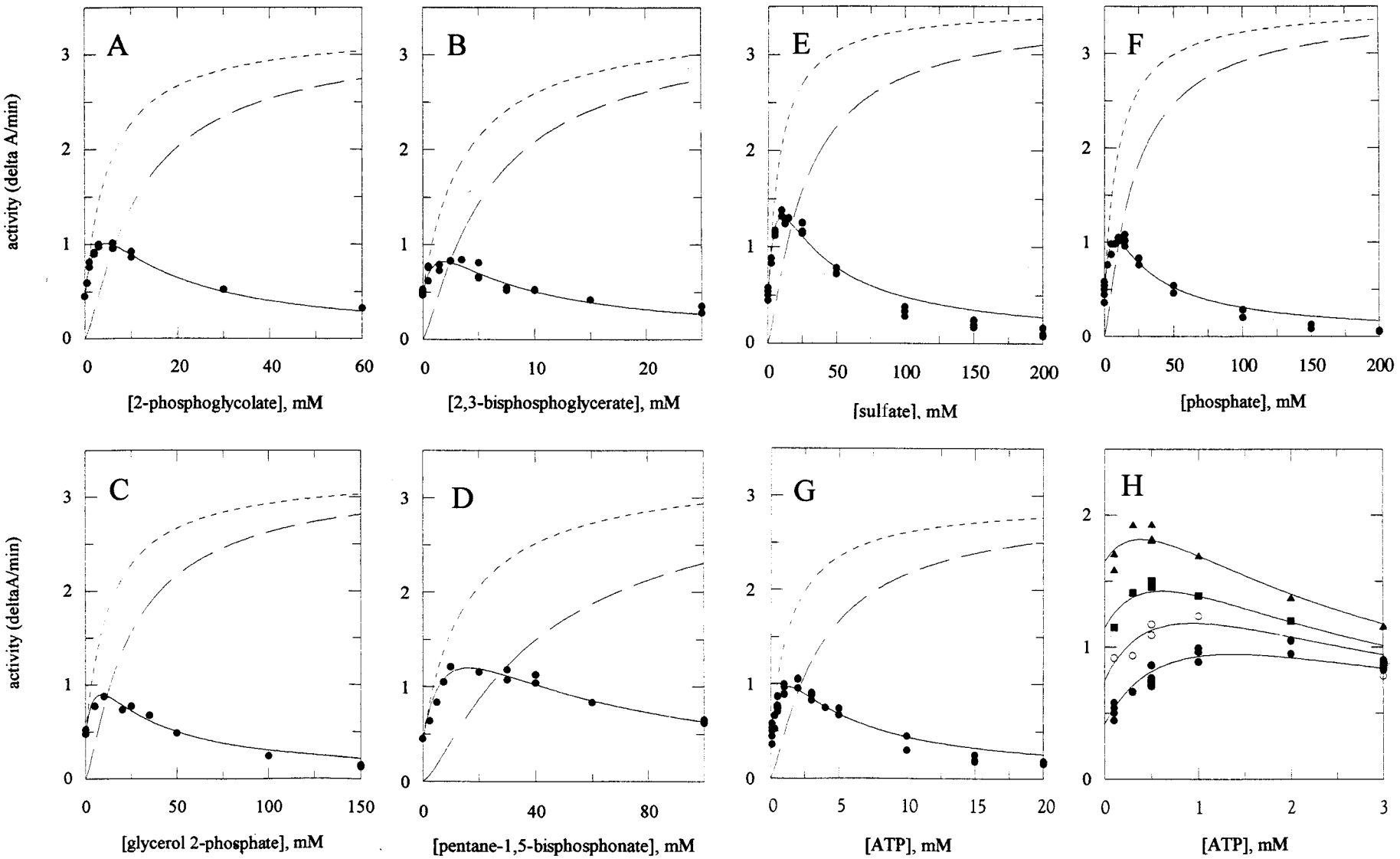


FIGURE 1: Activation and inhibition of PGK by various anions. The experiment was carried out with 40 nM enzyme, 0.2 mM 3-PG, and 0.5 mM MgATP. The latter was provided by 1 mM $MgCl_2$ and 0.6 mM ATP, which left only 0.1 mM $Mg^{2+}$-free ATP (A−F). In other cases (G and H), the $MgCl_2$ concentration was slightly varied between 0.5 and 1.0 mM as the ATP concentration changed from 0.6 to 20.5 mM. Enzyme activity was measured with varying concentrations of 2-phosphoglycolate (A), 2,3-bisphosphoglycerate (B), glycerol 2-phosphate (C), pentane 1,5-bisphosphonate (D), sulfate (E), and phosphate (F); the $Mg^{2+}$-free ATP concentration was varied in the absence (G) or in the presence of sulfate (H). In panel H, the sets of experimental points were obtained in the absence of salts (●), i.e., part of curve (G), and in the presence of 2.5 (○), 5 (■), and 15 mM (▲) sulfate. The curve fitting was carried out according to nonlinear regression analysis by using eq 4 of Materials and Methods. Dotted and dashed lines correspond to the activation and inhibition components of the fitted curves (solid line), respectively. When ATP and sulfate were present simultaneously (H), the following apparent $K_{d(act)}$ values were obtained as the best fitting variables at 2.5 (○), 5 (■), and 15 mM (▲) sulfate, in the same order: 0.97, 1.03, and 1.3 mM; for the other parameters, see Table 1.

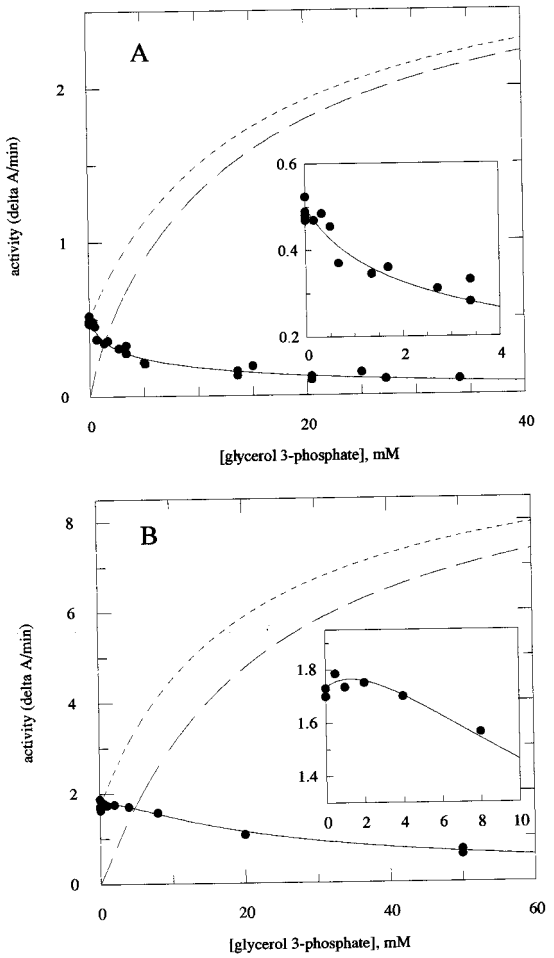


FIGURE 2: Effect of glycerol 3-phosphate on PGK activity. The experiment was carried out with 40 nM enzyme and 0.5 mM MgATP (provided by 1 mM $MgCl_2$ and 0.6 mM ATP as in case of Figure 1A−F) and with 0.2 (A) or 2 mM (B) 3-PG. Enzyme activity was measured as a function of glycerol 3-phosphate concentration. Curve fitting was carried out by using eq 4 of Materials and Methods. The components of the fitted curves (solid line) that correspond to activation (dotted line) and inhibition (dashed line) are also shown. The initial parts of the curves at low ligand concentrations are enlarged in the insets. For the variables that gave the best fit, see Table 1.

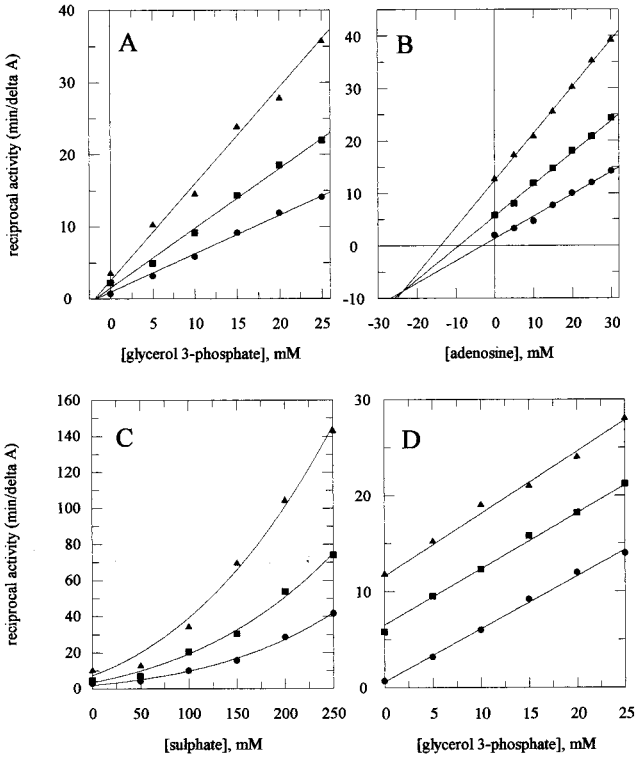


FIGURE 3: Double inhibition by various pairs of inhibitors. The experiment was carried out with 40 nM enzyme, 0.2 (B and C) or 0.3 mM (A and D) 3-PG, and 0.5 (B and C) or 2 mM (A and D) MgATP. The MgATP concentration was provided by 2.1 (A) or 0.5 mM (B and C) $MgCl_2$ and various excess concentrations of ATP or by 2.1 mM $MgCl_2$ and 4.0 mM ATP (D). Activity was determined as a function of glycerol 3-phosphate concentration in the presence of 2.0 (●), 11 (■), and 19.5 mM (▲) $Mg^{2+}$-free ATP (A), as a function of adenosine concentration in the presence of 2.1 (●), 11.7 (■), and 19.5 mM (▲) $Mg^{2+}$-free ATP (B), as a function of sulfate concentration in the presence of 10 (●), 15 (■), and 20 mM (▲) $Mg^{2+}$-free ATP (C), and as a function of glycerol 3-phosphate concentration in the absence (●) and in the presence of 150 (■) and 250 mM (▲) sulfate (D) and plotted according to Yonetani and Theorell (*55*).

slightly increased, while the extent of inhibition is greatly decreased (Table 1). These substrate-dependent changes allowed us to observe a very small, but significant, activation even by glycerol 3-phosphate (Figure 2B), which was not detectable at low substrate concentrations (Figure 2A).

In general, the maximum activation occurs at millimolar concentrations of the analogues, at 15−20 mM for sulfate or phosphate and at about 1 mM ATP. As most of the effectors are present in vivo, the similarity of these values to the physiological concentrations, at least for metal-free ATP [about 0.4 mM (*51*)] and for inorganic phosphate [about 8 mM (*52*)], indicates that activation may occur naturally.

These unusual kinetic effects of anions remained the same even after preincubation for 10−20 min of the enzyme with the ligands; thus, activation cannot be attributed to slow equilibration with the ligands. In case of rapid equilibration, however, the activation and inhibition by any ligand, L, cannot be described by a simple model of 1:1 binding stoichiometry. In a model of 1:1 stoichiometry, raising the concentration of ligand cannot first activate and later inactivate, since the ratio between species with the activating and inactivating (sub)sites filled is independent of ligand concentration. Thus, despite the absence of independent evidence in favor of it, a model of 2:1 binding stoichiometry must be assumed. According to this model, activation, caused by the occupation of the first site, turns into inhibition as soon as the second site becomes saturated. This type of model cannot distinguish, however, between independent and interacting sites, as pointed out earlier by Simms (*53*) and Dixon (*54*); in either case, the net dependence of the enzyme activity on ligand concentration is given by the curve that has a maximum. The experimental points in Figure 1A−G can indeed be satisfactorily fitted by theoretical curves calculated on this basis (eq 4 in Materials and Methods). The fitted parameters (Table 1) were obtained by keeping the initial estimates of the activation factor *a* close to each other for the anionic ligands with various chemical structures. This assumption was based on their closely similar extent of activating effects (Figure 1), indicating no particular specificity.

Figure 1 also shows activation and inhibition (dotted and dashed lines, respectively); the difference between them yields the actual dependence of activity on [L]. The

activation component, given by the first two terms of eq 4, represents a simple hyperbolic dependence on [L], whereas the inhibition component, given by the third negative term of eq 4, represents a more complex sigmoidal dependence on [L].

In some cases, e.g., with metal-free ATP or with sulfate, the fitted curve runs somewhat above the experimental points in the range of inhibition. This deviation can be attributed to the fact that each of these ligands may have more than one inhibiting site. In fact, the cooperative character of inhibition by metal-free ATP, as indicated by the parabolic Dixon plots, has been observed in a kinetic study (*15*).

It has to be emphasized that the values obtained for $K$ are not the real but apparent dissociation constants of the activating and inhibiting ligand complexes, because the substrate(s), present in the assay mixtures, may compete with the anions. This explains the variation of $K$ with the substrate concentration; at higher 3-PG concentrations, the values of the apparent $K$ constants are increased (Table 1). Knowing the substrate concentration, however, we can derive real $K_d$ values, independent of the substrate concentration, from the apparent ones (Table 1). The effect of substrate concentration is clearer for $K_{d(inh)}$ values. The real $K_{d(inh)}$ values closely resemble the corresponding $K_I$ competitive inhibitory constants, supporting the assumed competition between the anionic ligands and the substrate, 3-PG, i.e., the identity between the inhibiting site and the catalytic site for 3-PG. As for the constants $K_{d(act)}$, characteristic of activation, the effect of the substrate concentration is less pronounced. Hence, the ratio of $K_{d(act)}$ and $K_{d(inh)}$ depends on the substrate concentration. This makes activation somewhat more pronounced at higher 3-PG concentrations. This finding is demonstrated even better by the 3-PG saturation curves (see the section below and Figure 4A) determined both in the absence and in the presence of an anionic effector; at higher substrate concentrations the activation while at lower substrate concentrations the inhibition dominates, which makes the originally nonlinear double-reciprocal plot apparently a linear one (e.g., Figure 4A). The different effects of the substrate on activation and inhibition are entirely consistent with the assumption of two separate sites for activation and inhibition.

*Common Activator Site and Different Inhibitor Sites for the Various Anions.* Activations by ATP and by the other anions are mutually exclusive as demonstrated by separate experiments. Figure 1H for example shows that the extent of activation caused by ATP becomes smaller and smaller as the concentration of sulfate is increased; i.e., ATP and sulfate antagonize each other's activating action. In agreement, $K_{d(act)}$ for the first anion apparently increases as the concentration of the second anion is increased (legend of Figure 1). Thus, the mechanisms of activation by ATP and the other anions are very similar to each other, and they all may bind to the same regulatory site.

For the inhibiting site, we devised kinetic experiments for determining whether the inhibitory effects of any pair of effectors on the enzyme reaction were additive or exclusive. Such analysis, as originally described by Yonetani and Theorell (*55*), may be an indication of the existence of separate or common sites on the enzyme for the two inhibitors. The clearest results were obtained when one of the two inhibitors was glycerol 3-phosphate, which binds

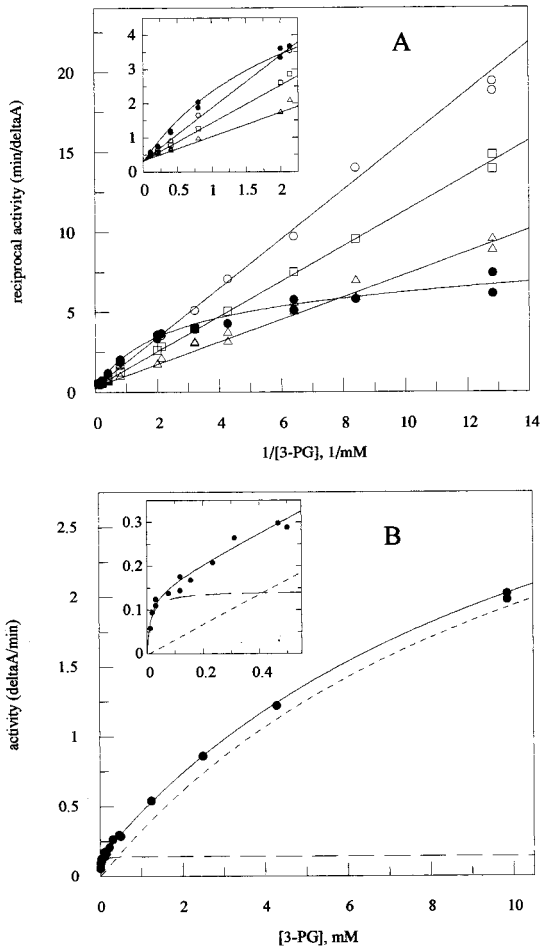


FIGURE 4: Variation of PGK activity as a function of 3-PG concentration in double-reciprocal (A) and linear (B) plots. The activity of 16 nM enzyme was measured in the presence of 0.5 mM MgATP (provided by 1 mM $MgCl_2$ and 0.6 mM ATP) and various concentrations of 3-PG. The double-reciprocal plot (A) shows the experiments carried out both in the absence (●) and in the presence of 50 (△), 75 (□), and 100 mM (○) glycerol 2-phosphate. The calculated competitive inhibitory constants for glycerol 2-phosphate ($K_I$) is 9.6 ± 2.0 mM. The linear plot (B) of the saturation of PGK by 3-PG shows only the experiment in the absence of inhibitor. The experimental points were fitted according to eq 5 of Materials and Methods. The components of the fitted curve (solid line) are also shown. The dotted and dashed lines correspond to saturation of the first (catalytic) and the second (regulatory) sites, respectively. In panels A and B, the insets show the enlarged initial parts of the plots.

firmly to the site of inhibition. In the presence of different, in each case constant, concentrations of metal-free ATP, straight lines intersecting on the abscissa were obtained as a function of glycerol 3-phosphate concentration in the Yonetani−Theorell plots (Figure 3A). Thus, the inhibiting effects of glycerol 3-phosphate and metal-free ATP are apparently additive, suggesting the existence of separate sites for these two inhibitors. As glycerol 3-phosphate is a good structural analogue of 3-PG and, as with the substrate itself, only the d-enantiomer has been shown to bind specifically to the enzyme (*37*), the most probable inhibitory site of this analogue is the site of 3-PG. For metal-free ATP, however, it is reasonable to assume a different type of nonionic interaction with the enzyme, e.g., through its adenosine part with the hydrophobic pocket of the nucleotide site, well defined by structural studies (*4*, *5*, *7*).

Table 2: Summary of the Results of Double-Inhibition Studies[a]

| inhibitor 1 | inhibitor 2 | type of inhibition |
|---|---|---|
| ATP | glycerol 3-phosphate | additive |
| | adenosine | mixed type |
| | sulfate | mixed type |
| | glycerol 2-phosphate | mixed type |
| adenosine | sulfate | additive |
| | glycerol 3-phosphate | additive |
| glycerol 3-phosphate | sulfate | exclusive |
| | glycerol 2-phosphate | exclusive |
| | 2-phosphoglycolate | exclusive |

[a] The experiments, like those in Figure 3, were carried out in the simultaneous presence of two inhibitors of different types. The data were plotted according to Yonetani and Theorell (*55*), i.e., the reciprocal activity vs concentration of inhibitor 1, at different, in each case constant, concentrations of inhibitor 2. In these plots, the additive character of the two inhibitors is reflected by a pattern of the straight lines intersecting each other on the abscissa, while in case of the exclusive nature of the two inhibitors, parallel lines were obtained.

However, the picture obtained in a similar double-inhibition experiment, but with adenosine in place of glycerol 3-phosphate, does not unequivocally support this assumption. Straight lines obtained approach each other at different concentrations of metal-free ATP with a possible intercept in the third quarter of the Cartesian coordinate system (Figure 3B). If ATP, as an inhibitor, were bound exclusively to the adenosine site, parallel lines should have been obtained as an indication of exclusive inhibition of the two ligands. Instead, the result shows that adenosine can only weaken ATP binding, but cannot abolish it. Thus, in addition to the adenosine site, the inhibiting metal-free ATP may have also other binding site(s) on the enzyme. Multiple sites for metal-free ATP, indeed, were also postulated by Larsson-Raznikiewicz and Schierbeck (*15*).

We searched for a possible anionic site for the metal-free ATP by double-inhibition experiments with sulfate as a second inhibitor (Figure 3C). Because of the nonlinearity of the plots, indicative of cooperative binding, the results are not as conclusive as above. Despite the uncertainties, one can observe that the curves at different concentrations of metal-free ATP approach each other, rather than remaining parallel, indicating that sulfate may weaken the binding of ATP, but cannot completely replace it. A similar picture was also obtained in a separate experiment with another anionic effector, glycerol 2-phosphate (not shown). At the same time, a control double-inhibition experiment with sulfate and adenosine yielded straight lines (as a function of adenosine concentration) at different sulfate concentrations; these clearly intersected each other on the abscissa (not shown), indicating additive character of their inhibition, as expected on the basis of independent sites. On the other hand, double-inhibition experiments with glycerol 3-phosphate and sulfate (Figure 3D) (or any of the other anionic analogues, except ATP) yielded clearly parallel lines, indicative of their exclusive binding. The results of all double-inhibition experiments are summarized in Table 2.

These experiments, on one hand, support the existence of a common inhibiting anionic site(s) for all of the inhibitors, except for metal-free ATP; on the other hand, these studies raise the possibility of the existence of two different type of inhibiting sites for ATP. One of these two sites may be a relatively weak anionic site (where sulfate and other anions may also be able to bind, in addition to their main inhibiting site), and the other one may be the nonionic adenosine site. Testing of these suggestions by direct equilibrium dialysis binding studies is underway.

*Activation of PGK by the Excess Substrate*. Figure 4 illustrates a typical dependence of the rate of the PGK-catalyzed reaction upon saturation by the substrate, 3-PG, determined at low ionic strength. In the absence of any anionic effector, the usual double-reciprocal plot of the kinetic data does not yield a simple straight line; activation by the excess substrate has been revealed (Figure 4A), in agreement with earlier observations (*13*, *16*, *17*). Thus, the curves of velocity versus substrate concentration (Figure 4B) cannot be fitted by a single hyperbola. This non-Michaelian character of the substrate saturation has been interpreted long before any structural information was available about the enzyme, by assuming the existence of two independently functioning catalytic sites. Indeed, the experimental points of Figure 4 could be fitted by a theoretical curve of the sum of two hyperbolas (not shown). This assumption, however, cannot be held in light of the X-ray structural data showing the presence of only one catalytic site (*5*, *7*).

An anomalous substrate saturation curve might also be a consequence of slow formation of the active enzyme−substrate complex, relative to the rate of the catalyzed reaction, often coupled with a protein conformational change. For PGK, indeed, a large-scale hinge-bending domain closure was assumed a long time ago, as an essential requirement for the occurrence of the enzyme reaction (*1*), supported by small-angle X-ray scattering studies (*56*, *57*) and by more recent crystallographic data (*3*−*5*, *7*). We have found, however, that preincubation for 10−20 min of PGK with both of its substrates (3-PG and MgATP) had no influence on the measured rate of the catalyzed reaction. This result clearly shows fast equilibration with the substrates, as with the anionic effectors. Even if domain motion occurs before the catalytic reaction, this protein isomerization step, which probably accompanies substrate binding, must also be faster than the enzymatic reaction itself. Consistent with this, Geerlof et al. (*24*) had already proposed that the putative hinge-bending domain motion occurred before establishment of the steady state. Thus, the anomalous substrate saturation curve cannot be due to slow equilibration with the substrates.

Here we show that activation by the excess of the substrate and activation by various anionic effectors are related phenomena and both can be described by a similar two-site model. Accordingly, the excess of the substrate, which is itself an anion, can bind not only to the catalytic site but also to the same regulatory site where the other activating anions bind. The experimental data in Figure 4, indeed, can be satisfactorily described by eq 5 in Materials and Methods, on the basis of this model. For a better illustration, enlargements of the initial parts of both the double-reciprocal (A) and the linear (B) plots are also shown as insets in these figures. The parameters that gave the best fit are shown in Table 1. Thus, the enzyme molecules, in which only the catalytic site is filled by the substrate, may function only with a moderately low activity. With a large excess of substrate, the weaker regulatory sites may also be filled, with a concomitant great enhancement of activity.

*Activation of the Reaction with 3-PG by a Weak Substrate*. The above model assumes simultaneous binding of two 3-PG molecules to the enzyme (one to the catalytic and one to the

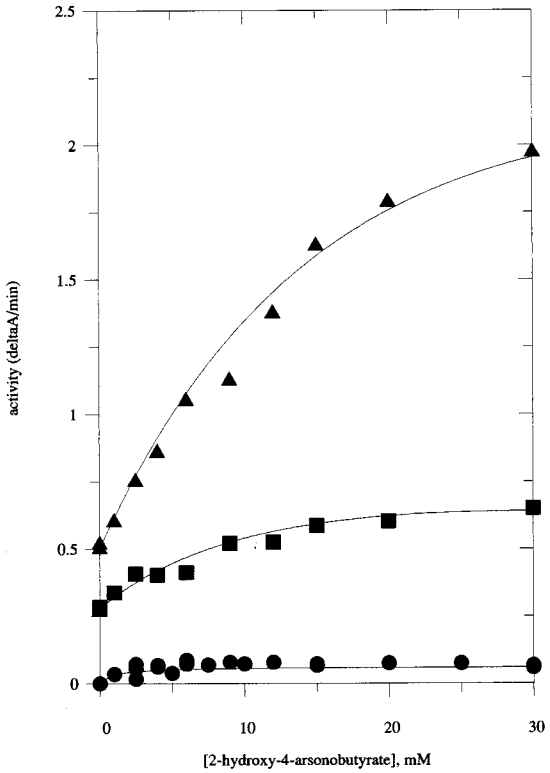


FIGURE 5: Effect of 2-hydroxy-4-arsonobutyrate on the reaction of PGK with 3-PG. The activity of 40 nM enzyme was measured in the presence of 0.5 mM MgATP (provided by 1 mM $MgCl_2$ and 0.6 mM ATP) in the absence (●) and in the presence of 0.05 (■) and 0.2 mM (▲) 3-PG as a function of 2-hydroxy-4-arsonobutyrate concentration. Curve fitting was carried out by using eq 11 of Materials and Methods. For variables that gave the best fit, see Table 1.

regulatory site), for which direct evidence is still lacking. Neither structural nor binding studies indicate binding of a second substrate molecule to PGK. The hypothesis that a separate regulatory site exists is strongly supported by the following experiment.

We have investigated whether a weak substrate, the arsonomethyl analogue of 3-PG, i.e., 2-hydroxy-4-arsonobutyrate, can activate PGK in the reaction with its physiological good substrate, 3-PG. Keeping the concentration of 3-PG at a constant, nonsaturating level, we progressively increased the concentration of the weak substrate. A large acceleration of the enzyme reaction was detected, although the activity with the weak substrate itself is clearly negligible under these conditions (Figure 5). This activation can only be due to binding at another site, since any binding at the catalytic site would have caused inhibition by gradual replacement of the good substrate, 3-PG, by the increasing concentration of the poor substrate. The experimental data on Figure 5 can, indeed, be described by eq 11 in Materials and Methods, which assumes activation, and also takes into account the competition of the two substrates for the catalytic site. This finding provides direct evidence in favor of the existence of a separate regulatory site.

*Modeling of Binding of Regulatory Anions to PGK*. To search for the location of the regulatory site in the three-dimensional structure of the PGK molecule, we have inspected the available X-ray structural data of PGK. The highly conserved structure of this enzyme allows comparison of data of different origins. Up to now, several structures of various complexes with substrates are known at high resolution (*1*, *3*−*5*, *7*), but none of them shows binding of a second substrate molecule. Thus, apparently, no information has been provided about the location of the postulated regulatory anionic site. This site could not be identified even from structural data obtained in the presence of high concentrations of sulfate (*1*), although in this structure all of the possible anionic sites, including the activating one, should have been occupied by sulfate. As mentioned in the introductory section, three different anionic sites could be observed from X-ray and NMR data, but none of them was clearly separated from the substrate sites or from the active center area.

We suggest therefore that the regulatory site may not exist originally in the structure of the substrate-free enzyme but may only be formed as a consequence of the conformational change, which occurs upon binding of both substrates. The recently published crystal structure of the *T. brucei* PGK complexed with MgADP and 3-PG revealed a new conformation of the enzyme, basically different from all of the previously known structures, showing a large extent of domain rotation (*7*). We have attempted to model (see Materials and Methods) the possible binding of the various anionic effectors, both to the closed conformation of this ternary complex of the *T. brucei* enzyme and to the previously determined open conformation of the 3-PG binary complex of pig muscle PGK, into which crystals of an ATP analogue, MnAMP-PNP, have been diffused (*5*).

Panels A and B of Figure 6 illustrate the results of docking of an orthophosphate ion to these structures. A new anion-binding site can indeed be seen in the structure of the closed conformation (Figure 6A), while in the open conformation (Figure 6B), only a part of this site exists. This newly detected site is composed of the basic residues Lys-215 (219) from the C-domain and Arg-65 (65), among other basic patch residues, e.g., Arg-170 (172) and His-169 (171) from the N-domain. Besides the sequence numbering of the muscle enzyme, the corresponding numbering of *T. brucei* PGK is marked in parentheses. These residues are completely conserved in all of the known PGK sequences (*58*).

The role of the basic patch residues in binding 3-PG is known from high-resolution structural studies (*3*, *5*). Here we show that, in addition to their involvement in binding of this substrate, they still have remaining capacities to bind electrostatically a further anion. A regulator anion, which can be the phosphate ion, as in Figure 6A,B, or any of the anions investigated, or even the substrate itself, can bind to the outer surface of the basic residues, while these residues create a closed cavity with the bound substrate inside. It is notable that the role of the entirely conserved Lys-215 (219), which is situated on the open channel between the two domains (*5*), has not been recognized earlier.

By similar molecular modeling, all of the substrate analogues now investigated and even the metal-free ATP can be docked successfully by their phosphate groups into this anion-binding site (not shown). This indicates a low specificity for the regulatory site, consistent with the kinetic data (Table l). In general, these docking studies essentially supported our assumption that a regulatory anionic site may be formed only as a consequence of substrate binding and the accompanying hinge-bending domain closure.

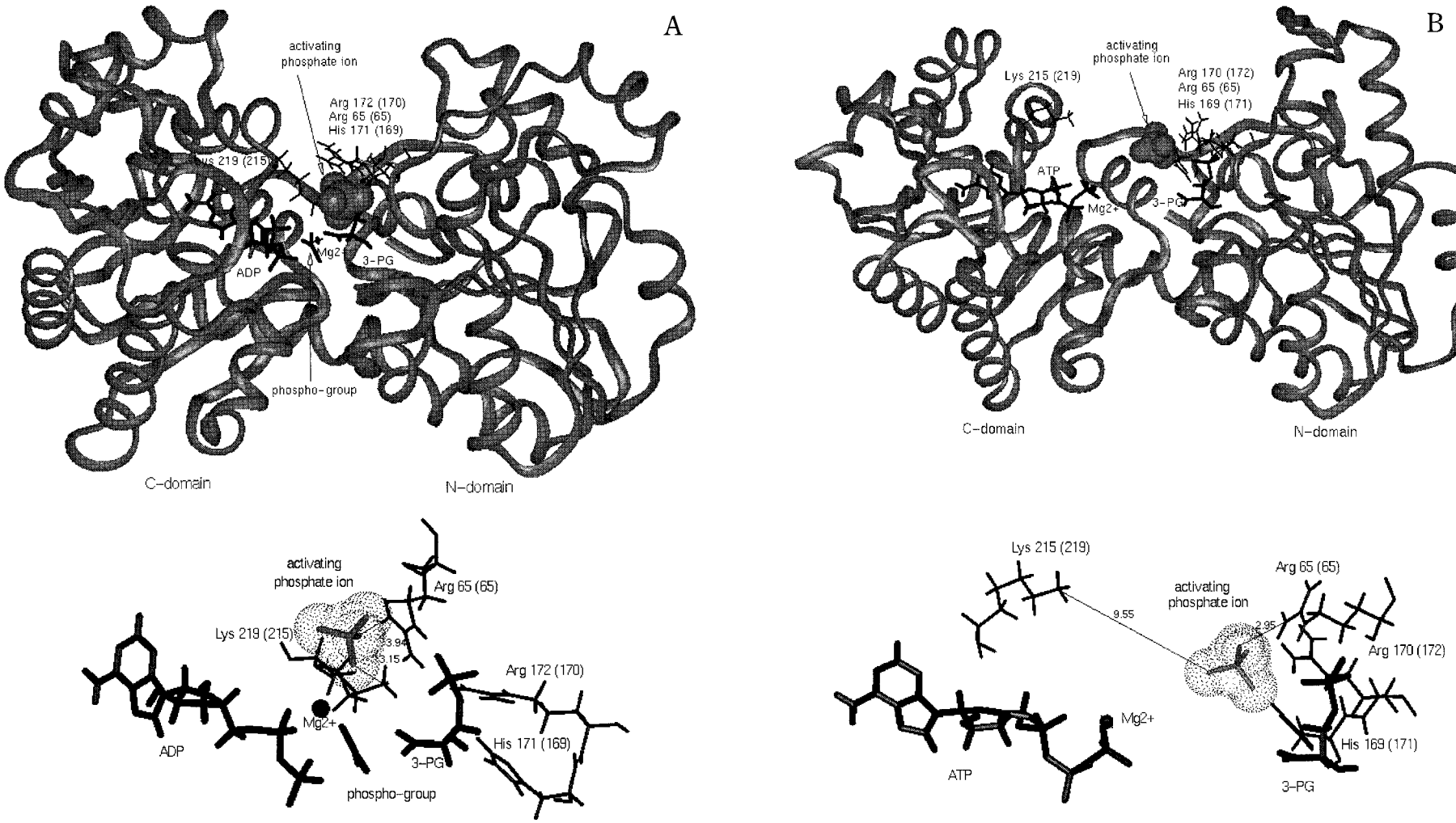


FIGURE 6: Modeling of phosphate ion binding to the closed (A) and open (B) conformations of PGK. Phosphate ion is shown by the van der Waals space-filling model at the proposed activatory anion binding site both in the closed structure of *T. brucei* PGK (A) and in the open structure of the pig muscle enzyme (B) (for X-ray coordinates, see Materials and Methods). The environment of this site is enlarged in the lower parts of both figures. The distances of phosphate oxygens from one of the guanidino N atoms of Arg-65 and from the amine N atom of Lys-219 (A) or of Lys-215 (B) are shown in angstroms. Residue numbering in brackets refers to the numbering of the other structure; i.e., in the structure of *T. brucei* PGK (A), the bracketed numbers designate the numbering of the corresponding residues in the structure of pig muscle PGK (B) and vice versa.

## DISCUSSION

The nature of kinetic cooperativity and regulation by anions of the monomeric PGK has long remained controversial because of the uncertainties about the roles and the spatial positions of the multiple anion binding sites of this enzyme. These kinetic results together with modeling studies, based on the existing structural data, including the recently published closed conformation of the enzyme (*7*), have led to a comprehensive picture about the location of the various anion binding sites of PGK.

Three possible sites for anions have been identified by early X-ray and NMR structural studies: two sulfate binding sites (one at the basic path region of the N-domain and one near the α-phosphate of the bound nucleotide in the C-domain (*11*, *39*) and a selenate binding site at the N-terminus of the α-helix (number 13 in the muscle enzyme), close to the assumed phosphotransfer area (*27*). Later structural studies with the enzyme−substrate complexes revealed the possible functional role of these three sites. The basic patch region creates a strong anionic site, which has been shown to be involved in binding of 3-PG (*3*). The anionic sites at Lys-219 (the numbering refers to the muscle enzyme) are involved in the binding of the α-phosphate, while the N-terminus of helix 13 is involved in the binding of *β*-phosphate of the nucleotide substrates (*4*, *5*). These anion sites are probably weaker than the site created by the basic path. In addition to these, the existence of a further anionic site between the N-terminus of helix 14 and Arg-38 (the numbering refers to the muscle enzyme) has been proposed to stabilize the transition state of the catalyzed reaction (*5*) and supported by modeling (*7*). Figure 6A shows the modeled transferable trigonal phospho group in this position. The existence and location of a hypothetical regulatory site responsible for activation by various anions or by excesses of substrates, however, remained an open question.

Our kinetic data provide strong evidence for the existence of a separate regulatory site; the docking of various anions to the known structures shed light on the structural basis of a unique mechanism of anion activation. According to this, the regulatory site does not exist in the open conformation, but is formed by the participation of certain residues from both N- and C-domains, as a result of the domain closure that occurs only in the presence of both substrates. This conclusion may also eliminate the apparent long contradiction between the kinetic and structural data of this enzyme. For example, it interestingly supports both different suggestions about the location of the activating site being at the basic patch (*11*, *21*) and at the area near the terminal phosphate of the nucleotide substrate (*18*).

As for the inhibiting sites, our double-inhibition kinetic studies allowed us to distinguish between at least two sites for anions, one strong and one possibly weaker. The main inhibiting site is proposed to be identical with the phosphate site for 3-PG. The existence of a secondary inhibiting anion site has been indicated by the finding that the inhibiting metal-free ATP does not interfere with other anions in binding to the main inhibiting site, while it partially interferes with binding of the less specific inhibiting sulfate and with the binding of adenosine. The most possible candidate for this inhibiting site is the site where the phosphates of the nucleotide substrate have been shown to be bound, including Lys-219 and the N-terminus of helix 13 (*4*, *5*).

Naturally, the site for activation holds much greater interest than the sites for inhibition, as the mechanism of activation has not yet been clarified. The formation of the activating site as a result of domain closure may suggest a close relationship between anion activation and the hinge-bending relative motion of the domains. It cannot be decided, however, whether anion activation is only a consequence of domain closure or anion binding itself mediates the closure by connecting the two domains. Anion binding to the activating site may also lower the energy barrier between the open and closed conformations of the enzyme and thereby may promote both closing and opening. Accordingly, even if the rate-limiting step is not domain closure, but the release of the very tightly bound product, 1,3-bisphosphoglycerate, as was shown by Geerlof et al. (*24*), activating anions may promote either directly or indirectly this step. Easier opening of the structure would indirectly assist in product dissociation. Furthermore, interaction of an activating anion with residues Arg-65 (65), His-l69 (171), and Arg-170 (172) (see Figure 6A,B) will shield part of the positive charges of the basic patch and therefore may directly weaken the product binding. Elucidation of the details of such an interesting activation mechanism is a great challenge for future research.

The problem of anion activation has a more general in vivo relevance in the regulation of the physiologically widespread enzymes that handle the transfer of the phopsho group among various anionic metabolites (from the intermediate metabolism to more complex regulatory mechanisms such as the cell response to external stimuli). Our investigations with PGK will contribute to general knowledge about the action and regulation of this type of enzyme that is being widely studied (*59*−*61*).

## NOTE ADDED IN PROOF

While this paper was being reviewed, another closed structure of PGK from *Thermotoga maritima* has been published (*62*). Its X-ray coordinates were kindly sent to us by G. Auerbach. Surveying the active site region of this structure may also allow the recognition of the corresponding anion binding site (here it is constituted by residues Lys-197 and Arg-62), suggested by this study. Auerbach et al., however, have suggested that Lys-197 is involved in stabilization of the transition state phospho group; i.e., a direct catalytic role, different from the presently suggested regulatory role, has been attributed to this conserved side chain (the corresponding residues are Lys-215 and Lys-219 in PGKs from pig muscle and *T. brucei*, respectively). Further studies are required to decide between these two proposals or to determine whether they are really alternatives.

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