Orientation of 1,3-Bisphosphoglycerate Analogs Bound to Phosphoglycerate Kinase*

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We have previously reported dissociation constants for a range of bisphosphonate analogs of 1,3-bisphosphoglyceric acid binding to yeast phosphoglycerate kinase. Data for the unsymmetrical analogs were difficult to interpret because it was not clear in which of the two possible orientations these ligands bound. Here we report a novel NMR method for quantifying orientation preference based on relaxation effects induced by titration with CrADP, which is applied to these ligands. It is shown that all ligands can bind in both orientations but that the driving force for the orientational preference is to put the α,α-difluoromethanephosphonate group in the “basic patch” (nontransferable phosphate) position. The relevance to the design of phosphoglycerate kinase inhibitors is discussed.

Phosphoglycerate kinase (PGK)1 (EC 2.7.2.3) is a glycolytic enzyme that catalyzes the following reaction, where MgADP and MgATP represent the magnesium complexes of ADP and ATP, respectively.

\[ 1,3\text{-Bisphosphoglycerate}^{+} + \text{MgADP}^{-} \rightarrow 3\text{-phosphoglycerate}^{3-} + \text{MgATP}^{2-} \]

**REACTION 1**

It is an attractive drug design target because trypanosomes rely on glycolysis as their sole source of energy, and hence inhibition of PGK could provide effective treatment for trypanosomal infections. The enzymes from a wide variety of sources have been compared (1). They show high sequence and structure similarity and consist of monomeric proteins of 45 kDa with two distinct domains connected by a hinge (Fig. 1). The N-terminal domain (which also contains the last 10 residues at the C terminus) has a “basic patch” containing a number of arginines and histidines that is the binding site for the glycolytic substrates 1,3-BPG and 3-PGA. The C-terminal domain is the binding site for ATP/ADP. The enzyme has a number of crystal structures (2–13). In many of the two domains are in an open conformation with the substrates −11 Å apart, which is much too far for direct phosphotransfer. Crystal structures also exist in which the enzyme forms a closed conformation with the two substrates close enough for direct nucleophilic associative phosphotransfer (14). A mechanism has been proposed based on these crystal structures of which the key features are that Arg-38 binds to the transferred 1-phosphate of 1,3-BPG and locates it close to the nucleophilic oxygen of ADP in the closed form, whereas Lys-213 (Lys-197 in *Thermotoga maritima*) (9) also binds to the phosphate, stabilizing the negative charge that develops on it as it transfers, thereby catalyzing the phosphoryl transfer. (Note: yeast PGK numbering has been used throughout.) The hinge bending has been studied in detail. Another subject of intense study is the kinetics of the reaction, which is activated by anions (15). Although the issue has not been resolved, it is likely that an anion binding site is formed when the two domains close (16).

The crystal structures have provided considerable information on how the substrates bind, both to the closed and to the open conformations. 1,3-BPG itself is very unstable, so no structures exist of bound 1,3-BPG. Crystals of 3-PGA show it bound with the 3-phosphate close to Arg-168, Arg-121, and Arg-65 in the basic patch, with the carboxylate close to Arg-38 and pointing toward the nucleotide. Somewhat surprisingly, in crystals of *Trypanosoma brucei* PGK grown in the presence of 3-PGA and MgADP from 2.5 mM potassium phosphate, two of the four subunits in the asymmetric unit have a phosphate anion bound in the basic patch but close to Arg-38, i.e. close to the transferable phosphate site (11).

We have previously reported the synthesis and dissociation constants of PGK ligands based on 1,3-BPG (17, 18), which inhibit PGK (19). The phosphate linkages were replaced by non-scissile methanephosphonates, giving ligands that bind as much as 50 times more tightly than 3-PGA. It was observed that replacement of methanephosphonate by α,α-difluoromethanephosphonate increased the affinity, and it was suggested that this was because of the lower pKₐ induced by the fluorines (18, 20). However, replacement of both methanephosphonates by difluoromethanephosphonates led to no further increase in affinity, suggesting that only one end of the molecule required the lower pKₐ and consequent greater charge density at neutral pH. Affinities could not be interpreted further because it was not possible to tell in which orientation the unsymmetrical ligands were binding. We have therefore developed a method to distinguish the orientation of the ligands and report its application with an analysis of the implications for design of PGK ligands.
lignand, here denoted $\alpha$ and $\beta$, and use the relative broadening to measure the distance to each nucleus and hence the orientation of the ligand.

Chromium(III) is a high spin (3d$^3$ ion) dipolar relaxation probe. The contribution to the nuclear transverse relaxation rate ($1/T_2^p$) from the paramagnetic probe is given by equations derived by Solomon and Bloembergen and presented by Mild-van and Gupta (Ref. 25 and references therein) as Equation 1.

$$\frac{1}{T_{2,obs}^p} = f_q F(r) (\frac{C}{\rho})$$  \hspace{1cm} (Eq. 1)

$f_q$ is the mole fraction of nuclei bound, $C$ is a constant dependent on nuclear and electronic magnetic moments, $r$ is the distance from $\text{Cr}^{3+}$ to the relaxing nucleus, and $F(r)$ is a function of $r$ (correlation time for dipolar relaxation) and $\omega$ (Larmor frequency of phosphorus). The line width at half-height ($\Delta \nu_{1/2,obs}$) in the presence of the paramagnetic ion is inversely proportional to the observed $T_2$ ($T_{2,obs}$). Equation 2.

$$\Delta \nu_{1/2,obs} = \frac{2\pi}{T_{2,obs}}$$  \hspace{1cm} (Eq. 2)

Under conditions of fast exchange of the $\text{Cr}^{3+}$ on and off the protein, the observed relaxation rate can be considered to arise from two contributions (Equation 3), namely, the relaxation rate in the absence of the paramagnetic ion $1/T_{2,abs}^p$ and the relaxation rate arising from dipolar interaction with the bound paramagnetic ion, $1/T_{2,gp}^p$.

$$\frac{1}{T_{2,obs}^p} = \frac{1}{T_{2,abs}^p} + \frac{1}{T_{2,gp}^p}$$  \hspace{1cm} (Eq. 3)

Combining Equations 1, 2, and 3 for a $^{31}$P nucleus $\alpha$ gives Equation 4 ($r_\alpha$ is the assumed constant).

$$\Delta \nu_{1/2,obs} = C \Delta \nu_{1/2,abs} + k$$  \hspace{1cm} (Eq. 4)

$k$ is a constant assuming all $1/T_{2,abs}$ values are the same for all observed cases.

A plot of peak width at half-height versus $[\text{CrADP}]$ has a gradient of $C_{\alpha} r_\alpha^{-6}$. Thus, a ratio of gradients allows us to estimate the relative distances of the $^{31}$P nuclei $\alpha$ and $\beta$ ($r_\alpha/r_\beta$) in the basic patch from the paramagnetic probe $\text{CrADP}$ in the nucleotide binding site. The ratio of gradients is calculated according to Equation 5.

$$\text{gradient}_\alpha = \frac{C_{\alpha} r_\alpha^{-6}}{C_{\beta} r_\beta^{-6}}$$  \hspace{1cm} (Eq. 5)

because $C_{\alpha} = C_{\beta}$. Thus, the $^{31}$P signal that broadens most on the addition of $\text{CrADP}$ is the one closest to the $\text{Cr}^{3+}$.

If the enzyme remains in its open conformation when the ligands bind, then the ratio $r_\alpha/r_\beta$ is fixed and can be estimated from crystal structures (3) as 1.8. Any ratio of gradient/gradient$^{-6}$ less extreme than $1/r_\alpha/r_\beta = 1.34$ can therefore be interpreted as caused by the ligand binding in both possible orientations. If the ligand binds in conformation I with probability $p$...
and in conformation II with probability \(1 - p\) (Fig. 2), then the results are determined by Equations 6 and 7.

\[
\frac{1}{T_{2,\alpha}} \propto \frac{p}{r_{\beta}^p} + \left[\frac{(1 - p)}{r_{\beta}^p}\right] \quad \text{(Eq. 6)}
\]

\[
\frac{1}{T_{2,\mu}} \propto \frac{p}{r_{\beta}^p} \left[\frac{(1 - p)}{r_{\beta}^p}\right] + \left[\frac{(1 - p)}{r_{\beta}^p}\right] \quad \text{(Eq. 7)}
\]

Substituting Equations 6 and 7 into Equations 2 and 3, we obtain Equation 8.

\[
\frac{\text{gradient}}{\text{gradient}_\alpha} = C_r \left(\frac{p}{r_{\beta}^p} + \frac{(1 - p)}{r_{\beta}^p}\right) \quad \text{and} \quad C_r \left(\frac{p}{34r_{\beta}^p} + \frac{(1 - p)}{r_{\beta}^p}\right)
\]

(Eq. 8)

After rearrangement and using Equation 5, the apparent distance ratio is given by Equation 9, thereby allowing us to estimate conformational preference.

\[
r_{\alpha}/r_{\beta} = \frac{\left[34p + (1 - p)\right]}{p + 34(1 - p)} \quad \text{(Eq. 9)}
\]

This function is shown in Fig. 3, which shows that relatively small proportions of binding in the alternative conformation drastically reduce the apparent distance ratio.

If the enzyme conformation is altered in the direction of the closed form when the ligands bind, then both \(r_\alpha\) and \(r_\beta\) are reduced and the ratio \(r_{\alpha}/r_{\beta}\) increases. The conformational preference derived above (Equation 9) is therefore a maximum limit, and any closed form population brings the conformational preference closer to 50%.

RESULTS

\(^{31}\text{P}\) NMR spectra were acquired for a range of PGK ligands in 1:1 complexes with PGK, and line widths were measured as a function of added CrADP. The addition of CrADP caused a monotonic increase in line width as expected. From the \(^1\text{H}\) spectra acquired on titration of the ligands with PGK, it is clear that no large conformational changes occur in PGK, and thus the enzyme is expected to remain in the “open” conformation seen in the majority of crystal structures. Typical spectra are shown in Figs. 4 and 5 for a ligand containing only methanephosphonates and a ligand containing both a methanephosphonate and an \(\alpha,\alpha\)-difluoromethanephosphonate, respectively. Differential broadening exists of the two \(^{31}\text{P}\) signals, which indicates a conformational preference of the ligand. Line widths were measured by line fitting, and the gradients of line width versus CrADP were compared to derive an effective distance ratio of the two phosphorus atoms from the chromium, as in Equation 5. A typical result is shown in Fig. 6 for compound 6 (data from Fig. 5). The results are summarized in Table II, which also presents the affinities measured previously. None of the distance ratios measured approached the theoretical limit of 1.8 as presented above (assuming that the open enzyme conformation is the same as that in the crystal). The largest ratio found was 1.49. We therefore used the distance ratios to calculate the approximate conformational preference of the ligands, which are listed in Table II. Conformational preferences range between 29 and 94%, with some analogs showing almost no orientational preference. We note that nonspecific binding of the ligand to PGK would reduce the ratio toward a

![Fig. 3. Plot of Equation 9.](image-url)

![Fig. 4. CrADP titration of 1.](image-url)
50% orientational preference; therefore, the values given in Table II represent minimum values for the conformational preference.

The ligands were designed to have a similar electronic distribution to 1,3-BPG and 3-PGA in having either a simple ketone or an amide group in analogous positions to the substrates (Table I). Therefore it was expected that the non-symmetrical ligands would bind such that the carbonyl oxygen would occupy a location similar to that of the carboxylate of 3-PGA. The results presented in Table II for the nonfluorinated analogs 1, 4, and 5 show that they do indeed bind preferentially in the expected orientation but that the conformational preference is not strong.

We have previously shown that substitution of the methanephosphonate by the α,α-difluoromethanephosphonate group almost always gave a large increase in affinity for PGK (18). (One of the few exceptions is 7, which is mainly in the diol form and therefore has steric problems in binding.) This was true whether the fluorines were placed at what was expected to be the 1-phosphate end (2) or the 3-phosphate end (3), yet substitution at both ends gave binding that was no stronger and indeed often weaker. The results (Table II) indicate that in all cases the difluoromethanephosphonate group is positioned at the 3-phosphate end. This finding makes sense of the affinities as discussed below.

**DISCUSSION**

A quantitative use of paramagnetic relaxation as done here is only applicable if certain conditions are met. Many of the NMR-specific requirements have been addressed by Gupta and co-workers (25, 26), who showed in a study of pyruvate kinase that CrADP can be used as a substitution-inert paramagnetic analog of MgADP and that the relaxation effects are dominated by the paramagnetism of the chromium in a simple $r^{-6}$ relationship. CrATP has been shown to be a suitable analog of MgATP when binding PGK, and its effects on longitudinal relaxation rates were used to probe substrate binding (27). It is also of course necessary that CrADP binds at a single site on PGK. In this case chromium is in a very similar position to magnesium (28, 29). Considerable debate
has occurred as to whether 1,3-BPG and its analogs bind at
more than one site on PGK (16), and it is now reasonably
clear that at least in the open form it has only a single major
binding site (13). Finally, by using a low concentration of both
enzyme and CrADP along with tight binding PGK ligands we
can ensure that essentially all of the paramagnetic relaxation
of the ligand occurs while bound to the enzyme, and no
significant relaxation occurs in a CrADP-ligand binary
complex. This fact has been confirmed by measurements in
the absence of enzyme (data not shown).

Apparent $^{31}$P-chromium distance ratios were obtained from
measuring the change in line width of the $^{31}$P signal with the
addition of CrADP. In all cases the ratios were substantially
smaller than the theoretical limit of 1.8. This result could in
principle be explained if the geometry of the bound 1,3-BPG
analog relative to CrADP is very different from that of 1,3-
BPG so that the $^{31}$P-chromium distance ratios become more
similar. This circumstance is unlikely. In PGK crystal struc-
tures (2–13) and in a modeling study (30) the only significant
change is a complete or partial closure of the hinge between
the two domains that brings the transferable 1-phosphate closer
to the bound nucleotide while maintaining the same orientation
and therefore makes the ratio $r_{1}/r_{p}$ greater not smaller. The
two most likely explanations for the reduced distance ratios are
either that CrADP or the analogs bind weakly in different
binding sites or that the 1,3-BPG analogs can bind in the
correct binding site but with both possible orientations. The
first option can be ruled out as discussed above. Thus, binding
in two possible orientations is the most likely explanation for
the low $r_{1}/r_{p}$ ratio.

Equation 9 provides a crude but useful characterization of
the approximate conformational preference of the 1,3-BPG an-
alogs tested. The most striking result is that all of the $\alpha,\alpha$-
difluormethanophosphate analogs bind with the more highly
charged difluormethanophosphate at the 3-phos-
teine end. This finding is perhaps surprising in view of the
location of the phosphate anion at the 1-phosphate end in the
$T$. brucei crystal structure, but it allows us to interpret
the ligand affinities in a straightforward manner. We assume
that the greater charge density induced by the difluoro sub-
stitution is the main driving force for the conformational prefer-
ence seen. It may also be the case, however, that the fluorines
can act as mimics of the lone pairs of the phosphate-bridging
oxygen of the substrate and participate in hydrogen bonds to
the enzyme, as seen in a crystal structure of glycerol phosphate
bound to AMP-PCP-P (31). However, the result does agree with
the long standing findings that the 3-phosphate groups should
be doubly ionized to bind effectively (32) and that the binding of
the carboxylate of 3-PGA is less important (33). We note that
the strongest conformational preference was for 3, which has
the amide functionality in a "substrate-like" location.

The results indicate that the location of the difluormetha-
nosphonate dominates the orientation of binding and that
any functionalization of the carbon chain between the phos-
phate atoms has only a secondary effect. Thus, a difluormetha-
nosphonate group at the 3-phosphate end is always favor-
able and will make the ligand bind in this orientation even if
this means disrupting other substrate-like interactions else-
where. The effect can be seen from a comparison of 2 and 3,
both of which bind with the fluorines at the 3-phosphate end,
despite putting the carbonyl group of 2 in the "incorrect"
location. However, such "incorrectness" only reduces the affinity
from 2 $\mu M$ (3) to 6 $\mu M$ (2). This conclusion makes sense of our
previous study (18), which showed that substitution by a single
difluormethanophosphate always improved affinity but
that substitution of difluormethanophosphonates at both ends of
the ligands conferred no additional affinity and often re-
duced the affinity. We infer that the presence of a diflu-
romethanophosphate group at the transferred 1-phosphate
position must be unfavorable either for steric reasons or be-
cause the greater charge density on the difluromethanophos-
phonate cannot easily be stabilized in the open form of the
enzyme. The enzyme functions by stabilizing the developing
negative charge on the transferring phosphate in the closed
form (11), and therefore we suggest that inhibitors should only
have a charge of $-1$ at the 1-phosphate end but a charge of $-2$
at the 3-phosphate end.

Our results suggest that the basic patch is important mainly
as a region of high positive charge to locate the 3-phosphate of
the substrate (34). This finding may explain why mutations in
the basic patch have relatively little effect on the kinetics of
the reaction (1, 35).

In summary, we have shown that the results obtained on the
orientation of PGK ligands allow us to interpret the affinities
measured previously. We suggest that an effective PGK inhib-
itor should have a high charge density at the non-transferred
3-phosphate end but a lower charge density at the transferred
1-phosphate end. Steric restrictions at the 3-phosphate end are
not strong. We have not identified strong requirements for
functionality within the ligand, although substrate-like func-
tionality provides weak additional affinity.

Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>$r_{1}/r_{p}$</th>
<th>Preference for 3-PG orientation</th>
<th>Dissociation constant $K_{D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>1.80</td>
<td>$-$</td>
<td>$110$</td>
</tr>
<tr>
<td>1</td>
<td>1.05 ± 0.1</td>
<td>58</td>
<td>$675$</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1.49 ± 0.2</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1.12</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>1.02</td>
<td>53</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>0.89</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>$&lt;1$</td>
<td>$&lt;$50</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ Calculated from Equation 9.
$^b$ Taken from Ref. 18.
$^c$ Determined from the crystal structure (3).
$^d$ Unknown, except that a crystal structure of 3-PGA bound to the
R654 mutant of yeast PGK had "a significant population" of molecules
bound in the opposite orientation (8).
$^e$ Large uncertainty makes the exact number hard to quantify.

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