Critical Role of Human Bisphosphoglycerate Mutase Cys^{22} in the Phosphatase Activator-binding Site*  

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The enzymatic activities catalyzed by bisphosphoglycerate mutase (BPGM, EC 5.4.2.4) have been shown to occur at a unique active site, with distinct binding sites for diphosphoglycerates and monophosphoglycerates. The physiological phosphatase activator (2-phosphoglycolate) binds to BPGM at an undetermined site.  

BPGM variants were constructed by site-directed mutagenesis of three amino acid residues in the active site to identify residues specifically involved in the binding of the monophosphoglycerates and 2-phosphoglycolate. Substitution of Cys^{22} by functionally conservative residues, Thr or Ser, caused a great decrease in 2-phosphoglycolate-stimulated phosphatase activity and in the $K_m$ value of the activator, whereas it caused no change in other catalytic activities or in the $K_m$ values of 2,3-diphosphoglycerate (2,3-DPG) and glycerate 3-phosphate (3-PG, EC 1.1.1.12), indicating that Cys^{22} is specifically involved either directly or indirectly in 2-phosphoglycolate binding.  

Kinetic experiments showed that the $K_m$ of the cofactor and the $K_m$ of 3-PG were affected by the substitution of Ser^{20} indicating that this residue is necessary for the fixation of both 3-PG and 2-phosphoglycolate. The R89K variant has previously been shown to have a modified $K_m$ value for monophosphoglycerates, however, its affinity for 2-phosphoglycolate is unaltered, suggesting that Arg^{89} is specifically involved in monophosphoglycerates binding.  

CD spectroscopic studies of substrates and cofactor binding showed that 2,3-DPG induced structural modifications of normal and mutated enzymes which could be due to protein phosphorylation. Addition of 2-phosphoglycolate to phosphorylated proteins with normal affinity for the cofactor produced spectra with the same characteristics as unphosphorylated species.  

In summary, monophosphoglycerates and 2-phosphoglycolate have partially distinct binding sites in human BPGM. The specific implication of the Cys^{22} residue in 2-phosphoglycolate binding is of great significance in the design of analogs of therapeutic benefit.

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1 The abbreviations used are: BPGM, bisphosphoglycerate mutase; 3-PG, glycerate 3-phosphate; 1,3-DPG, glycerate 1,3-diphosphate; 2-PG, glycerate 2-phosphate; 2,3-DPG, 2,3-diphosphoglycerate; MPGM, monophosphoglycerate mutase; MPGM, monophosphoglycerate mutase; 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, glycerate 3-phosphate; 1,3-DPG, glycerate 1,3-diphosphate; 2-PG, glycerate 2-phosphate.
great benefit in the chemical synthesis of analogs capable of specifically modulating one of the three activities of BPGM. A prokaryotic expression vector constructed in our laboratory (23) was used to synthesize wild-type and mutant forms of BPGM in Escherichia coli. Analysis of the catalytic properties of variants have shown that Arg89 is specifically involved in the monophosphoglycerate-binding sites (24) as has been reported on a natural human BPGM variant (25–27). In contrast, we have shown that Gly13 is specifically involved in the diphosphoglycerate-binding sites and that replacement of Gly13 by a positively charged amino acid residue greatly activates the phosphatase reaction, whereas the synthase and mutase reactions are greatly reduced (28).

Further amino acid substitutions were made to determine which residues are actively involved in the catalytic reactions. Initially we focused on Cys22 as sulfhydryl treatment of human BPGM in (29) was used to study the effect on the secondary structure and on the catalytic activity of the protein. The Cys22 residue was replaced by Leu, Ser, or Thr by changing the BPGM cDNA sequence. The purified enzymes were stored in sodium phosphate buffer containing 20% glycerol at −80 °C until used. Protein concentration was determined using the method of Lowry et al. (35).

Synthase and Mutase Assays—Synthase activity was measured using a simplified method recently published (37). Mutase activity was measured as described previously (25). The catalytic constants of synthase and mutase activity were calculated using the $\varepsilon$ of NADH of 6.22 mM cm$^{-1}$ at 334 nm and by fitting experimental points to the Michaelis-Menten equation with a nonlinear iterative regression program.

Phosphatase Assay—Phosphatase activity was measured using a radioactive procedure (37). Following the synthesis of the radioactive substrate $[\text{32P}]2,3$-DPG, the phosphatase reaction was monitored by measuring the radioactivity of monophosphorylated reaction products (P, and 3-PG). Two types of phosphatase reaction were studied. The first was hydrolysis of 2,3-DPG in the presence of activator ions: chloride (100 mM) and phosphate (10 mM) (25). This reaction was called the ion-stimulated phosphatase activity. The second reaction was 2,3-DPG hydrolysis in the presence of both anions and the physiological activator 2-phosphoglycerate (50 μM). This reaction was called the 2-phosphoglycerate-stimulated phosphatase activity.

Circular Dichroism Spectroscopy—All far-UV CD spectra were recorded on a Jasco 700 spectropolarimeter in a 0.1-cm cell at 20 °C, at enzyme concentrations between 5 and 10 μM. Near-UV spectra were measured using 1-cm quartz cells at concentrations 10-fold higher. The average of 10 runs was taken corrected for solvent distortion. Samples were prepared in 10 mM sodium phosphate buffer at pH 7.0. The concentration of samples was determined from the UV absorption at 280 nm using $\varepsilon$ of the BPGM monomer of 50 mM cm$^{-1}$. The CD data are given as mean residue ellipticity in units of degree cm$^2$ dmol$^{-1}$.

RESULTS AND DISCUSSION

Purification of Wild-type and Mutant BPGMs Expressed in E. coli—In the human, murine, and rabbit BPGMs, the Cys22 and Ser23 residues are strictly conserved (Table I), however, at the position of Ser23 in BPGMs there is always a Gly in the MPGMs from human muscle and brain and from yeast. In contrast, whereas Cys22 is conserved in human muscle MPGM, it is substituted by Ser in human brain MPGM and by Thr in
2-Phosphoglycolate-binding Site in Bisphosphoglycerate Mutase

TABLE I
Alignment of the amino acid sequences (one-letter code) of the human BPGM, rabbit BPGM, murine BPGM, human MPGM type M (MPGM-M), human MPGM type B (MPGM-B), and yeast MPGM. Residue numbers refer to the human BPGM amino acid sequence.

| Enzyme           | Amino acid residues |
|------------------|---------------------|
| Human BPGM       | F  C  S  W          |
| Rabbit BPGM      | F  C  S  W          |
| Murine BPGM      | F  C  S  W          |
| Human MPGM-M     | F  C  G  W          |
| Human MPGM-B     | F  S  G  W          |
| Yeast MPGM       | F  T  G  W          |

TABLE II
Steady-state kinetic parameters of synthase activity of wild-type and mutant forms of BPGM

| Enzyme       | k_{cat} | K_{m} 3-PG | k_{cat}/K_{m} |
|--------------|---------|------------|---------------|
| WT           | 13.63 ± 0.30 | 28.70 ± 0.90 | 0.47 ± 0.02  |
| C22T         | 8.48 ± 0.85  | 23.86 ± 4.03 | 0.36 ± 0.10  |
| C22S         | 9.94 ± 0.91  | 20.98 ± 0.93 | 0.47 ± 0.06  |
| S23G         | 4.63 ± 0.19  | 51.90 ± 5.20 | 0.09 ± 0.01  |

* 3-PG as substrate.

experiments were conducted in near- and far-UV regions for all the purified enzymes, to detect aberrant protein folding or large-scale structural modifications induced by the amino acid substitutions.

The far-UV circular dichroism spectrum (185–250 nm) is a sensitive probe for protein secondary structure. The wild-type spectrum is characterized by two negative peaks at 208 and 222 nm and by a positive one at 192 nm (Fig. 2A). The relative intensities of these peaks and the global shape of the spectrum are characteristic for α/β protein three-dimensional structure (38). The amplitude of the negative peak at 222 nm is directly related to the structure of the α-helix. The mutants analyzed in this study (C22L, C22T, C22S, S23G, and R89K) all had similar CD spectra (data not shown), suggesting that the secondary structure was not significantly perturbed by the amino acid substitutions.

The near-UV CD spectrum (250–300 nm) gives a qualitative measure of changes in tertiary structure as it is sensitive to asymmetry of aromatic side chains (Trp, Tyr, and Phe). All the variants had similar near-UV spectra to the wild-type BPGM (Fig. 2B).

The enzymes studied here were found to be stable in solution under the experimental conditions used. Taken together with the CD results, this strongly suggested that the secondary and global tertiary structure of the mutant enzymes was unaltered by the amino acid substitutions. The alterations in enzyme activity are therefore likely to be due to the local changes induced by specific amino acid substitutions.

Functional Analysis—The Michaelis constants were determined for each substrate. In addition, the affinity constant for 2-phosphoglycolate was also determined (Tables II-IV). A detailed analysis of all the catalytic activities enabled the functions of Cys^{22}, Ser^{23}, and Arg^{39} in monophosphoglycerates and phosphatase activator binding to be distinguished.

Cys^{22} Is Specifically Involved in 2-Phosphoglycolate Binding—When Cys^{22} was substituted by Ser, a polar residue of similar size, only the 2-phosphoglycolate-stimulated phosphatase reaction was significantly reduced (Tables II-IV). The presence of another polar residue (Thr) in this position caused an additional decrease in cofactor-stimulated phosphatase reaction, indicating that residue 22 and its specific polarity are important in this catalytic activity. An increase in the size of the side chain from Ser to Thr caused a moderate alteration of all the activities (Tables II-IV), suggesting that the bulkier moiety reduced enzymatic activity. This supposition was reinforced by the functional analysis of the C22L variant which had lost all catalytic activities (data not shown), probably due to the large size of the leucine side chain.

Further analysis of the catalytic reaction parameters may
facilitate an understanding of the molecular basis of the reactions. The steady-state kinetic parameters of the synthase, mutase, and ion-stimulated phosphatase activities were not significantly altered in C22S and C22T (Tables II-IV), whereas the affinity for 2-phosphoglycolate was drastically reduced, with $K_a$ values 22-fold higher for C22T, and 16-fold higher for C22S than for the wild-type enzyme (Table IV). In contrast, the $K_m$ values for 2,3-DPG and 3-PG of the Cys22-mutated enzyme was not significantly altered.

The enzymatic data provides good evidence that Cys22 is necessary for 2-phosphoglycolate-stimulated phosphatase activity probably via activator binding. These results are consistent with those of Diederich et al. (39) who reported that the phosphatase reaction of yeast MPGM which has a Thr side chain at position 22, was not activated by 2 mM 2-phosphoglycolate, whereas this catalytic activity was stimulated 9-fold in MPGM from human skeletal muscle, which has a Cys residue in the position corresponding to Cys22 in human BPGM. Furthermore, MPGM from human erythrocytes (brain type) which has a serine residue at position 22, has very little phosphatase activity, even in the presence of 1 mM 2-phosphoglycolate (40), indicating the lack of activation by this cofactor.

Our results, in agreement with Hg2+-inhibition experiments on BPGM activities (30), did not implicate Cys22 in 2,3-DPG binding, indicating that cysteine oxidation did not impede this binding. All the data support the conclusion that Cys22 is specifically involved, directly or indirectly, in 2-phosphoglycolate binding of human BPGM.

Ser23 Is Involved in the Binding of Both Monophosphoglycerates and 2-Phosphoglycolate—The properties of the S23G variant were very similar to MPGM: the mutase activity was highly effective whereas the synthase reaction was significantly reduced (Tables II-IV). Substitution of Ser by Gly did not affect either of the 2,3-DPG hydrolysis reactions whereas 2-phosphoglycolate stimulated activity was greatly reduced.

A detailed analysis of the catalytic reactions indicated a 3-fold increase in the Michaelis constant for 3-PG in the mutase reaction and a 4-fold decrease in the affinity for 2-phosphoglycolate in the cofactor stimulated phosphatase reaction, however, no significant modification of the apparent affinity for 2,3-DPG was observed as provided by absence of its $K_m$ modification (Tables II-IV).

Two other proteins, mutated at position 23 were also studied. The kinetics showed that the replacement of Ser23 by Ala did not produce any significant change in the enzymatic reactions, however a variant with a Thr residue at position 23 was too unstable to be studied (data not shown).

These enzymatic results all provide strong evidence for a direct or indirect active role of Ser23 in 3-PG and 2-phosphoglycolate binding. The presence of a glycine residue at position 23 as in all MPGM species, may explain the increase in mutase activity.

Arg89 Is Specifically Involved in the Monophosphoglycerate-binding Site—Previous results obtained in our laboratory strongly suggested that the Arg89 residue of human BPGM is specifically involved in the 3-PG-binding site (24, 27). We completed the functional studies on the R89K mutant by investigating the ion-stimulated phosphatase activity and measuring the affinity constant for 2-phosphoglycolate. This substitution has no significant effect on the catalytic constant or the activator binding (Table IV).

As previously reported (24), the R89K mutated protein presents increased Michaelis constants for monophosphoglycerates, whereas the 2,3-DPG one is not affected. Our present results demonstrated that its affinity for 2-phosphoglycolate was also unchanged, indicating that Arg89 is only involved, directly or indirectly, in the monophosphoglycerate-binding sites. By these properties Arg89 allows a discrimination between monophosphoglycerates and 2-phosphoglycolate-binding sites.

### Table III

| Enzyme | $k_{cat}$ | $K_a$ | $K_m$ | $k_{cat}/K_m$ |
|--------|----------|-------|-------|--------------|
| WT     | $0.050$  | $29.41$ | $6.55$ | $0.0016$     |
| C22T   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |
| C22S   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |
| S23G   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |

$^a$ Determined with different concentrations of 2,3-DPG substrate.  
$^b$ Determined with different concentrations of 3-PG substrate.

### Table IV

| Enzyme | $k_{cat}$ | $K_a$ | $K_m$ | $k_{cat}/K_m$ |
|--------|----------|-------|-------|--------------|
| WT     | $0.050$  | $29.41$ | $6.55$ | $0.0016$     |
| C22T   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |
| C22S   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |
| S23G   | $0.054$  | $29.41$ | $6.55$ | $0.0016$     |

$^a$ Reaction occurred in the presence of 10 mM KH$_2$PO$_4$, and 100 mM KCl.  
$^b$ Reaction occurred in the presence of 10 mM KH$_2$PO$_4$, 100 mM KCl, and 50 mM 2-phosphoglycolate.  
$^c$ ND, not determined in this study but already published (24).
CD Spectroscopic Investigation of the Substrates and Cofactor Binding—The far-UV region of the CD spectrum was used to monitor possible structural changes of the enzymes upon ligand binding. Addition of 2,3-DPG to the native BPGM solution (protein monomer: 2,3-DPG molar ratio = 1:10) induced spectroscopic changes. A small but significant and reproducible decrease in the negative band at 208 nm was detected, whereas the α-helix-type band at 222 nm was practically unchanged (Fig. 3). Phosphorylation of the protein by 2,3-DPG is thought to be the first step in the mutase and phosphatases activities of BPGM (11) and CD spectral modification may be a result of this phosphorylation. The lack of resolution of the method precludes a precise structural explanation of the decrease in the 208 nm negative band, however, the observation is compatible with a relative decrease in the random coil spectral component (negative band at 197 nm) in favor of more regular structures. The stabilization of the COOH-terminal structure of the enzyme by 2,3-DPG fixation and subsequent phosphorylation (11) would explain the spectroscopic data. Similar CD experiments with yeast MPGM failed to detect any effect of 2,3-DPG (41). This may be due to differences between the COOH-terminal structures of MPGM and BPGM.

Similar results were obtained for the C22S, C22T, S23G, and R89K variants probably due to their comparable binding parameters for 2,3-DPG. Monophosphoglycerate alone and 2-phosphoglycolate alone did not produce any spectroscopic modification.

The addition of 2-phosphoglycolate to the phosphorylated wild-type and R89K mutant led to restoration of the spectroscopic characteristics of the unmodified enzymes (Fig. 4). Structurally, this may correspond to dephosphorylation of the enzyme after stimulation of its phosphatase activity by the cofactor. This hypothesis is further supported by the fact that the two phosphorylated variants, S23G and C22T, which have lower 2-phosphoglycolate affinity than the wild-type and decreased cofactor-stimulated phosphatase activity, show no additional CD modification in the presence of the cofactor.

In contrast, addition of 3-PG to the phosphorylated native and mutated BPGMs did not produce a significant spectral change, probably because the enzymes maintain the phosphorylated state. The presence of 3-PG induces the mutase activity without any reduction in the 2,3-DPG level. Under these conditions, the transient phosphorylated state of the enzymes may be maintained.

CONCLUSIONS

The present results show that monophosphoglycerates and 2-phosphoglycolate have partially distinct binding sites in the active site of BPGM. Cys22 is involved specifically, either directly or indirectly, in 2-phosphoglycolate binding, whereas Arg89 is specific for 3-PG binding, demonstrating that the binding sites are distinct. On the other hand, Ser23 seems to be implicated in both binding sites. Delineation of the specific roles of amino acid residues in the active site is of great importance in the design of substrate or cofactor analogs of potential pharmacological benefit. Analogs of 2-phosphoglycolate modulating the 2,3-DPG level in red blood cells, without changing 3-PG binding, would be of great therapeutic advantage. Discrimination between the monophosphoglycerates and 2-phosphoglycolate-binding sites also prevents perturbation of red blood cells MPGM activity by avoiding cofactor analogs affecting the 3-PG-binding site. This is a critical requirement in therapeutic design since the integrity of MPGM and its interaction with 3-PG in the glycolysis, is critical for cell survival.

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