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Active Human-Yeast Chimeric Phosphoglycerate Kinases Engineered by Domain Interchange

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Phosphoglycerate kinase (PGK) is a monomeric protein composed of two domains of approximately equal size, connected by a hinge. Substrate-induced conformational change results in the closure of the active site cleft, which is situated between these two domains. In a study of the relations between structure and function of this enzyme, two interspecies hybrids were constructed, each composed of one domain from the human enzyme and one domain from the yeast enzyme. Despite a 35% difference in the amino acid composition between human and yeast PGK, catalytic properties of the hybrid enzymes are very similar to those of the parental proteins. This result demonstrates that the evolutionary substitutions within these two distantly related molecules do not significantly affect formation of the active site cleft, mechanism of domain closure, or enzyme activity itself.

IN MANY ENZYMES, DOMAINS (*1, 2*) CAN be viewed as functional units, involved in binding of substrates or cofactors. Binding of ligands frequently induces conformational changes within the domains, as well as relative domain movements (*3*). The importance of domain-domain interactions is particularly apparent for enzymes such as phosphoglycerate kinase (PGK), which have active sites located at the domain interfaces (*2*).

Phosphoglycerate kinases from yeast and horse muscle have very close structural homology (*4–6*) despite their 35% difference in amino acid sequence. Both proteins are composed of two globular domains of approximately equal size (22 kD), corresponding to the NH_2- and COOH-terminal halves of the molecule. Examination of a crystal structure of the yeast enzyme revealed a distance of about 10 Å between the inferred binding sites for the substrates, adenosine triphosphate (ATP) and 3-phosphoglycerate, each located on a different lobe of the enzyme (*4, 5*). It has been proposed that binding of substrates initiates a hinge-bending motion of the two domains, which results in the closure of an active site cleft (*5, 7*). A substrate-induced domain closure has also been postulated for other kinases (*8*). A crystallographic structure of phosphoglycerate kinase in the presence of both substrates is not available, and the nature of interactions involved in maintaining the closed conformation of the enzyme remains unknown. A precise arrangement of the amino acid residues on or near the interacting surfaces of both domains seems necessary for the formation of a functional catalytic center. Furthermore, many subtle intradomain and interdomain interactions may contribute to the mechanism of domain movement.

To study the importance of domain-domain interactions for (i) enzyme catalysis, (ii) protein folding, and (iii) protein stability, we have constructed two interspecies hybrids of phosphoglycerate kinase, each composed of a combination of one human and one yeast protein domain (Fig. 1). This approach exploits the 145 naturally occurring amino acid substitutions between the species. Studying protein function by interchanging protein domains is a potentially powerful approach that exploits the natural variability of protein structure. Construction of hybrid α subunits of tryptophan synthetase, produced by in vivo recombination of *Escherichia coli* and *Salmonella typhimurium* genes, has been reported (*9*), but in vitro recombinant DNA techniques have not previously been used for this purpose. We report here that protein domains that have been evolutionarily separated for as much as 2 billion years (divergence of plants and animals) can still interact to yield enzymes with almost full activity.

A high-copy-number plasmid, YEp9T (*10*), was used as a vector for the construction of the chimeric PGK genes—yeast-human PGK (yhPGK) and human-yeast PGK (hyPGK)—and for the expression of the chimeric and parental [yeast PGK (yPGK) and human PGK (hPGK)] genes (*11*). The yeast-human gene was constructed with the use of synthetic DNA (yeast sequence) to span a gap between the NH_2-terminal fragment of the yeast PGK gene, which had been cleaved at the Kpn I site, and the Nco I site in the human PGK coding sequence. The human-yeast hybrid gene was made with synthetic DNA (yeast sequence) to span a gap between the Nco I site (human PGK gene) and the Hpa II site in the yeast PGK gene. Therefore, these two constructions have the same junction point in the third base pair of the codon for a serine residue at position 172 in yeast PGK (174 in human PGK). This junction site in both hybrids is located within a highly conserved stretch of 16 amino acids (Fig. 2). The intact

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hinge is a part of the COOH-terminal domain of the parental protein (Fig. 1).

The expression vector for yeast and human PGK and the hybrid genes, YEp9T, contains the yeast 2-μm origin of replication and yeast *trp1* gene for complementation of the *trp1* mutation in *Saccharomyces cerevisiae* strains. For expression of hPGK, yPGK, and yhPGK, the recipient was the PGK^- yeast strain XSB44-35D (*a pgk1 trp1 ade1 leu1 gal1*). hyPGK was expressed in the 20B-12 strain (α *trp pep4–3*), because of difficulties with transformation of XSB44-35D. The yeast was transformed (*12*) and grown at 30°C in selective medium YNB + CAA (Trp selection) (*10*). Cells were collected by centrifugation, resuspended in buffer A containing 0.1*M* KCl, and homogenized in a glass bead homogenizer (Biospecific Products). Buffer A contains 0.01*M* MOPS (3-[*N*-morpholino]propanesulfonic acid) (*p*H 7.5), 1 m*M* dithiothreitol (DTT), 0.2 m*M* EDTA, and 0.1 m*M* phenylmethylsulfonyl fluoride.

The cell extract was treated with 2% protamine sulfate to precipitate nucleic acids. Proteins in the supernatant were concentrated by ammonium sulfate precipitation (85% final concentration). The precipitate was redissolved in buffer A, desalted by Sephadex G-25 gel filtration, and applied to a DEAE-cellulose column (2.7 × 18 cm) equilibrated with the same buffer. The unbound fractions were pooled and loaded onto a Cibacron Blue–Sepharose column (1.2 × 18 cm) equilibrated with buffer A. PGK was eluted with a linear gradient of 0 to 0.1*M* KCl. Pooled fractions containing PGK were concentrated with an Amicon ultrafiltration cell equipped with a YM10 membrane and fractionated by size on a Sephacryl S-200 column (2.5 × 51 cm) equilibrated with 0.01*M* MOPS (*p*H 7.5) plus 1 m*M* EDTA. All purification steps were performed at 4°C.

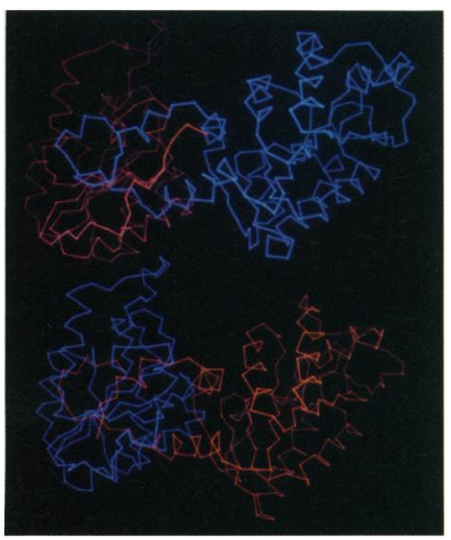

Fig. 1. Computer graphics representation of the structures of interspecies hybrids of phosphoglycerate kinase. (Top) Yeast PGK; (bottom) horse muscle PGK. Hybrid yhPGK consists of the NH_2-terminal yeast and COOH-terminal human domains (red); the hybrid hyPGK is composed of the NH_2-terminal human and COOH-terminal yeast domains (blue). The known structure of the horse muscle enzyme was used to represent human PGK. Both mammalian enzymes exhibit 98% sequence homology (*19*).

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Y   1  SLSSKLSVQDLDLKDKRVFIRVDFNVPLDGKKITSNQRIVAALPTIKYVLEHHPRYVVLA  60
H   1  ***N**TLDK**V*G***VM*******MKNNQ**N****K**V*S**FC*DNGAKS***M  60

Y  61  SHLGRPNGERNE-KYSLAPVAKELQSLLGKDVTFLNDCVGPEVEAAVKASAPGSVILLEN  119
H  61  ******D*VPMPD****E***V**K*******L**K********K*CANP*A********  120

Y 120  LRYHIEEEG-SRKVDGQKVKASKEDVQKFRHELSSLADVYINDAFGTAHRAHSSMVGFDL  178
H 121  **F*V****KGKDAS*N****EPAKIEA**AS**K*G***V*****************VN*  180

Y 179  PQRAAGFLLEKELKYFGKALENPTRPFLAILGGAKVADKIQLIDNLLDKVDSIIIGGGMA  238
H 181  **K*G***MK***N**A****S*E******************N*M****NEM*******  240

Y 239  FTFKKVLENTEIGDSIFDKAGAEIVPLKMEKAKAKGVEVVLPVDFIIADAFSADANTKTV  298
H 241  ***L***N*M***T*L**EE**K**KD**S**EKN**KIT*****VT**K*DEN*K*GQA  300

Y 299  TDKEGIPAGWQGLDNGPESRKLFAATVAKAKTIVWNGPPGVFEFEKFAAGTKALLDEVVK  358
H 301  *VAS******M***C****S*KY*EA*TR**Q******V****W*A**R*****M*****  360

Y 359  SSAAGNTVIIGGGDTATVAKKYGVTDKISHVSTGGGASLELLEGKELPGVAFLSEKK     415
H 361  ATSR*CIT*********CCA*WNTE**V****************V****DA**NI      416
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Fig. 2. Sequence homology between human and yeast phosphoglycerate kinase. Yeast PGK (*19*) and human PGK (*20*) exhibit 65% sequence homology (asterisks). The junction in the hybrid proteins (residues 172–173) is indicated by an arrow and is situated on the NH_2-terminal side of the α-helix that constitutes a covalent link between the domains (residues 185–199). The ATP binding site involves residues 211, 213, 217, 311, 334–341, 371, and 372. A hypothetical 3-phosphoglycerate binding site contains residues 21, 38, 62, 167, 168, 170, and 391–394 (*4*).

An additional step was included in the purification of hyPGK to separate it from the wild-type enzyme, yPGK, present in the 20B-12 strain. Good separation of the two enzymes was achieved on a CM-cellulose column (2.5 × 23 cm) equilibrated with 0.02*M* 2-(*N*-morpholino)ethanesulfonate buffer (*p*H 7.5) and eluted with a linear gradient of 0 to 0.1*M* KCl (Fig. 3A). The presence of both enzymes was monitored on denaturing polyacrylamide gels (*13*). Despite their nearly identical molecular weights, all four enzymes studied have significantly different mobilities on SDS-PAGE (Fig. 3B). We estimate that all proteins used to determine kinetic parameters were at least 98% pure.

Typically, 10 to 50 mg of protein were obtained from a 10-liter yeast culture grown to an optical density at 660 nm of 1.3. The composition of the hybrid enzymes was confirmed by amino acid analysis. The most characteristic difference was in cysteine content. Cysteic acid determination showed 1, 7, 5, and 3 cysteines for yPGK, hPGK, yhPGK, hyPGK, respectively, the expected numbers.

A comparison of the kinetic parameters for both hybrids, as well as for yeast and human phosphoglycerate kinases, is shown in Table 1. The Michaelis constants for ATP were almost identical for yPGK, hyPGK, and yhPGK. About a 45% increase in K_s for

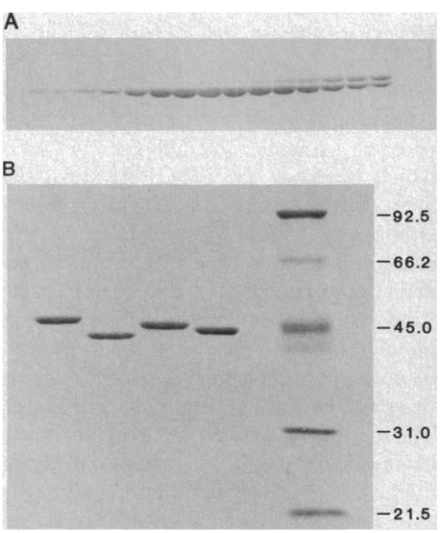

Fig. 3. SDS-polyacrylamide gel electrophoresis of human, yeast, and the interspecies hybrids of phosphoglycerate kinase. (A) Separation of hyPGK and yPGK on a CM-cellulose column. The first half of the protein peak contained pure hyPGK (faster migrating bands). The final preparation contained about 1% of contaminating yPGK (slower migrating bands). (B) Purified preparations of phosphoglycerate kinases. (Left to right) yPGK, hPGK, yhPGK, hyPGK (1 μg of each protein), low molecular weight standards (Bio-Rad), whose sizes are shown in kilodaltons. The gels (10% polyacrylamide) were stained with Coomassie blue.

Table 1. Comparison of kinetic parameters for yPGK, hPGK, yhPGK, and hyPGK. Steady-state kinetic parameters for the yeast, human, and interspecies hybrids of phosphoglycerate kinases were determined at a constant (1 m*M*) concentration of free magnesium ions (*17*). Kinetic parameters were investigated for the formation of 1,3-diphosphoglycerate by a conventional assay coupled to the glyceraldehyde 3-phosphate dehydrogenase reaction (*18*). Enzymatic activity was measured at 25°C by monitoring a decrease of optical density at 340 nm, which reflects the consumption of reduced nicotine adenine dinucleotide (NADH). Assays were performed in 0.02*M* triethanolamine buffer adjusted to *p*H 7.5 with acetic acid, and containing 0.05*M* Na_2SO_4, 1 m*M* magnesium acetate and 5 m*M* dithiothreitol. Glyceraldehyde phosphate dehydrogenase (150 μg/ml) and NADH (0.02 m*M*) were present in the assay solution. Michaelis constants (K_s) for ATP and 3-phosphoglycerate were determined at constant 3-phosphoglycerate concentration (10 m*M*) or constant ATP concentration (5 m*M*), respectively. The concentration of the other substrate varied. The values of kinetic parameters for each enzyme are the means ± SD obtained for at least two different preparations.

Substrate	3-phosphoglycerate*			ATP†		
Enzyme	$K_s \times 10^3$ (*M*)	$k_{cat} \times 10^{-4}$ (min^{-1})	k_{cat}/K_s (M^{-1} min^{-1})	$K_s \times 10^3$ (*M*)	$k_{cat} \times 10^{-4}$ (min^{-1})	k_{cat}/K_s (M^{-1} min^{-1})
yPGK	0.42 ± 0.04	2.96 ± 0.25	7.05×10^7	0.30 ± 0.02	3.59 ± 0.26	1.20×10^8
hPGK	0.38 ± 0.02	2.49 ± 0.21	4.93×10^7	0.32 ± 0.01	2.85 ± 0.12	1.02×10^8
yhPGK	0.61 ± 0.01	3.01 ± 0.16	6.55×10^7	0.30 ± 0.01	3.25 ± 0.14	0.93×10^8
hyPGK	0.95 ± 0.05	3.58 ± 0.36	3.77×10^7	0.44 ± 0.05	4.12 ± 0.40	0.94×10^8

*ATP concentration, 5 m*M*; 3-phosphoglycerate concentration, 0.1 to 8.8 m*M*. †3-Phosphoglycerate concentration, 10 m*M*; ATP concentration, 0.1 to 9.1 m*M*.

ATP was observed for hyPGK. The catalytic efficiency (k_{cat}/K_s) for all four enzymes was very similar (~1×10^8 M^{-1} min^{-1}). The values of the Michaelis constants for 3-phosphoglycerate were higher for the hybrid enzymes. A twofold increase was observed for hyPGK and about a 50% increase for yhPGK. The k_{cat}/K_s values for the four enzymes remained within the same order of magnitude (Table 1).

These results demonstrate that both combinations of yeast and human domains of phosphoglycerate kinase resulted in the formation of enzymatically active species, with catalytic properties similar to those of the parental proteins. The affinity for ATP, which binds to the COOH-terminal domain, is almost the same for both hybrid enzymes. A cluster of positively charged residues located on the NH_2-terminal domain has been suggested as a binding site for 3-phosphoglycerate (*5*). Binding of 3-phosphoglycerate was shown to induce a large conformational change, resulting in a more compact structure of the molecule (*7, 14*). The decreased affinity for this substrate, observed for both interspecies hybrids, might indicate that the optimum alignment of the residues located on the interdomain surfaces and involved in binding of 3-phosphoglycerate has been perturbed in hybrid molecules.

We conclude that the 35% differences in the amino acid composition of yeast and human phosphoglycerate kinases have only a small effect on substrate binding and conformational changes occurring during catalysis. Two-thirds of these mutations are conservative or neutral (*15*). Strong selective pressures probably have conserved the essential interdomain surfaces, catalytic site, and hinge structure. Preliminary studies indicate that hyPGK is less stable, suggesting a significant contribution of the domain-domain interactions, localized in the hinge region, to the stability of PGK. The formation of fully active chimeric species is consistent with the hypothesis that both domains of phosphoglycerate kinase fold independently of one another (*16*).

The results described here further establish that domains can be exchanged and retain their properties. In the future, it may be possible to create new enzyme specificities by combining functional domains from different proteins. Molecular modeling and site-directed mutagenesis experiments should facilitate optimization of interdomain interactions.

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