The Primordial High Energy Compound: ATP or Inorganic Pyrophosphate?*

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The pyrophosphate-dependent phosphofructokinase (PPi-PFK) of Entamoeba histolytica displays a million-fold preference for inorganic pyrophosphate (PPi) over ATP (calculated as the ratio of \( k_{cat}/K_m \)). The introduction of a single mutation by site-directed mutagenesis changes its preference from PPi to ATP. The single mutant has an 8-fold preference for ATP whereas a related double mutant shows a preference exceeding 10,000-fold. The results suggest the presence of a latent nucleotide binding site aligned for a catalytic role in PPi-PFK. It is proposed that the ancestral PFK was an ATP-dependent enzyme and that PPi-PFKs are a later evolving adaptation.

In the mid-1960s Calvin and Lipmann (1, 2) independently first suggested a role for pyrophosphate as a high energy bond donor in the primeval earth. They proposed that the reactions found in primitive life forms evolved from prebiotic systems and that contemporary primitive organisms could retain the ability to employ inorganic pyrophosphate (PPi) as a high energy compound. Suggestions that PPi could serve as an energy source found later support in the discovery of a number of instances in which polyphosphates or pyrophosphate could substitute for ATP in reactions of glycolysis: glucokinase, pyruvate kinase, phosphoglycerate kinase, and phosphofructokinase (see reference 3 for review). In most instances these enzymes were found in anaerobic bacteria and presumptive primitive protists, such as Giardia and Entamoeba, supporting the idea that the kinases employed ancient mechanisms. Of the PPi-dependent enzymes, phosphofructokinase (PFK) has been studied most extensively. Sequence data have shown the pyrophosphate-dependent PFK (PPi-PFK) to be homologous to the ATP-dependent PFK, but with amino acid identities being less than 35% even when all positions that are not shared among all PFKs are eliminated (4). In a recent study of a PPi-PFK from the archaeon, Thermoproteus tenax, Sibers et al. (5) suggested that the archenal PPi-PFK represents the most ancient lineage of PFKs. In the current study we demonstrate the presence of a latent nucleotide binding site in the PPi-PFK of Entamoeba histolytica, suggesting that the nucleotide-dependent enzyme is the more primitive phosphofructokinase.

EXPERIMENTAL PROCEDURES

The plasmid bearing the E. histolytica PPi-PFK gene cloned into the prokaryotic expression plasmid pALTER-Ex1 (Promega) at the NdeI and XhoI restriction sites has been described previously (6). Site-directed mutagenesis was performed by using the Altered Sites II In Vitro Mutagenesis System (Promega). Recombinant wild type and mutant plasmids were transformed into DF1020 (pwo-8, Δpho201, recA56, Δrho-pka200, endA1, hsdR17, supE44) Escherichia coli and plated onto Luria broth agar plates (100 μg/ml ampicillin). Freshly transformed single colonies were inoculated into Luria broth medium (100 μg/ml ampicillin) and grown at 37 °C until A500 reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.4 mM, and incubation was continued at 30 °C for an additional 12–18 h. The cells were harvested by centrifugation at 5000 × g for 5 min. Wild type and mutant E. histolytica PFKs were then purified and assayed as described previously (6). Homogeneity of the preparations was demonstrated by SDS-polyacrylamide gel electrophoresis.

Enzyme activity of PPi-PFK was assayed spectrophotometrically at 30 °C in an assay solution that contained 150 mM Tes/KOH (pH 7.2), 3 mM MgCl₂, 1 mM EDTA, 0.2 mM NADH, the indicated concentrations of sodium PPi, and Fru-6-P, and 2–6 units each of aldolase, triose-phosphate isomerase, and glycerol-3-phosphate dehydrogenase. These auxiliary enzymes were dialyzed against 50 mM Tes/KOH (pH 7.2), 1 mM EDTA prior to use.

Enzyme activities for the ATP-dependent PFK were determined at 30 °C and at pH 7.2. The assay contained 150 mM Tes/KOH, 1 mM EDTA, 0.2 mM NADH, the indicated concentrations of Fru-6-P and ATP, 1 mM dithiothreitol, 0.6 unit of aldolase, and 0.3 unit each of triose-phosphate isomerase and glycero-3-phosphate dehydrogenase. MgCl₂ was added at a concentration 4 mM higher than the concentration of ATP under all conditions to ensure that virtually all of the ATP existed as the MgATP complex. For both ATP-dependent and PPi-dependent PFKs, 1 unit of enzyme was defined as the amount of enzyme producing 1 μmol of fructose 1,6-bisphosphate per min under the standard assay conditions. Kinetic parameters were calculated with the GraFit graphical analysis program.

RESULTS

Despite the low sequence identity between ATP- and PPi-dependent PFKs, the residues surrounding the substrate binding sites of the two enzymes bear much greater similarity (8). Inspection of two highly conserved sequence motifs in the subunit where phosphoryl transfer occurs reveals subtle differences between the two types of PFK. In the E. coli ATP-PFK, the last two residues (Asp-104 and Gly-105) of the conserved GGDG motif are directly involved in binding the phosphates of ATP (9). Whereas the last residue in the motif is Gly in ATP-PFKs, in most PPi-PFKs including that of E. histolytica, the last residue is Asp. In the other highly conserved active site motif, P(G/K)(TIDX), a Gly resides in the second position of this sequence in ATP-PFKs whereas in PPi-PFKs it is Lys. We have mutated both critical Gly residues at positions 105 and 124 in E. coli ATP-dependent PFK to Asp and Lys, respectively, to seek activity with PPi as a phosphoryl donor. While each of the mutations individually or together effectively eliminated activi...
Altered Phosphoryl Donor Specificity of Phosphofructokinase

TABLE I
Kinetic parameters of E. histolytica PFK wild type and mutant enzymes using PP\textsubscript{i} or ATP as a phosphoryl donor

|          | K\textsubscript{m} (mM) | K\textsubscript{m} Fru-6-P | k\textsubscript{cat} \textsubscript{PPi} | K\textsubscript{m} \textsubscript{PPi} | K\textsubscript{m} \textsubscript{ATP} | K\textsubscript{m} Fru-6-P | k\textsubscript{cat} \textsubscript{ATP} | K\textsubscript{m} \textsubscript{ATP} |
|----------|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|----------------|----------------|
| WT       | 0.026           | 0.101           | 341            | 13,100          | 2.9             | 2.0             | 0.037          | 0.013          |
| D175G    | 0.034           | 0.104           | 0.045          | 1.3             | 0.158           | 0.28            | 6.9            | 44             |
| K201G    | 0.019           | 0.088           | 0.073          | 3.8             | 1.4             | 0.082           | 0.142          | 0.101          |
| D175G/K201G | ~              | ~               | ~              | ~              | <4 \times 10\textsuperscript{-4} | 2.9             | 0.088          | 3.2            | 1.1            |

\* No activity was detected.

On the other hand, very striking changes in phosphoryl donor specificity were observed when the corresponding mutations were carried out with the PP\textsubscript{i}-dependent PFK from E. histolytica. As shown in Table I, changing either residue to Gly reduced k\textsubscript{cat} with PP\textsubscript{i} as the phosphoryl donor by 4 orders of magnitude while having a limited effect on the apparent PP\textsubscript{i} affinity of the residual activity. The wild type PP\textsubscript{i}-dependent enzyme was found to have a trace of activity with ATP, having a k\textsubscript{cat} that was nearly 10\textsuperscript{4} lower than that with PP\textsubscript{i} and a k\textsubscript{cat}/K\textsubscript{m} for ATP that was 10\textsuperscript{5} lower than that determined with PP\textsubscript{i} as a substrate. Mutation of Asp-175 or Lys-201 to Gly brought about increases in apparent affinity for ATP and increases in the k\textsubscript{cat} with ATP as substrate. The D175G mutant in particular showed a nearly 200-fold increase in k\textsubscript{cat} with ATP as phosphoryl donor and a nearly 4000-fold increase in activity at low concentrations of ATP. The double mutant (Table I) with both active site residues converted to Gly had no detectable activity with PP\textsubscript{i} as substrate. This mutant had activity with ATP but was not as active as the single mutant, D175G.

In a study of altering the coenzyme use of isocitrate dehydrogenase by site-directed mutagenesis, Chen et al. (10) defined k\textsubscript{cat}/K\textsubscript{m} as enzyme performance and the ratio of k\textsubscript{cat}/K\textsubscript{m} with one substrate to k\textsubscript{cat}/K\textsubscript{m} for the second substrate as enzyme preference. The enzyme preference of the wild type and mutant enzymes, (k\textsubscript{cat}/K\textsubscript{m})\textsubscript{PPi}/(k\textsubscript{cat}/K\textsubscript{m})\textsubscript{ATP}, are shown in Fig. 1. The single mutation of Asp-175 \rightarrow Gly changed substrate preference by a factor of nearly 10\textsuperscript{7}. The mutation produced a decrease in k\textsubscript{cat}/K\textsubscript{m} for PP\textsubscript{i} of approximately 2200-fold and an increase in k\textsubscript{cat}/K\textsubscript{m} for ATP of 3800-fold. This represents an extraordinary change in substrate preference brought about with a modest change in primary structure. No comparable change in substrate preference has been described as the result of single mutation. Chen et al. (10) shifted the preference of E. coli isocitrate dehydrogenase from NAD to NADP by >10\textsuperscript{6}, but this required seven mutations in the enzyme.

The D175G and the D175G/K201G mutants were also capable of using other nucleotides as substrates in the PFK reaction (see Table II). Both mutants actually exhibited greater specificity with GTP as a phosphoryl donor than ATP, as the performances with GTP in both mutants were twice that with ATP. The apparent affinity for GTP in the D175G mutant (K\textsubscript{m} = 0.024 mM) was slightly better than its apparent affinity for PP\textsubscript{i} (K\textsubscript{m} = 0.034 mM) of the residual PP\textsubscript{i} activity and was nearly identical to the apparent PP\textsubscript{i} affinity of the wild type enzyme (K\textsubscript{m} = 0.025 mM). UTP was used by both mutants as well, although the performance with UTP was only one-third that of ATP. Both mutant enzymes have a greater performance value with any of the nucleotides examined than that with PP\textsubscript{i}.

Despite the fact that the recombinant PP\textsubscript{i}-PFK and its mutants were expressed in a puf\textsuperscript{K} strain of E. coli, there could remain concern that the relatively low rates of catalysis seen with alternative substrates resulted from contamination or by catalysis at an alternative site on the enzyme. To eliminate these possibilities, PP\textsubscript{i} was tested as an inhibitor of the ATP-utilizing pathway in a puf\textsuperscript{K} strain of E. coli. Assays were performed at pH 7.2 with a saturating concentration of the second substrate as described under “Experimental Procedures.” It indicates that the value could not be calculated because no activity could be detected. However, the indicated value represents a maximum activity based on the level of sensitivity of the assay with 10 mM PP\textsubscript{i} and using the assumption that the apparent K\textsubscript{m} for PP\textsubscript{i} is no lower than seen with the other mutants. All standard deviations were less than 10%.

\* X. Wang and R. G. Kemp, unpublished data.
PKF activity of the D175G mutant. The data were analyzed by a Dixon plot (not shown). PP, indeed acts as a competitive inhibitor with a $K_i$ of about 1 $\mu M$, actually slightly lower than the apparent $K_m$ value calculated when used as a substrate. The $K_i$ more closely represents a true dissociation constant whereas the $K_m$ is likely to involve other rate constants. Nonetheless, it is clear that the mutation of $E. histolytica$ PP-PFK has brought about a change in specificity of the enzyme due to an alteration in the substrate binding site.

To further demonstrate the existence of a nucleotide binding site in the mutant that was not present in the wild type enzyme, binding to $N^\omega-[(6$-aminohexyl)carbamoyl methyl]-ATP coupled to Sepharose was employed. The D175G mutant (15 $\mu g$) was incubated in 100 $\mu l$ of packed ATP-Sepharose pre-equilibrated with 20 mM Tris/HCl (pH 7.2), 0.1 mM EDTA, 14 mM $\beta$-mercaptoethanol. The suspension was mixed by rotation for 30 min at 20 $^\circ C$. The ATP-Sepharose was then washed three times with 1.5 ml of the same buffer. No enzyme was detectable by SDS-polyacrylamide gel electrophoresis or by measuring activity in the washes. All of the enzyme activity was eluted with 300 $\mu l$ of 2.5 mM ATP in the same buffer. Under identical conditions, the wild type $E. histolytica$ PFK did not bind to the $N^\omega-[(6$-aminohexyl)carbamoyl methyl]-ATP-Sepharose. It is clear that the mutation of $E. histolytica$ PP-PFK has exposed a latent nucleotide binding site.

**DISCUSSION**

The most striking conclusion of this study is that a latent ATP binding site aligned for a catalytic role is present in the PP-PFK of $E. histolytica$ and possibly all PP-PFKs. It is difficult to imagine how the active site of PP-PFK of $E. histolytica$ could efficiently accommodate the comparatively large ATP molecule with the slight changes brought about by a single mutation without a pre-existing nucleotide binding site. Because the active site motifs are well conserved in all PFKs, it is possible that all PFKs have remnants of a nucleotide binding site and that only subtle structural alterations are required for a change in phosphoryl donor specificity. Recent studies of the adenosine subsite of $E. coli$ ATP-dependent PFK have shown the importance of an aromatic ring at position 41 of that enzyme for the high affinity binding of ATP (11). Although alignments of PP-PFKs with ATP-PFKs depend upon somewhat subjective judgments in this region of the enzyme, almost all PP-PFKs have an aromatic amino acid at this position (4). Also supporting the idea that major structural changes are not involved for phosphoryl donor selectivity is that the ATP-PFK of *Streptomyces coelicolor* and the PP-PFK of *Amycolaptosis methanolica* share 71% identity and that all of the residues at positions corresponding to those of the ATP binding site of the *E. coli* enzyme are identical (12).

This study supports the concept that ATP or a closely related nucleotide was the original high energy source in the primeval earth. A primordial role for ATP in early biotic or even prebiotic processes is not surprising in light of the now established role of nucleic acids as catalysts. The earliest forms of life were probably self-replicating nucleic acids, most likely RNA molecules, that eventually developed the ability to direct the synthesis of DNA and proteins. In this early RNA world, nucleic acids could have recruited proteins to assist in catalytic processes, such as seen in ribonuclease P. Later evolution would have led to decreasing complexity in the nucleic acid component and increasing complexity in the protein component of these catalysts. By the time enzyme systems for carbohydrate metabolism had evolved, usage of nucleic acids and nucleotides by proteins in catalysis must have been well established. Consistent with this proposal is the fact that many enzymes in addition to kinases, sugar transferases, and thiol transferases, employ nucleotides in important catalytic roles. As our study suggests, PFK originally evolved as an enzyme that used ATP as a high energy bond donor, and the utilization of PP, as a phosphoryl donor was a late evolving adaptation.

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