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EVOLUTION OF PHOSPHOFRUCTOKINASE(U) UNIVERSITY OF
HEALTH SCIENCES/CHICAGO MEDICAL SCHOOL DEPT OF
BIOLOGICAL CHEMISTRY AND STRUCTURE R G KEMP 31 JUL 87

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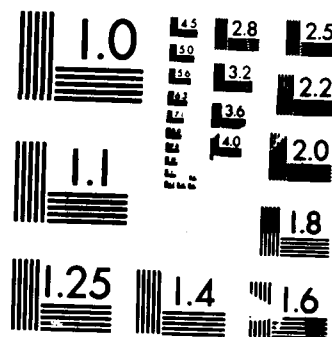
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Comparative sequence data suggest that eucaryotic phosphofructokinase (PFK) has evolved from a procaryotic precursor by gene duplication, fusion, and mutation of previous catalytic sites into new regulatory ligand binding sites. We are attempting to duplicate these processes by recombinant DNA technology. The <u>E. coli</u> pfk gene is being recloned into a convenient plasmid. Using a synthetic oligo-nucleotide that matches the mammalian "link" region, two <u>E. coli</u> pfk genes will be joined. The properties of the expressed "duplex" enzyme will be analyzed. Utilizing site-directed mutagenesis, conversion of one of the two catalytic sites to a regulatory site will be carried out.			
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PROJECT TITLE: EVOLUTION OF PHOSPHOFRUCTOKINASE

Annual Report for the period of 8/1/86 - 7/31/87

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The *pfk* gene along with a strong upstream promoter was removed from pLC16-4 by cleavage with *SinI* and *HpaI*. *HindIII* linkers were ligated to the fragment and the fragment was inserted into the *HindIII* site of pUCmp8. Very "sick" colonies were produced and the only successful M13 insertion involved extensive deletions from the 5' end of the gene as indicated by restriction maps. After numerous failures, we decided that the strong promoter present with the fragment was leading to a transcript that was somehow disturbing the host cell's normal transcription. The vector pKK232-8 has a promoter-less chloramphenicol transacetylase gene along with unique transcription terminators. We cloned into the *HindIII* site of this vector and generated an excellent PFK overproducer that grew in the presence of ampicillin and chloramphenicol. This plasmid has been named pSLJ-3. PFK was overproduced by approximately 10-fold when this vector was used to transform *E. coli* LE392.

Oligonucleotides to be used to assemble the "linker" region between two *pfk* genes were synthesized. Unfortunately, at this point we discovered numerous errors in the published *pfk* sequence. These errors have now been corrected and a new oligonucleotide is being synthesized.

The *HindIII* linked *pfk* has been circularized, restricted at the *BglII* site and inserted into pSLJ-7, which was constructed by putting an additional *BglII* site into a *BamHI*/*HindIII* site of pKK232-8. The new construct is called pSLJ-2. This will serve as a vector for manipulations of the "linker" and will then be inserted into the *BglII* site of pSLJ-3 to create the "duplex".

We are now completing the assembly of the newly designed double stranded oligonucleotide that codes for the link between the two *pfk* genes. This will be assembled and inserted into pSLJ-2 in place of the terminator, initiation, and upstream promoter site located in the middle of the inverted *pfk* gene. This modified gene will be restricted with *BglII* and inserted into pSLJ-3. The result should be the duplex product.

(2) A second area of investigation has been the production of site-directed mutants that will be useful in altering one of the two catalytic sites of our duplex. A mutation was made by the method of Mandecki, which involves introducing a single restriction site cleavage into the gene and transforming with the cut plasmid plus an oligonucleotide that bridges the cut and contains one or more mismatches near the restriction site. We cleaved pSLJ3 with *BglII* and added a 65-mer that removed the restriction site and changed G174 to E. Although this experiment was largely a student exercise, it did have a rational basis. G174 is three residues from critical catalytic residue involving ATP and seems to be important for the folding of the pocket. The kinetic properties of the mutant enzyme are fairly similar to native enzyme except for a low V_{max} . This low V_{max} may be an artifact due to the high ability of the enzyme. Preliminary studies indicate that the mutant enzyme is much less stable than the native enzyme.

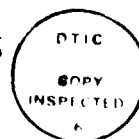
pSLJ3 is an excellent overproducer of PFK, but we still have the problem of cloning into M13 which is useful for most of the techniques of mutagenesis. The approach that we are taking is

to subclone a large fragment into M13, mutagenize it, and then reinsert it into pSLJ3 at the appropriate restriction sites. We utilized a double digestion with BfII and HindIII to produce two large fragments, 710 and 769 bp, which were put into M13 mp8 and mp9 restricted with Bam HI and HindIII. Although we thought we may only be able to clone the 3' fragment because of the problem with promoters, it turned out that each fragment has been successfully cloned into M13 in both directions. Thus, we now have a fragment that can be used for oligonucleotide-directed mutagenesis in M13.

Mutagenesis will be carried out using the procedure of Kunkel which has a high efficiency of mutagenesis. This technique has quickly gained in popularity and was recently introduced in kit from Bio-Rad. Efficiency may even be greater by using a second primer in addition to the oligonucleotide containing the mismatch. Briefly, the procedure involves generating the single-stranded DNA substrate in dut- ung- mutants of E. coli. The phage DNA will have significant amounts of uracil substitutions. After annealing and extending the primers, the DNA is transfected into wild type E. coli which will destroy the uracil-bearing primer, leaving only the mutant form free for replication. The mutagenized fragment will be reinserted into pSLJ3 and the plasmid will be transformed into DF1020, a mutant lacking both pfk has been introduced. Obviously any mutations must be confirmed by mapping and sequencing. Oligonucleotides have been prepared for the preparation of replacements at both the ATP and fructose-6-P binding sites.

(3) Further clues to the evolution of allosteric properties may be found in other comparative studies. We wished to examine the structure of PPi-dependent PFK to see its structure would tell us something about the catalytic site. The protein has been purified to near homogeneity from potatoes by a new procedure. The preparation of PPi-dependent PFK will be scaled up to produce 3 to 5 mg of enzyme. This will be used for peptide mapping to determine the relationship to the E. coli enzyme. If the enzyme is related, plans will be made for determining the complete sequence of the protein (probably from a cDNA sequence).

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