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Comparative sequence data suggests that mammalian 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 examining this process by two approaches. First, we are attempting to duplicate these processes by recombinant DNA technology. The *E. coli* pfk gene is being recloned into a convenient plasmid. Using a synthetic oligonucleotide that matches the mammalian "link" region, two *E. coli* pfk genes will be joined. The properties of the expressed "duplex" enzyme will be analyzed. In the second approach, we are looking at several other PFKs to determine whether their evolution has followed a similar path. Those being examined include $PP_i$ dependent PFKs.

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PROJECT TITLE: EVOLUTION OF PHOSPHOFRUCTOKINASE

Annual Report for the period of 8/1/87 - 7/31/88

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(1)

Work on the construction of the fused duplex PFK gene progressed to the point of complete assembly of the link between two *E. coli* PFK genes. Insertion of the link into the appropriate position has been deferred until additional, more convenient constructs of the gene are prepared, which is part of the second area of effort. Our initial construct, pSLJ3, was inconvenient for studies of site-directed mutagenesis because of difficulty in moving back and forth to M13. Our initial altered construct that was to solve this problem was reported last year but proved to be unsuccessful due to instability. We have now solved the problem by using a Bal digestion through the strong promoter that interfered with M 13 expression, readdition of linkers, and insertion into both pUC plasmids or M13.

(2)

We have characterized further our first site-directed mutant of *E. coli* PFK.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. 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 Vmax. Further studies have shown that the low Vmax was apparent and that the actual reason for the low values was the instability of the enzyme. Heat denaturation studies demonstrated that the mutant was denatured rapidly at 40 to 42°C whereas the wild-type enzyme denatured in the range of 60 to 62°C.

(3)

We extended our interest in the evolution of the PFKs to an evalutation of the place in the scheme of the PPi-dependent PFK of plants. A procedure was developed for the purification of inorganic pyrophosphate; fructose-6-phosphate 1-phospho-transferase (PPi-PFK) from potato tubers. The enzyme has the structure $\alpha_4\beta_4$ with a subunit of 68kDa and a $\beta$ subunit of 60 kDa. The structural relationship of the enzyme to other PFKs and to fructose

bisphosphatase was examined by imunoprecipitation and immunoblotting. Antibodies to the plant enzyme did not react with *E. coli* PFK. No crossreaction was seen among the following enzymes or their antibodies: yeast fructose bisphosphatase; rabbit PFKs A,B, or the enzyme from brain; and the two subunits of the potato PPi-PFK. On the other hand, antibody to *E. coli* PFK-1 strongly cross-reacts with the 60 kDa polypeptide but not 68 kDa peptide.

The data support the idea that the plant PPi-PFK has evolved from the same procaryotic progenitor that led to the major ATP-dependent enzyme of *E. coli* and to the mammalian PF-1-Ks. The structural identity of the second subunit, α, and its role in the reaction remain to be established. Three short tryptic peptides of the 60 kDal and one from the 68 kDal have been sequenced. Oligonucleotides are being synthesized on the basis of these sequences to probe a plant cDNA library.

In addition, we have recently partially purified the ATP dependent PFK of potato tubers. Using the Western blot technique, we have shown that the antibody to the 60 kDal subunit of the PPi-PFK is capable of recognizing a subunit of the ATP dependent enzyme.