DOE/ER/13923--4 #4

DOE GRANT (DE-FG06-88ER13923) FOR THE PROJECT

"ISOCITRATE LYASE AND THE GLYOXYLATE CYCLE"

A. Progress Report (February 16, 1992-February 15, 1993)

The results presented are organized into the same format as within the **Research Plan** in the application for renewal which was funded for the period of July 1, 1991-June 30, 1994. One minor modification in the Research Plan is described more fully under part 4 to follow.

1. Active-Site Modification of *Icl* from *Escherichia coli*

The use of group-specific reagents and affinity labels for isocitrate lyase (*icl*) was described in the last report and detailed in three publications cited in that report. Basically the findings indicated that *his* 266 and 306 and *ser* 319 and 321 were essential residues in *icl* from *E. coli* based on chemical modification.

The very recent alignment of *icl* sequences deduced from gene or cDNA cloning studies has enabled the identification of other amino acid residues that may be in the active site and are completely conserved in the enzyme from eight different sources (1). In the *E. coli* enzyme these are *lys* 193, *his* 184, *his* 197, *cys* 195 in a first highly conserved region. Ser 319 and 321 are completely conserved in part of a third highly conserved stretch from 310-324. Other completely conserved residues are found in a second highly conserved stretch from 348-356 in *E. coli icl*. Thus our use of an alignment algorithm has enabled the detection of numerous conserved amino acid residues in *icl* from such divergent eukaryotic sources as plants like castor bean, cotton and rape seed, as well as fungi such as *Emericella nidulans, Neurospora crassa, Saccharomyces cerevisiae* and *Candida tropicalis* and comparisons with the prokaryotic source, *E. coli* (1). In all eukaryotic enzymes a 100-residue stretch is inserted between 255 and 256 of the *E. coli* enzyme (1). This stretch may be of significance in the packaging of eukaryotic *icl* in microbodies [Matsuoka and McFadden, *J. Bacteriol.*, **170**, 458 (1988)] known to be absent in prokaryota. It is

remarkable that the amino acid sequences outside this 100-residue insertion show considerable similarity and four regions of homology. Given the strong evolutionary conservation of *icl* function [Vanni, Giachetti, Pinzauti and McFadden, *Comp. Biochem. Physiol.* **95B**, 431 (1990)] it is almost certain that the conserved residues identified (1) are involved in substrate binding or catalysis. Thus the way has been opened to direct probing of the function of *icl* by amino acid replacement using directed mutagenesis.

2. Directed Mutagenesis of the *Icl* Gene

Recently directed mutagenesis of the *icl* gene from *E. coli* has enabled the following *his* replacements: *his* 266 → *asp, glu, val, ser, lys* and *his* 306 → *asp, glu, val, ser.* These replacements resulted in almost no loss in enzyme activity (10-40% loss) and little change (<30%) in K_m for isocitrate. Clearly the indication that *his* 266 and 306, neither of which is conserved, were essential based upon chemical modification was incorrect. This research is being extended to replacement of *his* 184 and 197, both of which are completely conserved in an active site segment of primary structure.

Previous studies supported by this grant have established that *cys*195 is alkylated by brompyruvate, a substrate analog, and that the alkylated form is inactive [Ko and McFadden, *Arch. Biochem.*, **278**, 373 (1990)]. In directed mutagenesis studies, this residue has been replaced in the *E. coli* enzyme by *ser* leading to inactive enzyme. In this case, the results from directed mutagenesis corroborate the inferences from affinity labeling that *cys*195, which is completely conserved in all aligned *icl* sequences, is essential.

In other studies, using a newly developed double stranded mutagenesis technique, *his*(H), *glu* (E), *arg* (R) and *leu* (L) have been substituted for the *lys* 193 residue (K193) in *icl* from *E. coli.* The substitutions for this residue, which is present in a highly conserved, cationic region, significantly affect both the K_m for D_s-isocitrate and the apparent k_{cat} of isocitrate lyase (1). Specifically, the conservative substitutions, K193H and K193R, reduce catalytic activity by ca. 50-fold and 14-fold respectively and the non-conservative changes, K193E and K193L, result in assembled tetrameric protein that is completely

inactive. The K193H and K193R mutations also increase K_m of the enzyme of 5- and 2-fold, respectively. These results indicate that the cationic and/or acid-base character of K193 are essential for isocitrate lyase activity. In addition to the effects noted on enzyme activity, the effect of the mutations on growth of JE10, and *E. coli* strain which does not express isocitrate lyase, was observed (1). Active isocitrate lyase is necessary for *E. coli* to grow on acetate as the sole carbon source. It was found that a mutation affecting the activity of isocitrate lyase similarly affects the growth of *E. coli* JE10 on acetate when the mutated plasmid is expressed in this organism. Specifically, the lag time before growth increases over 7-fold and almost 2-fold for *E. coli* JE10 expressing the K193H and K193R isocitrate lyase variants. In addition, the rate of growth decreases by almost 40-fold for *E. coli* JE10 expressing the K193H and K193R isocitrate lyase variants. In addition, the rate of growth decreases by almost 40-fold for *E. coli* JE10 cells expressing form K193H and ca. 2-fold for those expressing the K193R variants. Thus the onset and rate of *E. coli* growth on acetate appear to depend on isocitrate lyase activity.

Collectively, these results illustrate the power of directed mutagenesis in studies of structure-function correlates for *icl*. In the coming grant period, directed mutagenesis will be extended to other conserved features of primary structure including *ser* residues 319 and 321.

3. Crystal Structure of *Icl* from *E. coli*

A study of the crystal structures of two forms of *icl* from *E. coli* continues at the University of Sheffield in the laboratory of D. Rice [Abeysinghe, Baker, Rice, Rodgers, Stillman, Ko, McFadden and Nimmo, *J. Mol. Biol.* **220**, 13 (1991)]. Dr. McFadden's laboratory continues to provide highly purified *icl* for these studies.

4. Cloning, Sequencing and High-Level Expression of cDNA for Watermelon *Icl*

Cloning, sequencing and high-level expression of cDNA for *icl* will be accelerated by studies of a higher plant cDNA for *icl* that has already been cloned. Recently, we have

obtained a plasmid from Professor Harada at U. C. Davis containing a cDNA insert of known sequence for *icl* from rapeseed (*Brassica napus*). Dr. Harada has encouraged us to develop a high-expression system and to characterize rapeseed *icl* including its structure and function through directed mutagenesis. Accordingly we have undertaken research on this interesting eukaryotic enzyme as it truly complements our studies of *icl* from *E. coli*. This effort on rapeseed *icl* replaces our objectives to clone, sequence, and express cDNA for watermelon *icl*.

1. Diehl P., and B. A. McFadden, "Site-Directed Mutagenesis of Lysine 193 in Isocitrate Lyase from *Escherichia coli* using Unique Restriction-Enzyme-Site Elimination," *J. Bacteriol.* **in press.**

END

DATE
FILMED
5/12/94