DOE/ER/13923--2

DOE GRANT (DE-FG06-88ER13923) FOR THE PROJECT "ISOCITRATE LYASE AND THE GLYOXYLATE CYCLE"

A. Progress Report (February 15, 1989-February 15, 1990)

1. The Structure and Catalytic Function of Isocitrate Lyase

Because knowledge of the catalytic mechanism for isocitrate lyase (*icl*) is essential to the present project, we have established the kinetic mechanism for the enzyme from *Escherichia coli* using inhibition patterns by products and dead-end inhibitors. (1). The mechanism for the cleavage of isocitrate is a steady-state ordered uni-bi reaction with succinate and glyoxylate being sequentially released. Moreover, itaconate, a tightly-bound succinate analog, also irreversibly inactivates *icl* in a reaction with a cysteinyl residue in the glyoxylate-binding domain (1). The use of ^{14}C-labeled itaconate should open a new way to label the active site of *icl*.

We have recently published information about the reversible interaction of various glyoxylate and succinate analogs with the binding sites for these substrates on *icl* of *E. coli*. (2) Specifically, phosphoenolpyruvate and *cis*-aconitate are bound at the succinate site and glycolate, oxalate, and 3-phosphoglycerate (3 PGA) at the glyoxylate site. However, the data suggest that bound 3 PGA overlaps the succinate site and that bound *cis*-aconitate overlaps the glyoxylate site. These results will facilitate the design of affinity labels to assist in the 3-dimensional mapping of the active site. Results with 3 PGA establish how this metabolite may regulate the activity of isocitrate lyase (2). In these studies as well as those of the kinetic mechanism, important contributions were made by Dr. P. Vanni, a Fulbright scholar and Visiting Professor of Biochemistry from University of Florence (1, 2).

During Professor Vanni's stay in Professor McFadden's laboratory, a long review article focussing on the structure and catalytic function of *icl* was completed. This review will be published in early 1990 (3).

2. Active-Site Modification and Proteolysis of *Icl*

Research on several active-site reagents for *icl* from *E. coli* continues. Bromomalonate modifies an active-site methionine. On the basis of cyanogen bromide cleavage fragments, it is likely that *met*-198 is the residue which is alkylated (Y. Ko and B. A. McFadden, unpublished observations). The group-specific reagent diethylpyrocarbonate modifies 1-2 histidines/subunit and inactivates *icl*. Present research in which ^{3}H-diethylpyrocarbonate is used [Y. Igarashi, T. El-Gul and B. A. McFadden, *Biochemistry*, *24*, 3957 (1985)] is promising in terms of the identification of the active-site histidine residue (Y. Ko and B. A. McFadden, unpublished observations).

The reagent 3-bromopyruvate inactivates *icl* in a highly specific manner by alkylating *cys*-195 in the glyoxylate binding domain (4). It is remarkable that modification by bromopyruvate imparts extreme resistance to proteolysis by trypsin, chymotrypsin, and V_8 protease as well as a major decrease in the intensity of tryptophan fluorescence (4, 5). This suggests that a major conformational change is induced by simple alkylation at *cys*-195 in the active site of *icl*. We presume, however, that this change may also be due to interaction of the pyruvyl carboxylate and carbonyl groups with the enzyme. The latter cannot be via a Schiff base with a lysyl residue because the pyruvyl moiety can be reduced with $NaB^{3}H_4$. In fact, this reduction has been used to generate the labeled active site peptide for sequencing (4).

Collectively, these studies of the active-site of *icl* add to our knowledge of the 3-dimensional structure of this domain. This knowledge coupled with information about the catalytic mechanism for *icl* (summarized in 3) is facilitating our design of directed mutagenesis of the *icl* gene (see next section). In this context, we are also interested in testing our hypothesis advanced in the first year of the present project [see M. Matsuoka and B. A. McFadden, *J. Bacteriol.*, *170*, 4528 (1988)] that *his*-266 is phosphorylated in activation.

3. Directed Mutagenesis of the *Icl* gene

The sequence of an active-site tryptic peptide from *icl* of *E. coli* (4) is shown below:

cys-gly-his-met-gly-gly-lys
195 200

As discussed in the preceding section, two affinity labels, 3-bromopyruvate and bromomalonate, have been used to label the active site at *cys*-195 and *met*-198, respectively. Also discussed was the use of ^{3}H-diethylpyrocarbonate in labeling an essential *his*. Reasoning that the active site residues *cys*-195 and *his*-197 may function in binding and/or catalysis, we are completing directed mutagenesis of the *icl* gene using synthetic deoxyoligonucleotides. The same approach has been used to ensure substitutions at the essential carboxyterminal *his*-429 and *his*-266, which is probably the phosphorylation site (M. Matsuoka and B. A. McFadden, ibid). Substitutions are shown below:

wide-type residue	substitutions
cys-195	*ala, ser*
his-197	*arg, val*
his-266	*asp, glu, ser, tyr, val*
his-419	*ala, arg, lys*

These substitutions are varied and ensure conservation of charge, structure, and/or function with appropriate changes also selected to ensure non-conservation of these parameters. In all cases, substitutions will presumably conserve secondary structure of the region altered. Finally, substitution at the phosphorylation site (*his*-266) will provide alternate acceptors such as *ser* and *tyr* or residues that structurally resemble phosphohistidine, *i.e., asp* and *glu*. The latter enzyme variants may be activated and unable to undergo inactivation.

For mutagenesis, the widely-used Batt technique is being used. Synthetic deoxyoligonucleotides have been prepared. In the Batt technique [M. A. Vandeyar, M. P. Weiner, C. J. Hutton and C. A. Batt, *Gene, 65*, 129 (1988)] the wild type strand in the filamentous bacteriophage M13 mp19 can be degraded after synthesis of the mutagenized strand. Infection of a suitable *E. coli* host by single-stranded mutant DNA then ensures intracellular production of double stranded circular M13 mp19 with the appropriate nucleotide substitution fixed in the complementary strand. We are now at the stage of isolating mutated bacteriophage and will soon insert each mutated *icl* gene in pUC18 for transfer to and expression in *E. coli*. Transformation of *E. coli* by single stranded M13 mp19 using the new method of PEG-dimethylsulfoxide has worked extremely well as have mutagenesis controls requiring successful application of the Batt technique.

Publications

1. Y. Ko, P. Vanni and B. A. McFadden, Isocitrate Lyase from *Escherichia coli*: Activation, Kinetic Mechanism and Interaction with Itaconate, to be submitted.
2. Y. Ko, P. Vanni and B. A. McFadden, The Interaction of 3-Phosphoglycerate and Other Substrate Analogs with the Glyoxylate- and Succinate-Binding Sites of Isocitrate Lyase, *Arch. Biochem. Biophys, 274*, 155 (1989).
3. E. Giachetti, G. Pinzauti, P. Vanni and B. A. McFadden, Comparative Structure, Function and Regulation of Isocitrate Lyase, an Important Assimilatory Enzyme, *Comp. Biochem. Physiol.*, in press.
4. Y. H. Ko and B. A. McFadden, Alkylation of Isocitrate Lyase from *Escherichia coli* by 3-Bromopyruvate, *Arch. Biochem. Biophys.*, in press.
5. Y. H. Ko and B. A. McFadden, Specific Alkylation of Isocitrate Lyase by 3-Bromopyruvate Induces Resistance to Proteolysis, *Abstr. Third Symposium of the Protein Society*, S76 (1989).

B. Renewal Proposal (July 1, 1990 - June 30, 1991)

In the original proposal a request was made for four years in the amount of $431,610. A 3-year grant of $220,500 was awarded starting July 1, 1988. In the original application, we proposed to compare the structure, regulation and catalytic function of isocitrate lyase (*icl*) from a bacterial source and higher plant. *Escherichia coli* and watermelon cotyledons were chosen as the sources of enzyme. Indeed our purification of *icl* from both sources was completed and published in the first project period (ref. 1, A, last report). After the application was submitted to the DOE, however, the cloning and sequencing of cDNA for higher plant *icl* (from castor bean) was published by Beeching and Northcote and the DOE was notified of this. Because of this valuable information about plant *icl* from another laboratory and the decreased term and level of our grant from DOE, we have reduced the scope of the research proposed in our original application. In the present 3-year period (July 1, 1988 - June 30, 1991), we will focus upon *icl* from *E. coli*. Progress upon the enzyme from this source is emphasized in **section A**. As outlined in the original application, we will continue to examine the structure, function and regulation of *icl* from *E. coli*.

Specifically, in the coming project period we will sequence up to five active site peptides labeled by various compounds including bromomalonate, itaconate, diethyl-pyrocarbonate, pyridoxal phosphate, and vanadate. In connection with the latter, we have recently discovered vanadate-dependent photo-oxidative inactivation of *icl* which is protected against by isocitrate. Remarkably, parallel studies of the enzyme ribulose bisphosphate carboxylase/oxygenase have established that a *ser* residue in the active site is modified [S. N. Mogel and B. A. McFadden, *Biochemistry, 28*, 5428 (1989)]. Thus in the coming project period, we may place *ser* as well as *lys,* (through use of pyridoxal phosphate), *his* and *met* in the active site of *icl* from *E. coli*. The identification of *cys*195 in the active site domain has already been mentioned as has the replacement of this residue by *ala* and *ser* by directed mutagenesis. This methodology is being used to test the consequences of replacement of *his*-197, *his*-266 and *his*-429 as described. These studies of directed mutagenesis of the *icl* gene will be completed in the coming project period.

As other active site residues such as *ser, lys, his* and *met* are firmly identified, these will be replaced by directed mutagenesis and the mutant *icl* proteins expressed and characterized. Collectively, the consequences of these and other amino acid replacements in the active and regulatory site domains will considerably enhance our understanding of the function and regulation of bacterial *icl*. Eventually, scientists will

gain a much better understanding of the regulation of fatty acid assimilation in bacteria. In the process, we will also learn much about the diversion of metabolites away from the energy-trapping tricarboxylic acid cycle into the carbon-conserving, energy-storing glyoxylate cycle.

In the coming project period, D. P. Singh, a doctoral candidate doing his research under Dr. McFadden, will begin to characterize mRNA for watermelon *icl*. The preparation was isolated in December of 1985 and shown to support the *in vitro* synthesis of watermelon *icl*. Because it has been stored at -70° C, the mRNA fraction may still be useful in making cDNA corresponding to the watermelon *icl* gene. Nevertheless it must be re-assayed and if inactive, prepared again. These studies will constitute the first step in synthesizing, cloning, sequencing and expressing watermelon cDNA for *icl* as described in the original proposal to DOE.

In the coming project period, we shall also initiate collaboration with Dr. D. Rice, a crystallographer at the University of Sheffield, who has proposed our collaboration in elucidating the crystal structure of *E. coli icl* which has been alkylated at *cys*-195. Dr. Rice will spend one month at Washington State University during which we will begin to grow diffraction-quality crystals.

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Isocitrate Lyase and the Glyoxylate Cycle
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Our objectives are to shed light upon the structure, regulation and catalytic function of isocitrate lyase (*icl*), an enzyme which catalyzes the first unique step in the glyoxylate cycle. In this cycle, lipids are converted to carbohydrates in a process which contributes to microbial growth on fatty acids and to the growth of oil-rich seedlings and animal embryos.

We have published data describing the cloning and sequencing of the *icl* gene of *Escherichia coli* [*J. Bacteriol., 170*, 4528 (1988)]. We have also described considerably improved purifications of *icl* from *E. coli* and watermelon cotyledons [*Prep. Biochem., 18*, 431 (1988)]. In the current project period, we have characterized the glyoxylate- and succinate-binding domains of *icl* [*Arch. Biochem. Biophys., 274*, 155 (1989)] and have alkylated the active-site residue, *cys*-195, (*Arch. Biochem. Biophys., in press*). In the coming project period, the amino acid sequences of up to five active-site peptides from the *E. coli* enzyme will be elucidated as will the flanking sequences for the phosphorylated *his*. Mutagenesis of the *E. coli icl* gene will be directed towards replacing hypothetically functional active-site residues that are conserved in the castor bean enzyme and towards replacing the *his* that is uniquely phosphorylated in bacterial *icl*.

These studies will provide basic information about *icl*. The function of this enzyme is vital to microbial growth (on fatty acids) and to the growth of varied plant seedlings and their subsequent utilization of solar energy.

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