Final Report

Role of Glycolytic Intermediates in Global Regulation and Signal Transduction

DE-FG03-95ER20205

TEXAS ENGINEERING EXPERIMENT STATION

May 8, 2000

**James C. Liao, PI
Department of Chemical Engineering
University of California, Los Angeles
Phone: (310) 825-1656, Fax: (310) 206-4107
e-mail: liaoj@ucla.edu**

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, make any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

I. Statement of Research Problem

The goal of this project is to determine the role of glycolytic intermediates in regulation of cell physiology. It is known that many glycolytic intermediates are involved in regulation of enzyme activities at the kinetic level. However, little is known regarding the role of these metabolites in global regulation and signal transduction. This project aims to investigate the role of glycolytic intermediates in the regulation of gene expression.

II. Summary of Progress

In our previous experiments, we have shown that overexpression of phosphoenolpyruvate (PEP) carboxykinase (Pck) or phosphoenolpyruvate synthetase (Pps) in *Escherichia coli* causes severe growth inhibition, and this effect is independent of carbon source used and cannot be reversed by any nutritional supplementation. The observation suggests that perturbation of PEP metabolism may cause metabolic imbalance, which leads to upset of various global regulation loops. To investigate these possibilities, we selected two global regulons, the Ntr regulon and the heat shock regulon, to probe the effect of such metabolic imbalance.

1. Overexpression of Phosphoenolpyruvate carboxykinase (Pck) affects Glutamine synthetase (GS) expression

The pair of isogenic strains, BW13711 and BW16464 *pta*, were used to probe the effect of Pck overexpression on the synthesis of GS. These strains were transformed with plasmid pCK601, which contains the *P_{tac}-pck* transcriptional fusion, and were cultured in media with glutamine (nitrogen limitation) or glutamine and ammonium (nitrogen rich) as the nitrogen source. The expression of *pck* was induced by 20 μ M of IPTG. We found that in the wild-type (BW13711) strain, the regulation of GS synthesis was not affected significantly by the overexpressed Pck. The total GS activity (with and without adenylation), as measured by the γ -glutamyl transferase assay increased about 6- to 10- fold in nitrogen limited culture. However, in the *pta* strain, BW16464, the synthesis of GS became poorly inducible by nitrogen limitation.

As a control, we showed that the plasmid vector (pJF118EH) without the *pck* gene showed no effect on the inducibility of GS synthesis. These results suggest the following possibilities: (i) the presence of excess PCK activity is responsible for the observed phenomenon, (ii) the presence of a large amount of unnecessary proteins interferes with the normal function of the cell, and (iii) the diversion of protein synthesis machinery to the plasmid-coded gene caused the observed effect. The second and third possibilities predict that the observed effect is not *pck*-specific. The third possibility further requires active de novo synthesis of an excess protein to create a metabolic burden. To test the last two possibilities, we overexpressed an altered-activity *pck* mutant, *pckm1*, which catalyzes the conversion from oxaloacetate (OAA) to pyruvate. Results show that overexpression of this mutant protein does not affect the inducibility of GS synthesis in the wild-type or *pta* strains. Furthermore, we showed that the overexpression levels of PCK and PCKm1 induced by 20 μ M IPTG were roughly the same. In addition, when BW16464/pCK601 induced with IPTG was shifted to a medium without IPTG, the inducibility of GS by nitrogen limitation was still impaired. These results suggest that the observed effect was attributed to PCK activity (PEP-forming), rather than the presence of any protein or the

metabolic burden caused by excess protein synthesis.

2. The effect of Pck on GS synthesis is exerted at the transcriptional level.

To further investigate the effect of PCK on GS synthesis, we used λ gln101 to lysogenize BW13711 and BW16464 *pta*. This λ phage contains the upstream control region (NRI-P binding sites, *glnAP1*, and *glnAP2*), which controls the transcription of the fused *lacZ* gene. The phage was integrated into the *E. coli* chromosome at the *att* site so that a merodiploid of *glnA* regulatory region is created. The activity of β -galactosidase was measured to determine the effect of Pck on the transcriptional regulation of *glnA*. As expected, the β -galactosidase activity paralleled the GS activity of the non-lysogenized strains. This result suggests that the effect of Pck is exerted at the transcriptional level.

3. GOGAT activity is not affected by Pck Overexpression

The induction of *glnA* by nitrogen limitation requires glutamate synthase (GOGAT), which is regulated by Lrp and perhaps other mechanisms. One possible explanation of the Pck effect is that metabolic imbalance caused by Pck overexpression directly or indirectly down regulates GOGAT. To probe this possibility, GOGAT activity was measured with or without Pck overexpression. Results show that Pck did not affect the activity of GOGAT (data not shown). Therefore, GOGAT activity cannot explain the observed Pck effect.

4. Intracellular metabolites

To probe possible mechanisms by which Pck affects the regulation of *glnA*, we measured the intracellular metabolite pools with or without Pck overexpression. Interestingly, we found that Pck overexpression did not change the glycolytic metabolite pools appreciably in the wild-type background. However, in the BW16464 *pta* background, overexpression of Pck increased both intracellular FDP and pyruvate pools. The increase in pyruvate pools also correlates with the reduced inducibility of the *glnA* gene under nitrogen limitation. It is therefore tempting to infer that these metabolites may play a role in signal transduction under nitrogen limiting conditions.

5. Overexpression of Pps caused a Pck-like effect.

According to results described above, the Pck effect is attributed to its activity, rather than the presence of excess protein or excess protein synthesis. The control experiment using Pck501, which exhibits the OAA decarboxylase activity, further suggests that the PEP-forming activity may be responsible for the Pck effect. If this hypothesis is true, other PEP-forming enzymes, such as PEP synthase (Pps) should show the same effect. To probe this hypothesis, we overexpressed Pps using plasmid pPS706 in BW13711 and BW16464 *pta*. Results show that Pps overexpression did show the similar effect as Pck overexpression (data not shown). However, pPS706 required higher concentration of IPTG to inhibit the induction of *glnA* synthesis. This difference is presumably due to the differences in PEP-forming activity and the expression efficiency of the genes.

6. Two-dimensional protein gel electrophoresis

To determine the global effect of Pck *E. coli* physiology, we used two-dimensional protein gel electrophoresis to detect the changes in protein expression patterns. The cells were pulse-labeled by ³⁵S-methionine for 1 minute and precipitated by trichloroacetic acid. The amounts of sample loaded to the gel were normalized by the radioactive material assimilated. After electrophoresis, the gels were imaged by autoradiography. Results show that overexpression of Pck caused a dramatic change in protein expression pattern. A typical protein expression pattern is shown in Fig. 1, where proteins changed by Pck overexpression were indicated. This result further confirm our hypothesis that overexpression of Pck perturbs global regulation.

7. Effect of Pck overexpression on the heat shock response

To probe the effect of Pck overexpression on heat shock response, we first used a λ phage harboring a *groEP_{hs}-lacZ* transcriptional fusion in a VJS676/pCK601. The β -galactosidase activity was measured after a temperature shift from 30 to 42 degree C. We found that without IPTG induction of *pck*, the strain exhibited the normal heat shock response. The β -galactosidase activity increased sharply after the heat shock, and reached a peak at about 20 min before leveling down to a new steady state, which was about 50% higher than the original one. However, when Pck is overexpressed by IPTG induction, the β -galactosidase activity was not induced by heat shock.

As a control, the overexpression of Pckm1, which is an altered-activity mutant, show no effect on heat shock response. These results suggest that heat shock response was abolished by Pck overexpression. To confirm these findings, we determined the synthesis rate of heat shock proteins, such as GroE and DnaK, by pulse-labeling with ³⁵S methionine and protein gel electrophoresis. Unexpectedly, we found that the synthesis rate of GroE and DnaK after heat shown was not affected appreciably by Pck overexpression. This result is still perplexing, and our current hypothesis is that the *groEP_{hs}-lacZ* fusion contains only the partial regulatory region of *groE* transcription. It is possible that the partial regulatory region on the *groEP_{hs}-lacZ* was repressed by overexpression of Pck. However, *E. coli* may possess other regulatory mechanism that can compensate for the metabolic imbalance caused by Pck overexpression. Therefore, the behavior of the cloned *groEP_{hs}-lacZ* gene is different from that of the chromosomal *groE* gene. Work is continuing in this direction to elucidate the detailed mechanism underlying the observed phenomenon.

8. Two additional heat-shock promoters are identified in the *groE* operon

We have found that overexpression of Pck abolishes the heat-shock induction of the *groE* promoter, as examined by the *groE-lacZ* promoter fusion. To examine whether this effect is due to the metabolic activity of Pck or is due to the overexpression of proteins, we used a non-functional protein, *luxA*, as a control. Results showed that overexpression of LuxA also abolishes the heat-shock induction of *groE-lacZ* fusion (Fig. 1). Therefore, this effect is attributed to the overexpression of protein, rather than the metabolic activity of Pck. However, the heat-shock induction of *rpoD_{hs}-lacZ* fusion was not affected by the protein overexpression. In addition, when examined by ³⁵S-pulse labeling experiments, the heat-shock induction of GroEL protein was not completely abolished by overexpression of proteins. Therefore, the effect appears to be specific to the *groE-lacZ* fusion.

We hypothesized that the *groE* operon contains more than one promoters, which are differentially regulated when a protein is overexpressed. Indeed, we identified from the nucleotide sequence that there are three potential promoters (p1, p2, and p3) in the *groE* operon region. Promoter p1 is the only promoter that has been positively identified in the literature. Promoter p2 is located within the *groES* gene, and promoter p3 is upstream of p1. We have cloned these promoters on a multicopy plasmid, pRS415, and showed that they contain the promoter activity, as demonstrated by the expression of the fused lacZ gene. We are currently constructing a lambda phage that contains the promoter-lacZ fusion so that a single copy promoter fusion can be integrated into the chromosome. These new construct will allow us to study the regulation of these promoters.

9. Overexpression of proteins reduces the expression of *rrnB* operon.

We have observed that overexpression of Pck in *E. coli* causes growth inhibition. To determine whether this effect is caused by the metabolic activity of Pck or by the burden of protein synthesis, we used LuxA as a control. It was found that overexpression of LuxA also results in growth retardation, and that the extent of growth inhibition is correlated with the amount of protein produced. Two possible mechanisms can potentially explain this phenomena. The first involves the stringent response, and the second is related to the growth media control. It is possible that overexpression of proteins depletes one of the internal amino acid pools and thus induces guanosine tetraphosphate (ppGpp) synthesis and stringent response. Alternatively, the growth rate is perhaps controlled by a mechanism independent of the stringent response in a similar way that the medium controls the growth rate. The expression of *rrnB* operon is influenced by these two mechanisms. The *rrnB* operon contains two promoters, *rrnBP1* and *rrnBP2*. Both of them are controlled by the stringent response, but only *rrnBP1* is controlled by the growth medium. We therefore used both *rrnBP1-lacZ* and *rrnBP2-lacZ* fusions to see if the growth inhibition caused by protein overexpression is regulated in the same way as in the stringent control or the growth medium control.

We showed that protein overexpression-induced growth inhibition is correlated with *rrnBP1* promoter activity. When LuxA protein is overexpressed by induction with IPTG, the growth rate decreased, and the transcription activity from *rrnBP1* decreased as well. This decrease in *rrnBP1* is independent of ppGpp, as the same phenomenon is found in a *relA* strain and in a *relA spoT* strain. Therefore, we conclude that the growth inhibition induced by protein overexpression is independent of ppGpp and is not due to the stringent response.

To examine whether the growth inhibition caused by protein overexpression is mediated through the growth medium control, we used *rrnBP2-lacZ* fusion. We showed that when protein overexpression decreases the growth rate, the transcription activity from *rrnBP2* is also reduced. However, when growth rate is decreased by growing in poor carbon sources, the transcriptional activity from *rrnBP2* is not reduced. Therefore, the growth rate regulation during protein overexpression is different from the growth medium control.

10. Reduction of Aerobic Acetate Production by *Escherichia coli*

As a related issue in determining the role of metabolite in signal transduction, we investigated the production of acetate during aerobic *E. coli* fermentation. Acetate excretion by *E. coli* during aerobic growth on glucose is a major obstacle to enhanced recombinant protein

production. We report here that the fraction of carbon flux through the anaplerotic pathways is one of the factors influencing acetate excretion. Flux analysis of *E. coli* central metabolic pathways predicts that increasing the fraction of carbon flux through the phosphoenolpyruvate carboxylase (PPC) pathway and the glyoxylate bypass reduces acetate production. We tested this prediction by overexpressing PPC and deregulating the glyoxylate bypass using a *fadR* strain. Results show that the acetate yield by the *fadR* strain with PPC overexpression is decreased more than four-fold compared to the control, while the biomass yield is relatively unaffected. Apparently, the fraction of carbon to anaplerotic flux is one of the factors that influences acetate excretion. These results confirm the prediction of our flux analysis and further suggest that *E. coli* is not fully optimized for the efficient utilization of glucose.

11. Engineering Metabolic Control

With the knowledge obtained above, we attempted to re-engineer the regulation in central metabolism for a desired purpose. In deed, our study of Ntr regulon has yielded such an opportunity.

Metabolic engineering has achieved encouraging success in producing foreign metabolites in a variety of hosts. However, common strategies for engineering metabolic pathways focus on amplifying the desired enzymes and deregulating cellular controls. As a result, uncontrolled or deregulated metabolic pathways lead to metabolic imbalance and sub-optimal productivity. Here we demonstrated the design and engineering of a regulatory circuit, in addition to amplifying the pathway genes, as the second stage of metabolic engineering effort. In particular, we recruited and altered one of the global regulatory systems in *Escherichia coli*, the Ntr regulon, to control the engineered lycopene biosynthesis pathway. The altered regulon, stimulated by excess glycolytic flux through sensing of the acetyl phosphate level, controls the expression of two key enzymes in lycopene synthesis in response to flux dynamics. This artificial regulon significantly enhanced lycopene and recombinant protein production and reduced the growth retardation caused by protein overexpression.

12. Application of DNA microarray to the study of central metabolism

During the project period, DNA microarray technology became available, which allows the simultaneous detection of transcript levels at a genomic scale. We applied this technology to a selected subset of *E. coli* genome which are involved in central metabolism and regulation.

The DNA microarray technology was applied to detect differential transcription profiles of a subset of the *Escherichia coli* genome. A total of 111 *E. coli* genes, including those in central metabolism, key biosyntheses, and some regulatory functions, were cloned, amplified and used as probes for detecting the level of transcripts. An *E. coli* strain was grown in glucose, acetate, and glycerol media and the transcript levels of the selected genes were detected. Despite extensive studies on *E. coli* physiology, many new features were found in the regulation of these genes. For example, several genes were unexpectedly up-regulated, such as *pps*, *ilvG*, *aroF*, *secA*, and *dsbA* in acetate and *asnA* and *asnB* in glycerol, or down-regulated, such as *ackA*, *pta* and *adhE* in acetate. These genes were regulated with no apparent reasons by unknown mechanisms. Meanwhile, many genes were regulated for apparent purposes but by unknown

mechanisms. For example, the glucose transport genes (*ptsHI*, *ptsG*, *crr*) in both acetate and glycerol media were down-regulated, and the *ppc*, glycolytic, and biosynthetic genes in acetate were also down-regulated because of the reduced fluxes. However, their molecular mechanisms remain to be elucidated. Furthermore, a group of genes were regulated by known mechanisms, but the physiological roles of such regulation remain unclear. This group includes *pckA* and *aspA*, which are up-regulated in glycerol, and *gnd* and *aspA*, which are down- and up-regulated, respectively, in acetate. The DNA microarray technology demonstrated here is a powerful yet economical tool for characterizing gene regulation, and will prove to be useful for strain improvement and bioprocess development.

Another problem we studied using the DNA microarray technology was the effect of protein overexpression on *E. coli* central metabolism. It has been commonly observed that gratuitous protein overexpression caused growth retardation due to both metabolic burden and specific biochemical toxicity. However, the molecular events involved in metabolic responses to gratuitous protein overexpression are still known. Here we used DNA microarrays to characterize the changes in gene transcription level due to protein overexpression. A non-toxic, soluble protein, α subunit of luciferase (coded by *luxA*), was used as a model system. A total of 132 *E. coli* genes, including those in the central metabolism, key biosynthesis pathways, and regulatory and respiratory functions, were cloned, amplified and used as probes for detecting the level of transcripts. Three *E. coli* strains, JM109, MC4100, and VJS676A, were used as hosts for protein overexpression. Upon induction, these strains showed several common responses, such as up-regulation of *glk* and heat shock genes as well as down-regulation of *fba*, *ppc*, *atpA* and *gnd*. In addition, several biosynthesis genes, *glnA*, *glyA*, and *leuA*, were commonly down-regulated. Different responses between media were also characterized. For example, many respiratory genes were up-regulated in minimal medium but down-regulated in complex medium during protein overproducing. These results indicate that growth retardation and metabolic flux perturbation caused by protein overexpression cannot be simply attributed to stoichiometric constraints. Apparently, significant actions at the gene expression level actively participate in the regulation of growth and metabolic flux during protein overexpression.

13. Publications.

Oh, M.K., and J.C. Liao, (2000) "Gene Expression Profiling by DNA microarrays and Metabolic Fluxes in *Escherichia coli*" *Biotechnol. Prog.* 16, 278-286.

Farmer, W.R. and J.C. Liao, (2000) "Improving Lycopene Production in *Escherichia coli* by Engineering Metabolic Control" *Nature Biotechnol.* 18, 533-537.

Oh, M.K., and J.C. Liao, "DNA Microarray Detection of Metabolic Responses to Protein Overproduction in *Escherichia coli*" *Metabolic Engineering*, in press