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**cDNA cloning of rabbit muscle-specific ( $\beta\beta$ ) enolase gene,  
site-directed mutagenesis (E417L) of the gene, expression  
of the wild-type and mutant genes in *Escherichia coli*.**

Shu-Xian Zheng

A Thesis in the Department of Chemistry and Biochemistry

Presented in partial fulfilment of the requirements

for the Degree of Master of Science at

Concordia University, Montreal, Quebec, Canada

March, 1995

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## ABSTRACT

### **cDNA cloning of rabbit muscle-specific ( $\beta\beta$ ) enolase gene, site-directed mutagenesis of the gene, expression of the wild-type and mutant genes in *Escherichia coli***

Shu-Xian Zheng, 1995

A cDNA encoding the complete open reading frame of rabbit muscle-specific  $\beta\beta$  enolase has been cloned and characterized. The 1441 bp cDNA (BEN) was isolated from a rabbit cDNA expression library screened with a 641bp Digoxigenin-labelled murine  $\beta\beta$  enolase cDNA probe. The whole nucleotide sequence of this cDNA fragment has been obtained by DNA sequencing. The open reading frame (ORF) encodes a protein of 433 amino acids, exhibiting 87%, 87%, and 86% sequence identity to human, murine and rat enolase mRNAs, respectively, and 64% sequence identity to *Saccharomyces cerevisiae* enolase A. 5'UTR region showed about 91% sequence identity with the same region of human enolase, 3'UTR region showed 70-78% sequence identity to its counterparts from other species. Site-directed mutagenesis was carried out converting Glu417 to Leu417, and was confirmed by DNA sequencing. Both wild type and mutant genes were inserted into expression vector pBKe and introduced into *E.coli* strain BL21(DE3)pLysS, protein products were isolated and partially purified with Q-sepharose chromatography. The recombinant proteins have been shown

to exhibit enolase enzymatic activity and are both eluted from Q-Sepharose chromatography at low salt concentration, similar to native rabbit  $\beta\beta$  enolase.

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## TABLES OF CONTENTS

List of Abbreviations.....	ix
List of Tables .....	x
List of Figures .....	x

### CHAPTER A-INTRODUCTION

A.1 Enolases .....	1
A.1.1 Yeast Enolase .....	2
A.1.2 Mammalian Enolases .....	2
A.1.3 <i>Escherichia coli</i> ( <i>E.coli</i> ) Enolase .....	3
A.2 Similarities and differences Among Enolases .....	4
A.2.1 Amino Acid Sequence Homology Between Yeast Enolase, Mammalian Enolases and Enolases From Other Sources .....	4
A.2.2 Catalytic Property Similarities .....	5
A.2.3 Physical Property Similarities and Differences .....	6
A.3 X-ray Structure of Yeast Enolase .....	7
A.4 Amino Acid Residues Involved in Subunit Interactions of Enolase Dimer .....	9
A.5 Site-Directed Mutagenesis Studies .....	11
A.6 Inactivation and Dissociation Studies of Enolase Isozyme .....	12
A.6 Outline of the Project .....	14

### CHAPTER B-MATERIALS

B.1 cDNA Library .....	16
B.2 cDNA Probe-3'UTRH .....	16
B.3 Oligonucleotide Primer For Mutagenesis .....	16
B.4 <i>E.coli</i> Strains .....	17
B.4.1 XL1-Blue Strain .....	17
B.4.2 CJ236 Strain .....	17
B.4.3 MC1066 Strain .....	18
B.4.4 BL21(DE3)pLysS Strain .....	18
B.4.5 SOLR Strain .....	19
B.5 The pBKe-Hb1 Expression Vector .....	19

### CHAPTER C-METHODS

<b>Part I General Protocols</b> .....	21
C.I.1 Preparation of Competent <i>E.coli</i> Cells .....	21
C.I.2 Transformation of Plasmid DNA Into <i>E.coli</i> Cells .....	21
C.I.3 Mini-Preparation/Large-Scale preparation of Plasmid DNA .....	22
C.I.4 Restriction Enzyme Digestion .....	23

C.I.5 Agarose-Gel Electrophoresis .....	23
<b>Part II Specific Methods</b> .....	24
C.II.1 Screening of cDNA Library For $\beta\beta$ Enolase Gene With cDNA Probe .....	24
C.II.1.1 Isolation and Purification of the cDNA Probe Fragment (3'UTRH) to be Labelled .....	24
C.II.1.2 DIG-Labeling of the cDNA Probe .....	25
C.II.1.3. Primary Screening .....	26
C.II.1.4 Secondary/Tertiary/Quaternary Screenings .....	27
C.II.2 <i>In vivo</i> Excision of the pBluescript Plasmid from the Lambda ZAPII Vector .....	28
C.II.3 Confirmation of the Positive Clone by Southern Blot of cDNA Inserts with the Probe .....	29
C.II.3.1 Agarose-Gel Electrophoresis of <u>EcoRI</u> Digested Clones .....	29
C.II.3.2 Transfer of DNAs from Agarose Gel to Nylon Membrane .....	29
C.II.3.3 Hybridization of the Membrane with the Labelled-Probe .....	31
C.II.3.4 Immunological Detection .....	31
C.II.4 Confirmation of the Positive Clone by DNA Sequencing .....	31
C.II.4.1 Sequencing Reactions .....	32
C.II.4.2 Electrophoresis for DNA Sequencing .....	32
C.II.5 Subcloning of the Full-Length Gene Fragment into pBluescript .....	33
C.II.6 Nested Deletion .....	34
C.II.7 Subcloning of the Full-Length Gene into Expression Vector .....	35
C.II.8 <i>In vitro</i> Site-Directed Mutagenesis of the Gene .....	37
C.II.8.1 Preparation of the Single-Stranded DNA Template .....	37
C.II.8.2 Phosphorylation of the Mutagenic Primer .....	37
C.II.8.3 Annealing of the Oligonucleotide Primer to the Template .....	38
C.II.8.4 Second Strand Synthesis .....	38
C.II.8.5 Transformation of the Mutagenic Product in MC1066 and XL1-Blue <i>E.coli</i> Cells .....	38
C.II.9 Expression of the Wildtype and Mutant Gene in <i>E.coli</i> Cells .....	39
C.II.10 Separation of $\beta\beta$ Enolase from <i>E.coli</i> Enolase by Chromotography on Q-Sepharose .....	39

## CHAPTER D-RESULTS AND DISCUSSION

D.1 Design and Logic of the Project .....	42
D.2 Cloning .....	46
D.2.1 cDNA Library Screening .....	46
D.2.2 Southern Blot Analysis .....	47
D.2.3 DNA Sequencing of the Clone and Subcloning of the Full-Length Gene into pBluescript .....	47
D.2.4 Conclusions on Cloning .....	52



D.3 Subcloning of the Gene into Expression Vector .....	52
D.4 Site-directed Mutagenesis .....	53
D.5 Expression of the Wild-Type and Mutant Proteins .....	56
D.6 Purification of the Wild-Type and Mutant Proteins .....	59
<b>CONCLUSION</b> .....	<b>60</b>
 <b><u>CHAPTER E APPENDIX</u></b>	
E.1 Media .....	61
E.2 Solutions .....	63
E.3 Antibiotics .....	68
E.4 Molecular Weight Markers .....	69
<b><u>REFERENCES</u></b> .....	<b>70</b>

## ABBREVIATIONS

AEP	—	3-aminoenolpyruvate phosphate
APS	—	ammonium persulfate
bp	—	base pairs
cDNA	—	complementary DNA
<i>E.coli</i>	—	<i>Escherichia coli</i>
IPTG	—	isopropyl- $\beta$ -D-thiogalactopyranoside
Kb	—	kilo bases
LB	—	Luria-Bertani media
MW	—	molecular weight
MUT	—	mutant
O.D.	—	optical density
OPF	—	open reading frame
PEP	—	phosphoenolpyruvate
PGA	—	2-phosphoglycerate
PhAH	—	phosphonoacetoxyhydroxamate
SSC	—	sodium chloride-sodium citrate buffer
TAE	—	tris-acetate-EDTA buffer
TBE	—	tris-borate-EDTA buffer
TE	—	tris-EDTA buffer
TEMED	—	N,N,N',N'-Tetramethylethylenediamine
TSP	—	D-tartronate semialdehyde phosphate
UTR	—	untranslated region
WT	—	wild type

## LIST OF TABLES

Table 1: Sequence Identity in Enolases .....	5
Table 2: Kinetic Properties of Enolases .....	6

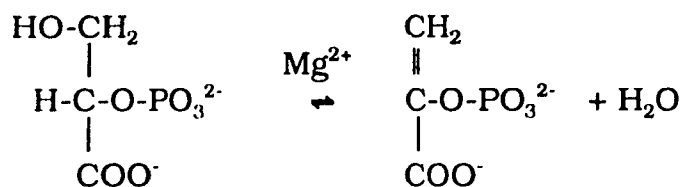
## LIST OF FIGURES

Figure 1: Stereoview of the cluster of ionic residues formed at the interface of the dimer .....	8
Figure 2: Schematic representation of the "charge shuttle" mechanism of enolase .....	9
Figure 3: Southern transfer of DNA from agarose gels .....	30
Figure 4: Construction of the expression plasmid pBKe-BEN .....	36
Figure 5: <u>SacI</u> enzyme digestion of plasmid DNAs isolated from the 35 colonies of transformed mutagenic product .....	54
Figure 6: <u>SacI</u> enzyme digestion of pBKe-BEN (WT) and pBKe-MUTBEN (mutant) plasmid DNA .....	55
Figure 7: SDS-PAGE electrophoresis of Q-Sepharose fractions of WT expression product .....	57

## CHAPTER A INTRODUCTION

### **A.1 Enolase**

Enolase (2-phospho-D-glycerate-hydrolase, EC4.2.1.11) is an enzyme which catalyzes the dehydration of 2-phosphoglycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway and the reverse reaction, the hydration of PEP to PGA, in gluconeogenesis.



Enolases from different organisms have been purified and shown to have similar catalytic properties. Most of the isozymes are dimers, with subunit molecular weights of 40,000 to 50,000 daltons (Mann et al, 1970). All known enolases exhibit an absolute requirement for certain divalent metal ions for catalytic activity, of which the natural cofactor  $\text{Mg}^{2+}$  gives the highest activity (Wold & Ballou, 1957; Brewer, 1985). Three metal ion binding sites per subunit were found in enolase. The first metal ion, traditionally called "conformational", binds in a high-affinity site I, the binding of which facilitates the binding of the substrate (Brewer and Weber, 1966); the second metal ion, "catalytic", binds in lower-affinity site II only in the presence of a substrate or substrate analogue, and produces catalysis only if the conformational metal ion is an activating one (Brewer and Collins, 1980). With higher concentration of metal ions, the third

cation referred to as "inhibitory" can also bind, and inhibit enzymatic activity (Faller et al, 1977).

### **A.1.1 Yeast Enolase**

There are two genes coding for yeast (*Saccharomyces cerevisiae*) enolase (gene Eno1 and gene Eno2); their product isozymes are called yeast enolase A and B, respectively (M.J.Holland et al, 1981). The primary structures of these two isozymes were determined by DNA sequencing of the two cloned genes from a yeast genomic DNA library, and by amino acid sequencing as well. It was found that the polypeptides encoded by these two genes differ in 20 out of 436 amino acids (Chin et al, 1981). Nearly all the work on yeast enolase, including that which is mentioned herein, has concerned enolase A. Yeast enolase A is the one which has been best characterized and most extensively studied, among all the enolase isozymes from different species. The enzyme as isolated is dimeric (Brewer & Weber, 1968) with two identical subunits of 44,350 daltons (Chin et al, 1978).

### **A.1.2 Mammalian Enolases**

Three major tissue-specific isoforms of enolase have been identified in mammals:  $\gamma\gamma$  or neuron-specific enolase (NSE), expressed primarily in neurons;  $\beta\beta$  or muscle-specific enolase (MSE), expressed in striated muscle; and  $\alpha\alpha$  or non-neuronal enolase (NNE), expressed in the embryonic tissues and other adult cell types. A development switch between  $\alpha$  and  $\gamma$  isoforms occurs in neurons and cells of neuronal origin, while a switch from  $\alpha$  to  $\beta$

enolase takes place in developing skeletal muscle and heart. Hetero-dimeric isoforms of the enzyme such as  $\alpha\beta$  and  $\alpha\gamma$  have also been found (Rider et al, 1974; Suzuki et al, 1980); the remaining  $\beta\gamma$  form has not been observed to occur naturally.

### **A.1.3 *Escherichia coli* (*E.coli*) Enolase**

*E.coli* enolase was purified by Spring and Wold in 1971. The molecular weight of the enzyme was found to be approximately 90,000 daltons. The subunit molecular weight estimated from SDS-PAGE electrophoresis was 46,000 daltons, suggesting that the enzyme is composed of two subunits of equal size. There are many functional similarities between *E.coli* enolase and other enolases studied, such as the dependence on  $Mg^{2+}$  for activity and the inhibition by fluoride in the presence of phosphate. Other catalytic parameters ( $K_m$ ,  $V_{max}$ , and pH optimum) are also similar, although the  $K_m$  of  $1 \times 10^{-4}M$  is somewhat higher than that observed for enolase from vertebrate sources ( $0.4-0.6 \times 10^{-4}M$ ), but identical with that for yeast enolase. The pH optimum of 8.1 is significantly higher than that for vertebrate enolases (pH7) and even somewhat above that of yeast and plant enolases (pH7.6-8.0). All these indicate that *E.coli* enolase is more closely related to yeast enolase than to vertebrate enolases. It was also found that *E.coli* enolase denaturation occurred in the absence of  $Mg^{2+}$ . Other enolases do not show this behaviour, although removal of  $Mg^{2+}$  has been found to greatly facilitate dissociation of both yeast and rabbit muscle

enolase into inactive monomers (Spring and Wold, 1971).

## **A.2 Similarities and Differences Among Enolases**

### **A.2.1 Amino Acid Sequence Similarities Between Yeast Enolases, Mammalian Enolases and Enolases From Other Sources**

The amino acid sequences of the two yeast genes are 95% identical within their respective coding regions. In addition, it was found that the primary structures of the 5' and 3' noncoding regions adjacent to the translational initiation and termination codons are approximately 70% identical (Chin et al, 1981).

The mammalian isozymes have very similar amino acid content also. Lebioda et al (1989) made a comparison of the amino acid sequences of enolases from different species, including yeast enolases A and B, *Xenopus laevis* enolase  $\alpha$ , duck enolase  $\alpha$ , human enolase  $\alpha$  and  $\gamma$ , rat enolase  $\alpha$ ,  $\beta$  and  $\gamma$ , mouse enolase  $\beta$  and chicken enolase  $\beta$ . They concluded that on average, there is a 62% sequence identity between yeast and mammalian enolases and a 82% sequence identity between the mammalian isozymes. Human  $\gamma\gamma$  shows only 7 amino acid replacements out of total 436 amino acids compared with the corresponding isozyme from the rat, and there are 27 changes between the human and rat  $\alpha\alpha$  isozymes, while 72 replacements exist between human  $\alpha\alpha$  and human  $\gamma\gamma$ . A table describing the sequence identity in enolases is presented as follows.

Table 1: Sequence Identity in Enolases (Lebloda et al, 1989).

	Yeast B	Rat $\alpha$	Rat $\gamma$	Human $\alpha$	Chicken $\beta$
Yeast A	95%	62	62	63	60
Yeast B		61	60	62	58
Rat $\alpha$			82	95	82
Rat $\gamma$				82	80
Human $\alpha$					81

*C.albicans* (asporogenic yeast) possess a single enolase gene and shows 78% and 76% amino acid sequence identity to yeast enolases A and B (Mason et al, 1993).

Not much information is available in the literature on plant enolases; Lal et al (1991, 1994) cloned genes encoding enolase from maize, and it was found that the maize cDNA encodes a protein of 446 amino acids with a high degree of similarity to enolase from other organisms (72% identity to yeast enolase and 82% identity to human enolase). Other sources of plant enolases have been purified or partially purified from potato tubers (Boser, 1959), from spinach leaves (Sinha and Brewer, 1984) and from castor oil seed (Miernyk et al, 1984); all these enolases showed similar catalytic properties.

The nucleotide sequence for the first 123 residues of *E.coli* enolase was determined by Weng et al (1986), and this segment showed 57% identity with yeast enolase (Weng et al, 1986), indicating that prokaryotic and eukaryotic enolases may have the same architecture and that we can regard yeast enolase as a good model for enolase structure.

### **A.2.2 Catalytic Property Similarities**



No significant difference has been found in catalytic properties of enolase isoforms, which agrees very well with the sequence similarity of isozymic enolases. In common, they all have an absolute requirement for certain divalent metal ions ( $Mg^{2+}$ ,  $Mn^{2+}$  or  $Zn^{2+}$ ) for substrate binding and catalytic activity (Wold, 1971). Most of them show inhibition by fluoride+phosphate (Warburg and Christian, 1942). Enolases from rabbit muscle, salmon muscle, yeast, and *E.coli* are all shown to be inhibited by substrate analogues such as TSP (D-tartronate semialdehyde phosphate), AEP (3-aminoenolpyruvate phosphate) (Spring and Wold, 1971), and PhAH (phosphonoacetohydroxamate) (Anderson et al, 1984). A table of the kinetic properties of enolases is presented as follows.

Table 2: Kinetic Properties of Enolases (Kornblatt and Klugerman, 1989)

	$\alpha\alpha$	$\gamma\gamma$	$\beta\beta$	Yeast
<b>K<sub>m</sub></b>				
PGA, x 10 <sup>-5</sup> M	5.7±0.4	5.8±0.3	8	15
PEP, x 10 <sup>-5</sup> M	51±10	45±0.7	-	-
<b>Optimum [Mg<sup>2+</sup>], mM</b>	1	1	1	1
<b>v<sub>H</sub>/v<sub>D</sub></b>	2.0	1.7	1.7	1.8

Kornblatt and Klugerman (1989) observed that all the enolases studied were inhibited by  $Na^+$  and  $Li^+$ , while the rabbit enolases, but not yeast enolase, were activated by  $K^+$ ,  $NH_4^+$ ,  $Cs^+$  and  $Rb^+$ ; rabbit enolase  $\gamma\gamma$ , was more susceptible to inhibition by excess  $Mg^{2+}$  than was the yeast enolase.

### A.2.3 Physical property similarities and differences

All of the enolases studied so far have similar physical properties,

although some differences in physical properties, such as susceptibility to inactivation by salt, pressure and temperature have been reported (Keller et al, 1981; Kornblatt et al, 1982; Marangos et al, 1978).

Keller et al (1981) observed that thermal stability at 55°C of semi-purified  $\alpha\alpha$  is weaker than that of  $\gamma\gamma$ , while  $\alpha\gamma$  has an intermediate stability.

Marangos et al (1977) found that  $\gamma\gamma$  enolase has a higher affinity for  $Mg^{2+}$  than does  $\alpha\alpha$  enolase. Later they also found that  $\alpha\alpha$  enolase was rapidly inactivated upon incubation in 0.5M KCl or KBr while  $\gamma\gamma$  enolase was minimally effected, and the hybrid has an intermediate stability (with 0.5 M KBr, all the  $\alpha\alpha$  activity was lost in 20 minutes, while  $\gamma\gamma$  retains more than 90% of its activity [Marango et al, 1978]).

### **A.3 X-ray Structure of Yeast Enolase**

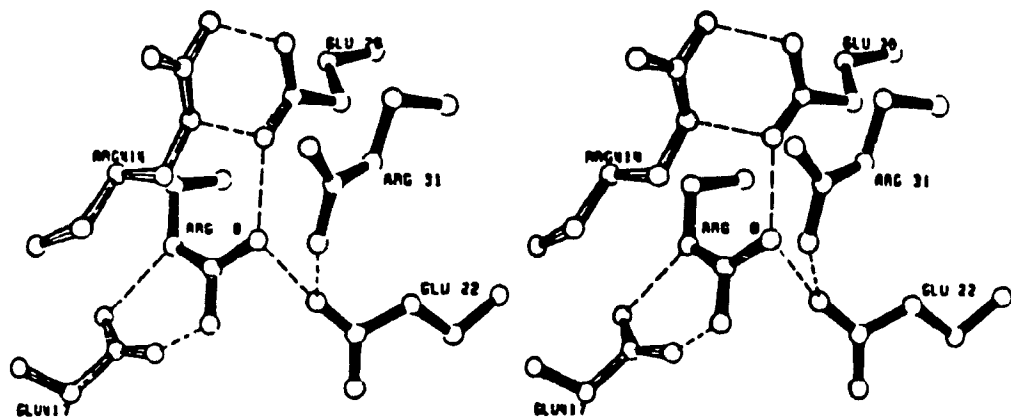
The X-ray structure of the yeast enolase was the last glycolytic enzyme structure to be published (Lebioda et al, 1989), and it was refined at 2.25Å resolution (Stec & Lebioda, 1990).

Each subunit of yeast enolase contains two domains: the main domain (Pro143 to Gly420) and a smaller N-terminal domain (Ala1 to Ser142), with a short C-terminal tail (Asp421 to Leu436).

The main domain is an 8-fold  $\alpha$  &  $\beta$  barrel, with a topology of  $\beta\beta\alpha\alpha(\beta\alpha)_6$ . The active site is located in a deep cavity at the carboxylic end of the  $\beta$ -barrel. The N-terminal domain has an  $\alpha$  &  $\beta$  structure based on a three-stranded anti-parallel meander and four helices. Most of the contacts at

the dimer interface are between the side chains of the last helix H (residues 404-419) of the barrel domain in one subunit and the atoms of the main chain of the meander (strand 9 and 10, residues 5-13 and 17-25, respectively) in the second subunit.

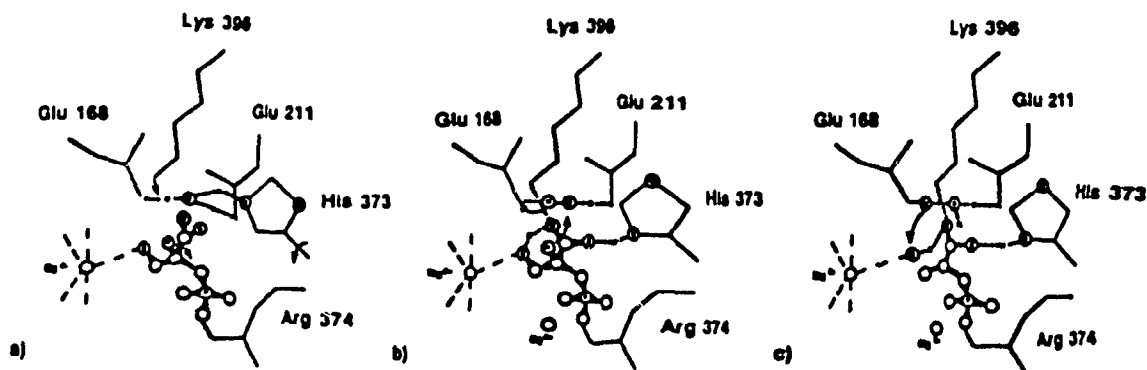
Figure 1: Stereoview of the cluster of ionic residues formed at the interface of the dimer. The residues from 2 subunits have different shading of the covalent bonds. The H bonds are shown with thin lines (Lebioda et al, 1990).



The "charge shuttle" mechanism for enolase catalyzed reaction involves Glu-168 accepting a proton from a water molecule which in turn accepts a proton from C2 of the substrate (Lebioda et al, 1991). The water molecule

molecule serves as the catalytic base. The mechanism is illustrated as in Figure 2.

Figure 2: Schematic representation of the "charge shuttle" mechanism of enolase (Lebioda and Stec, 1991b).



According to the mapping of the 3-D structure of enolase, Lebioda also reported the primary structure in the vicinity of the active site was highly conserved. The active site of enolase seems to be quite rigid, and no large conformational changes upon substrate binding were observed. Regions at which the sequence varies in a consistent way have only hydrophobic residues and none of them is in the active site. Three regions on the molecular surface with relatively high density of isozymic substitutions were considered as putative sites of contact with other macromolecules (Lebioda et al, 1989; Lebioda and Stec, 1991b).

#### **A.4 Amino Acid Residues Involved in Subunit Interaction of Enolase Dimer**

Three kinds of forces were thought to be involved in the interaction between the subunits of the dimer — hydrophobic interactions, ionic bonds and hydrogen bonds.

Previous studies gave controversial results. Brewer et al (1978) believed that hydrophobic interactions are the main forces between subunits. They made this conclusion on the basis of the observation that the pH dependence of the subunit dissociation constants is similar either at 0.05-0.1 ionic strength or in 1M KCl, which suggests that similar ligands are involved in the subunit interactions in both solvents. The temperature dependence of the subunit dissociation also appears identical at 0.05 ionic strength and in 1M KCl, which indicates that hydrophobic interactions predominate in producing subunit association. Another observation that is consistent with this conclusion is that subunit dissociation is pressure-dependent.

On the other hand, in describing the 3-D structure of yeast enolase, Stec et al reported that there were few hydrophobic contacts, which is in agreement with the observation that the association is not very strong, and in the absence of  $Mg^{2+}$ , the subunits dissociate (Brewer & Weber, 1968). In their analysis, they found that there is a large cluster of ionic side-chains forming an array with alternating charge (Lebioda et al, 1991a). Arg8 lies at the centre of the cluster. Two pairs of ion pairs Arg8-Glu417 and Glu20-Arg414 were shown to form bonds which strengthen the subunit

association. These four residues are all conserved in all amino acid sequences of the enolase isozymes from different organisms. Arg8 also forms contacts with the carboxylic groups of Glu-20 and Glu 22 from the same subunit, and Glu22 is in turn bonded to Arg31 from the same subunit. These residue interactions result in a large cluster of ionic side-chains and contribute to the dimer stability. In addition, there are several hydrogen bonds between the side-chains and two between the main-chains peptides across the subunit boundaries (Stec and Lebioda, 1990). Since the enzyme dimer has 2-fold symmetry, the number of hydrogen bonds between subunits is actually twice that stated.

#### **A.5 Site-Directed Mutagenesis Studies**

Site-specific mutagenesis is one of the most popular molecular biological techniques being used for the studies of structure-function relationship of polypeptides. By changing some amino acid residues of interest, and by comparing the differences between the wild type and mutant protein products in their physical, chemical or kinetic properties, we may get important information about the roles of these particular residues in the protein. There are numerous examples of the successful application of this technique in recent years. Since the interest of my study mainly concerns the residues involved in enolase subunit interactions, especially the residue Glu417, only some similar mutation examples are quoted as follows.

This technique was applied by Brewer et al (1993) to the preparation and

characterization of the E168Q mutant of yeast enolase 1. By simply replacing Glutamic acid 168, which was proposed to be in the active site centre and involved in the vital proton transfer reaction, with glutamine, they obtained a mutant having only 0.01% of the activity of native enolase. The mutant protein binds AEP and TSP, two substrate analogues, with affinities similar to those of the native enzyme.

White et al (1993) altered the subunit contact region of yeast tetrameric phosphoglycerate mutase, by replacing Lys 168 with a proline residue. They obtained a mutant enzyme which undergoes dissociation to dimers when the enzyme concentration was lowered from 200 µg/ml to 5 µg/ml, whereas the wild-type enzyme remains tetrameric over this concentration range.

Rafferty et al (1994) studied the effect of the mutation E172Q on catalytic activity of human O<sup>6</sup>-alkylguanine-DNA-alkyltransferase, since this residue was close to the active-site cysteine residues. They found that the mutagenesis completely inactivated the enzyme. Therefore they concluded that this Glu residue was crucial to the alkyl transfer from the substrate to the enzyme active-site Cys.

#### **A.6 Inactivation and Dissociation Studies of Enolase Isozymes**

It has been reported previously that yeast enolase is particularly sensitive to incubation with molar concentrations of KCl and KBr. It was suggested that these salts inactivated enolase by dissociating the enzyme into inactive subunits (Gawronski & Westhead, 1969); Brewer and Weber

(1968) found that subunits dissociated in the absence of magnesium ion (with excess EDTA present) were inactive. They suggested that the substrate binding site is altered in some way upon subunit association or dissociation in the absence of metal ion. Later Marangos et al (1978a) reported different sensitivities of enolase isozymes  $\alpha\alpha$ ,  $\alpha\gamma$ , and  $\gamma\gamma$  to salt inactivation. They found that  $\alpha\alpha$  is sensitive to 0.5 M KCl or KBr inactivation while  $\gamma\gamma$  is minimally effected under the same condition, and  $\alpha\gamma$  has an intermediate stability. The inactivation is temperature dependent and reversible by salt removal. They proved that salt-induced inactivation involves subunit dissociation by showing that renaturation of a mixture of  $\alpha\alpha$  and  $\gamma\gamma$  by removal of salts yields all three isozymes:  $\alpha\alpha$ ,  $\alpha\gamma$  and  $\gamma\gamma$ . The stability of  $\gamma\gamma$  towards salt and urea induced dissociation suggests that the subunits of the neuronal enzyme are more tightly associated than those of the  $\alpha\alpha$  protein. Results of the stability of each enzyme towards incubation at 50°C also substantiated this conclusion.

However, Keresztes-Nagy and Orman (1971) and later Holleman (1973) showed that at 40°C, in the presence of magnesium (II) and substrate, yeast enolase would dissociate into monomers which retained all the activity of the dimeric enzyme, and their data tended to prove that the active site is at least potentially complete on each subunit, that is, no parts of the active sites are shared between subunits. This proposal was confirmed by the X-ray structure of yeast enolase showing each subunit of the enzyme has an



isolated active site (Lebioda and Stec, 1991b).

Therefore, the monomer of yeast enolase is likely to be active, and the results of Brewer & Weber (1968) and Gawronski & Westhead (1969) can be attributed to the salt-induced disruption of the tertiary structure of the enzyme causing the formation of inactive monomers.

A lot of data has been collected from studies of dissociation and inactivation of mammalian enolases. Marangos et al (1978a) reported that inactivation of mammalian brain enolase in 1M KCl or KBr occurred accompanied by dissociation. Kornblatt et al (1982) demonstrated that inactivations of  $\alpha\alpha$ ,  $\alpha\gamma$ ,  $\gamma\gamma$  under pressure are partially reversible. Later it was found that the dissociation of the brain enolase is a two-step process — pressure-induced inactivation of the enzymes, followed by dissociation (Kornblatt et al, 1987). In 1990, Trepanier and Kornblatt (1990) studied the salt-induced dissociation and inactivation of rabbit  $\gamma\gamma$  enolase, and reported that  $\text{NaClO}_4$ , but not NaCl, dissociates and partially inactivates the enzyme, and that the rate of dissociation is faster than inactivation, which means an active monomer is formed via the dissociation (Trepanier et al, 1990). Further studies by Al-Ghanim and Kornblatt (unpublished results) showed that, for rabbit  $\beta\beta$  enolase,  $\text{NaClO}_4$ -induced inactivation of this enzyme occurs prior to dissociation, which means that this isozyme probably follows a different model from  $\gamma\gamma$  isozyme, and  $\text{NaClO}_4$  causes partial inactivation of dimeric enzyme preceding dissociation of the dimer

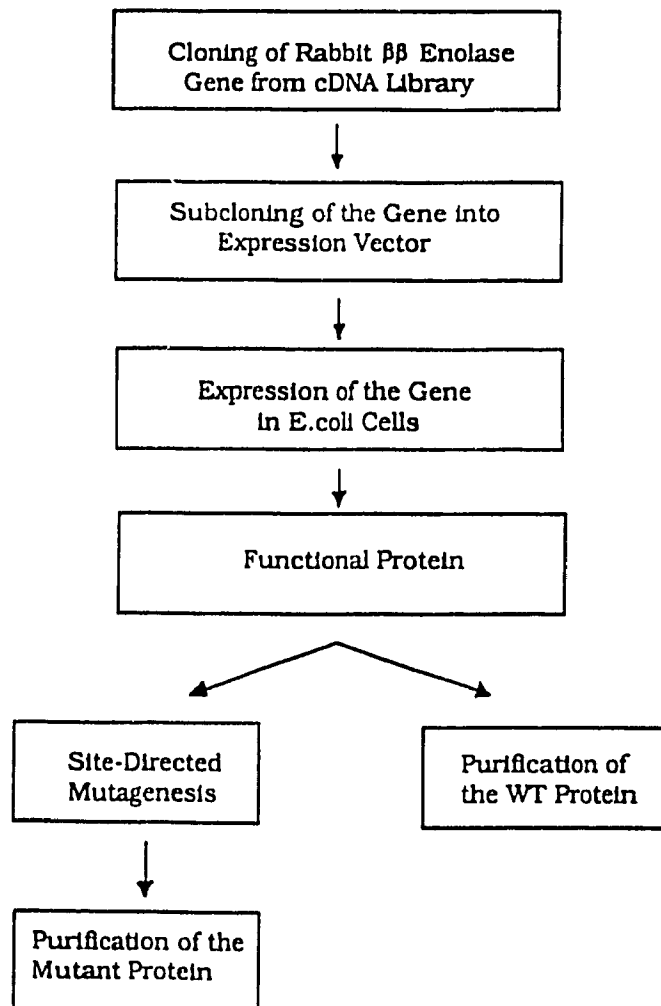
into inactive monomers.

### A.7 Outline of the Project

The aims of this study include:

- (1) To clone the gene for rabbit  $\beta\beta$  enolase.
- (2) To express this gene in a suitable host such that the enzyme can be purified and studied.
- (3) To use site-directed mutagenesis to modify the subunit contacts.

#### PROJECT OUTLINE



## **CHAPTER B MATERIALS**

### **B.1 cDNA Library**

The cDNA library used for screening was a rabbit muscle cDNA library in the lambda ZAPII vector from Stratagene (cat#936901). The library source was New Zealand White rabbit, 5-10 days old, fast twitch skeletal muscle. cDNA inserts were constructed with oligo dT and the cloning site was EcoRI in the polylinker region of the lambda ZAPII vector.

Lambda ZAPII is an insertion bacteriophage vector equipped with multiple cloning sites within plasmid sequences that can be excised *in vivo* and converted to pBluescript SK, a plasmid vector. It contains a polylinker region of pBluescript, which contains 21 unique cloning sites, and the region is flanked by the T3 and T7 promoters; in addition, there are 6 different primer sites for DNA sequencing with commercially available sequencing primers within this area.

### **B.2 cDNA Probe — 3'UTRH**

Murine muscle-specific enolase ( $\beta\beta$ ) cDNA probe was a generous gift from Dr D. Lazar at College de France. It is a 641 bp cDNA fragment from the 3'end of murine  $\beta\beta$  enolase, including the whole 3'UTR region and part of the coding region of this gene (Lamande et al, 1989).

### **B.3 Oligonucleotide Primer For Mutagenesis**

The oligonucleotide primer used for *in vitro* site-directed mutagenesis was a 27-base single stranded DNA fragment, with the same nucleotide

sequence as the coding strand of the rabbit enolase  $\beta\beta$  cDNA clone — BEN, from amino acid 413 to 421 except at amino acid 417, where the GAG coding for Glutamic acid was changed to CTC, which codes for Leucine.

413 414 415 416 417 418 419 420 421

Oligo sequence: 5' ..-ATG-AGG-ATC-GAG-CTC-GCT-CTT-GGG-GAC-..3'

BEN sequence: 5' ..-ATG-ACG-ATC-GAG-GAG-GCT-CTT-GGG-GAC-..3'

With this change, an additional restriction site of SacI (GAGCTC) at the mutation site was added which was used to screen for the mutant DNA on agarose gel.

The oligonucleotide was synthesized at Dalton Chemical Laboratories Inc.(North York, Ontario, Canada).

#### **B.4 *E.coli* Strains**

##### **B.4.1 XL1-Blue Strain**

Genotype:recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,[F'proAB, lacIqZDM15, Tn10(ter<sub>R</sub>).

It is a tetracycline resistant strain.

There is an uncharacterized mutation which enhances the alpha-complementation to give a more intense blue color on plates containing X-gal and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).

##### **B.4.2 CJ236 Strain**

Genotype: dut<sup>-</sup>, ung<sup>-</sup>, thi<sup>-</sup>, relA<sup>-</sup>, PCJ105(cmr).

It is a chloramphenicol resistant strain.

This *E.coli* strain is typically used for *in vitro* site-specific mutagenesis.

The *dut* mutation inactivates the enzyme dUTPase, therefore DNA synthesized in *dut*<sup>-</sup> mutant bacteria contains a number of uracils in thymine positions; the *ung* mutation inactivates uracil N-glycosylase and thus allows the incorporated uracils to remain in the DNA. The uracil-containing DNA strand synthesized within CJ236 cells can be used as the template for *in vitro* site-directed mutagenesis.

Both XL1 and CJ236 contain F' plasmid, which codes for the functions required to manufacture pili, and pili allows the helper phage to enter into the cell; The Tn10 tetracycline gene is also located on the F' episome in both strains, therefore, both of these two strains are tetracycline resistant.

#### **B.4.3 MC1066 Strain**

Genotype: *pyrF74::Tn5(km<sup>r</sup>)*, *leuB6*, *trpC9830*, *leu*, *hsdR-*, (*lacIP-OZYA*), *X74*, *galU*, *galK*, *strA<sup>r</sup>*.

MC1066 is used in the site-directed mutagenesis system, too. MC1066 is a *dut*<sup>+</sup>, *ung*<sup>+</sup> strain and thus has the ability to degrade uracil-containing DNA strand, and is usually used for transformation with mutagenesis product to get mutant plasmid DNA.

#### **B.4.4 BL21(DE3)pLysS Strain**

Genotype: *F-*, *ompT*, *rB-*, *mB-*.

As a B strain, this strain is deficient in the lon protease, it also lacks the *ompT* outer membrane protease that can degrade proteins during purification. Thus, at least some target proteins are expected to be more

stable in this strain than in host strains that contain these proteases.

This strain also contains a lysogen — DE3. Bacteriophage DE3 is a  $\lambda$  derivative that has the immunity region of phage 21, a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, the beginning of the *lac Z* gene, and the gene for T7 RNA polymerase. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by IPTG. Induction of T7 RNA polymerase by IPTG in turn transcribes the target DNA in the plasmid.

The presence of pLysS (a plasmid having the fragment of a clone of the T7 lysozyme gene under the promoter of tet in the BamHI site of pACYC184 is referred to as pLysE, a plasmid having the fragment in the opposite orientation is referred to as pLysS) increases the tolerance of BL21(DE3) for plasmids with gene products which are toxic to *E.coli*. A plasmid having pLysS has also the advantage of facilitating the preparation of cell extracts, since simply freezing and thawing allows the resident lysozyme to lyse the cells efficiently (Studier et al, 1990).

#### **B.4.5 SOLR Strain**

Genotype: *e14*<sup>-</sup>(*mcrA*), (*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC::Tn5(kan<sup>r</sup>)*, *uvrC*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, *lambdaR* [F<sup>+</sup>*proAB,lac/qZ M15*], *Su<sup>-</sup>* (non-suppressing).

SOLR cells were used as host cells during *in vivo* excision of phagemid containing the putative clones.

## **B.5 The pBKe-Hb1 Expression Vector**

The pBKe-Hb1 eukaryotic expression vector was a generous gift from Dr. R.E. MacKenzie at McGill University (Yang and MacKenzie, 1992). It is a 4.0 Kbp plasmid derived from pBluescript(KS+). It contains a T7 RNA polymerase promoter, a translational enhancer sequence, a ribosomal binding site, and an initiator ATG. It also includes a cDNA fragment Hb1, encoding the human bifunctional NAD-dependent dehydrogenase/cyclohydrolase, which was replaced with BEN, the cDNA sequence encoding rabbit  $\beta\beta$  enolase.

T7 RNA polymerase is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. When the pBke-BEN construct is introduced into the BL21(DE3)pLysS strain, the lacUV5 promoter on the DE3 lysogen genome will be triggered using IPTG as the inducer, and will promote the transcription of the T7 RNA polymerase gene on DE3; as a result, transcripts of BEN gene under the control of T7 promoter will be produced (Studier et al, 1990).

## **CHAPTER C METHODS**

- Recipes for media, solutions and buffers: refer to Chapter E Appendix.
- Types of antibiotics for different *E.coli* strains: refer to Chapter B Materials.
- Practical concentrations of antibiotics: refer to Chapter E Appendix.

### **Part I General Protocols**

The following methods of molecular biology frequently used in this work are described in Part I.

#### **C.I.1. Preparation of Competent *E.coli* Cells**

Preparation of competent *E.coli* cells was carried out following the protocol in Current Protocols in Molecular Biology (Ausubel et al, 1989), with the following modification:

Final competent cells were dispensed into Eppendorf tubes in 200  $\mu$ l aliquots, quickly frozen, and stored in  $-70^{\circ}\text{C}$  freezer.

#### **C.I.2. Transformation of Plasmid DNA Into *E.coli* Competent Cells**

Introduction of plasmid DNA into *E.coli* cells was achieved using heat-shock transformation protocol from Current Protocols in Molecular Biology (Ausubel et al, 1989), with minor modifications:

One tube of competent cells (200  $\mu$ l) was thawed rapidly by warming between hands and kept on ice. About 1  $\mu$ g of plasmid DNA was added to each tube of cells and mixed gently. The tube was placed on ice for 10 minutes. The cells were heat-shocked by placing tubes into  $45^{\circ}\text{C}$  water



bath for exactly 2 minutes, then put on ice to chill for 30 minutes. The cells were then transferred into 1 ml of pre-warmed LB medium in 15 ml tube and incubated at 37°C for 2 hours. 200 µl of the culture was spread on an LB plate with appropriate antibiotics and incubated overnight at 37°C.

### **C.I.3. Mini-Preparation/Large-scale Preparation of Plasmid DNA**

#### **(1) Minipreparation:**

The alkaline lysis method was used for small-scale preparation (minipreparation) of plasmid DNA (Birnboim and Doly, 1979, Ish-Horowicz and Burke, 1981).

The procedure was performed according to the protocol given in Molecular Cloning (Sambrook et al, 1989) with these modifications:

(a) Solution III used for lysis was 3M sodium acetate, pH4.6, instead of 3M potassium acetate, pH4.8.

(b) The optional step 5 (phenol:chloroform extraction) was always followed.

(c) Step 6 (ethanol precipitation): after adding 2 volumes of ethanol to the DNA solution, the mixture was allowed to stand for at least 1 hour at -20°C.

(d) In step 10, the purified DNA was redissolved in 50 µl of TE buffer (pH8.0) or dH<sub>2</sub>O without RNAase.

#### **(2) Large-Scale Plasmid DNA Preparation:**

Isolation of DNA from a 50ml culture or a larger scale preparation of

plasmid DNA was performed following the protocol in Protocols and Applications Guide, Promega, Fisher Scientific, with the following modifications:

(a) Instead of using a 250-ml culture for DNA preparation, a 50 ml-scale DNA preparation was carried out, Solution I,II, and III used were scaled down to 2 ml, 4 ml and 3 ml, respectively.

(b) At the last step, the purified DNA pellet was resuspended in 50  $\mu$ l TE buffer, pH8.0.

#### **C.I.4. Restriction Enzyme Digestion**

In an Eppendorf tube, for a final volume of 10  $\mu$ l digestion mixture, 1  $\mu$ l of 10x enzyme buffer (varies with different enzymes), 1  $\mu$ l of restriction enzyme, 1  $\mu$ l of 10x BSA, X  $\mu$ l of appropriate amount of DNA (normally 2  $\mu$ g), and (7-X)  $\mu$ l of dH<sub>2</sub>O were added and mixed. The mixture was incubated at 37°C for 1 to 4 hours.

#### **C.I.5. Agarose-Gel Electrophoresis**

In order to view the results of a restriction enzyme digestion, or to isolate a DNA fragment of interest, agarose gel for electrophoresis was prepared by dissolving agarose to 1% in 1xTBE buffer by boiling, and subsequently poured into the gel apparatus with comb in place to make loading wells. The gel was ready once it had polymerized; 6x stock solution of Agarose Gel Electrophoresis loading buffer was added to DNA samples and an appropriate volume of dH<sub>2</sub>O was added to make the final concentration of

the dye; samples were loaded into the wells on the gel. Molecular weight marker MWIII ( $\lambda$  DNA, EcoRI+HindIII cut fragments) from Boehringer Mannheim GmbH, Germany, or  $\phi\chi$ 174 (HaeIII digested) from NewEngland Biolabs, was also loaded on the same gel. With 1xTBE as the running buffer, electrophoresis was carried out for 2 hours at constant voltage of 100 volts. The gel was stained in ethidium bromide solution (stock concentration = 10mg/ml, staining solution contains 2-3 drops of stock solution in 100ml of dH<sub>2</sub>O) for 15 minutes, the bands were visualized under UV light.

## Part II Specific Methods

### **C.II.1 Screening of cDNA Library For $\beta\beta$ Enolase Gene With cDNA Probe**

#### **C.II.1.1 Isolation and Purification of the cDNA Probe Fragment (3'UTRH) To Be Labelled:**

128  $\mu$ g of 3'UTRH (6.4  $\mu$ g/ $\mu$ l) in pBluescript plasmid DNA was digested with EcoRI according to the protocol described previously in Part I. In order to achieve complete digestion, another aliquot of EcoRI was added after 2 hours' incubation at 37°C and the mixture was incubated at 37°C for 2 more hours. The digestion product was then run on a 1% agarose gel and the regions containing fragments of the correct size were cut out and put into Eppendorf tubes. Isolation and purification of the DNA fragment from agarose gels was performed following the protocol described in BioTechniques, with the following modifications. 250  $\mu$ l of phenol was

added, the tube was vortexed vigorously, kept at  $-70^{\circ}\text{C}$  for 30 minutes, and then thawed at  $37^{\circ}\text{C}$ . Another 250  $\mu\text{l}$  of phenol was added and the same procedure of vortexing-freezing-thawing was repeated two times. Finally, 100  $\mu\text{l}$  of  $\text{dH}_2\text{O}$  was added and the tube was vortexed and centrifuged for 20 minutes at 16,000g,  $4^{\circ}\text{C}$ . The supernatant was extracted twice with equal volume of each of the following: phenol, phenol-chloroform (1:1) and chloroform. The upper aqueous phase was kept and for each 250  $\mu\text{l}$  of upper layer, 25  $\mu\text{l}$  of 3M sodium acetate and 600  $\mu\text{l}$  of cold ethanol was added and the mixture was stored at  $-20^{\circ}\text{C}$  for 30 minutes. The tube was centrifuged at 16,000g for 20 minutes, the pellet was washed with 70% ethanol and centrifuged at 16,000g for 5 minutes. The supernatant was removed, the pellet was dried for 20 minutes under vacuum, and then redissolved in 15  $\mu\text{l}$  of  $\text{dH}_2\text{O}$  by vortexing (Bewsey *et al*, 1991).

#### **C.II.1.2 DIG-Labeling of the cDNA Probe**

The cDNA probe 3'UTRH was labelled with a nonradioactive DNA labelling system — DIG (digoxigenin) DNA Labelling and Detection Kit (Boehringer Mannheim, cat#1093657). The probe was generated with Klenow polymerase by random-primed incorporation of DIG-labelled deoxyuridine-triphosphate (dUTP-DIG). Following hybridization of the DIG-labelled DNA probe to target DNAs on a membrane, the hybridized DNAs were detected by enzyme-linked immunoassay using an antibody (against DIG) conjugated to alkaline phosphatase. An enzyme-catalyzed color reaction

with 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) and NBT (nitroblue tetrazolium salt) was used to produce an insoluble blue precipitate at the site of the DIG-labelled DNA.

1  $\mu\text{g}$  of purified (phenol/chloroform extracted and ethanol precipitated) cDNA probe fragment 3'UTRH (EcoRI fragment) was labelled following the protocol in the Instruction Manual for the DIG DNA detection kit. The mixture of DNA, hexanucleotides, dNTPs, and Klenow enzyme was incubated for 20 hours (in the protocol: at least 60 minutes) at 37°C.

### **C.II.1.3 Primary Screening**

The titering of the stock cDNA phage library and the screening procedure were performed following the Instruction Manual with the Pre-Made Lambda Phage Library from Stratagene, with several minor modifications:

The library was titered to determine the concentration of the phage. To a series of dilutions of the stock phage in SM buffer, 200  $\mu\text{l}$  of XL1-Blue host cells ( $\text{OD}_{600}=0.5$ ) were added per tube. Following incubation at 37°C for 15 minutes, 3 ml of top agar at 48°C were added to each tube and the mixture was plated on 100 mm LB plates.

For each plate for screening, 50,000 pfu of stock phages (as determined above) were mixed with 600  $\mu\text{l}$  of  $\text{OD}_{600}=0.5$  XL1-Blue cells and incubated at 37°C for 15 minutes. 6.5 ml of top agar was added and plated on 150 mm plates, the plates were incubated at 37°C for 8 hours. In total 3 plates were prepared, and chilled by refrigeration overnight at 4°C to prevent top

agar from sticking to the nylon membrane.

After 8 hours' incubation, about 50,000 1mm-diameter plaques were seen on each plate. Phage DNA was transferred onto nylon filters by placing the membranes carefully on to the agar surface and removing them after 1 minute. The membranes were placed plaque side up on sterile filter paper first, then left on a pad of absorbent filter paper soaked in denaturing solution for 7 minutes; later, the membranes were placed plaque side up, on a pad of absorbent filter paper soaked in neutralizing solution for 7 minutes. The membranes were then washed with 2xSSC (NaCl-sodium citrate), and transferred to dry filter paper, air dried, and then baked in an oven at 120°C for 30 minutes. After the DNAs were fixed on the membrane, the membrane was ready for hybridization with DIG-labelled 3'UTRH probe. The hybridization and DNA detection steps were performed according to the DIG DNA Labelling and Detection Kit Instruction Manual. The concentration of DIG-labelled 3'UTRH probe in the hybridization solution was 1 µl (10ng) of labelled probe /ml of hybridization solution. A dot blot with positive control (3'UTRH plasmid DNA) and negative control (pBluescript DNA) was done at the same time.

The nylon membrane used was Hybond-N nylon membrane from Amersham, cat#RPN.132N for large plate (150mm) and cat#RPN.82N for small plates (100mm).

#### **C.II.1.4 Secondary/Tertiary/Quaternary Screenings**

After the DNA detection procedures, several strong "putative" clones showed up on the filters. The filters and the stock plates were lined up, and square centimetre "windows" of agar were cut out of the stock plate at the positions where putative clones lined up with filter spots, and each was put into 1 ml of SM buffer. Clone phages were titered with host cells and rescreened. Two 100 mm small plates were used per clone; one plate had about 50 plaques and the 2nd had about 450 plaques. The plates were incubated overnight at 37°C. The next day, lifts from the two pre-chilled plates were made as in primary screening and prehybridization and hybridization were performed as before.

Tertiary and quaternary screenings were carried out until isolates of positive plaques could be picked and 100% of the plaques showed positive signals on the membrane. The putative clones on the agar plate were "cored" out and stored at 4°C in SM buffer with 2% CHCl<sub>3</sub>.

#### **C.II.2 *In vivo* Excision of the pBluescript Plasmid from the Lambda ZAPII Vector**

The Lambda ZAPII vector has been designed to allow excision and recircularization to form a phagemid containing the cloned insert. *In vivo* excision requires superinfection of a strain of *E.coli* with both the lambda vector and a filamentous helper phage (f1 bacteriophage). The process is outlined in the section "*in vivo* excision of pBluescript from Lambda ZAPII

vector" in Stratagene's Instruction Manual for Lambda ZAPII Vector.

*In vivo* excision of the cloned pBluescript phagemids from the lambda ZAPII was carried out using the ExAssist<sup>TM</sup>/SOLR<sup>TM</sup> system. In this system, SOLR cells were used as the host cells instead of XL1-Blue cells. Instead of R408 helper phage, ExAssist helper phage was used. The ExAssist helper phage contains an amber mutation that prevents replication of the helper genome in a non-suppressing *E.coli* strain such as SOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of co-infection from the ExAssist helper phage.

### **C.II.3 Confirmation of the Positive Clone by Southern Blot of cDNA Inserts with the Probe**

#### **C.II.3.1 Agarose-Gel Electrophoresis of EcoRI Digested Clones**

Plasmid DNAs of putative "positive" clones C1, C2 and C3 (c1b1w, c2a1w and c3a1w respectively) and pBluescript(SK+) vector (negative control) were cut with EcoRI as described before, and electrophoresed on a 1% agarose gel; molecular weight marker Phix174 (HaeIII digested) was also run on the side; duplicate set of DNAs were run on the same piece of gel. After electrophoresis, the gel was cut in half, one half was placed in Ethidium Bromide solution to be stained and a photograph of the cut DNA fragments was taken; the other half was used for Southern Transfer.

#### **C.II.3.2 Transfer of DNAs from Agarose Gel to Nylon Membrane**

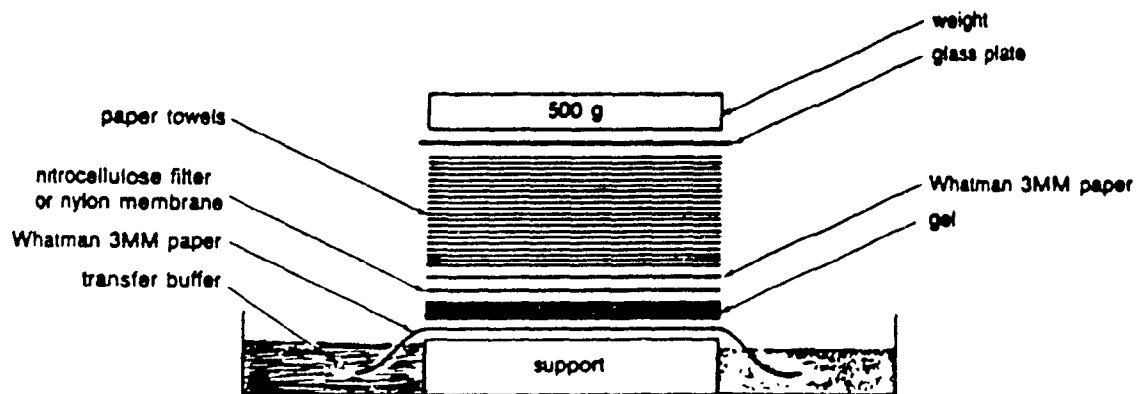
After electrophoresis was completed, half of the gel was inverted and



placed on a glass baking dish, and the unused area was trimmed away. The apparatus for DNA transfer was then set up as in Figure 3. A tray was filled with transfer buffer, a glass plate supported on two sides of the tray, and a thick pad of filter paper soaked in transfer buffer draped over the glass plate with two ends dipping into the solution. The gel was placed on the pad of filter paper, and on top of the gel the nylon membrane was placed and covered with a piece of filter paper and a stack of absorbent paper (paper towels) on top of the filter paper. The whole stack was weighed down with a thick textbook.

The nylon membranes used were Boehringer Mannheim Nylon Membranes, positive charged (cat#120929). The transfer buffer was 0.4M NaOH. Transfer was performed overnight at room temperature.

Figure 3: Southern transfer of DNA from agarose gels (Sambrook et al, 1989).



### **C.II.3.3 Hybridization of the Membrane with the Labelled-Probe**

After DNAs on the agarose gel were transferred onto nylon membrane, the membrane was baked for 30 minutes at 120°C to fix the DNAs to the membrane. Then the membrane was prehybridized in a sealed plastic bag with 20 ml hybridization-solution (with 50 ng of herring sperm DNA as heterologous nucleic acid to reduce the background colouring) per 100 cm<sup>2</sup> of membrane at 68°C in a shaker for 1 hour. Afterwards, the solution was replaced with about 2.5 ml per 100cm<sup>2</sup> membrane of hybridization solution containing 1 µl (10ng) of labelled cDNA probe fragment/ml of hybridization solution. The membrane was incubated overnight at 68°C with shaking.

When hybridization was completed, the filter was washed with SSC solutions, following the protocol in the Instruction Manual with the DIG DNA Detection Kit, and used directly for detection of hybridized DNA.

### **C.II.3.4 Immunological Detection**

The DIG-system DNA detection was carried out following the protocol supplied with the kit. For the last step, the filter was incubated with 10 ml color solution in a 15 cm Petri dish in the dark. The colour precipitate started to form within several minutes to 1 hour. The reaction was left overnight and then stopped by washing the membrane for 5 minutes with 50 ml of Buffer 4 (see Appendix).

### **C.II.4 Confirmation of the Positive Clone by DNA Sequencing**

In order to confirm that the clone is ββ enolase, double-stranded DNA of

the cloned plasmid was sequenced. Sequencing was done by the dideoxy method (Sanger et al, 1978) using bacteriophage T7 DNA polymerase (Tabor & Richardson, 1985) with the T7 Sequencing kit (Pharmacia Biochemical Co.Ltd, cat#27-1682-01).

#### **C.II.4.1 Sequencing Reactions**

The template plasmid DNA to be sequenced was isolated and purified according to the "DNA miniprep" protocol described before.

The concentration of the template was adjusted so that 32  $\mu$ l contain 1.5-2  $\mu$ g of DNA. The whole procedure was performed exactly as described in the T7 Sequencing Kit Instructions from Pharmacia Biotech.

The radioisotope used was  $\alpha^{35}\text{S}$ -dATP from New England Nuclear (cat#NEG 034S).

'A','C','G' and 'T' Mix-Short solutions were used to read less than 500 nucleotides of the sequence starting from the primer binding site; 'A', 'C', 'G' and 'T' Mix-long solutions were used to read 50-1000 nucleotides from the primer binding site.

The samples were stored at  $-20^{\circ}\text{C}$  before loaded on the sequencing gel.

#### **C.II.4.2 Electrophoresis for DNA Sequencing**

8.55g of acrylamide, 0.45g of bis-acrylamide and 63g urea were added to 30ml 5xTBE and  $\text{dH}_2\text{O}$  was added to give the final volume of 150ml. The solids were allow to dissolve before the mixture (6% acrylamide solution) was degassed for 15 minutes. 25 ml of the 6% acrylamide solution was

placed in an 25 ml flask and 125  $\mu$ l of freshly prepared 25% ammonium persulfate (APS) and 125  $\mu$ l of TEMED were added to the solution and mixed briefly. The mixture was poured into the assembled apparatus to seal the bottom of the sandwich chamber and allowed to polymerize for 10 minutes. 125  $\mu$ l of 25% APS and 125  $\mu$ l of TEMED was added to the remainder of the acrylamide solution and the mixture was poured into the assembled sandwich and the comb was inserted. When the gel had polymerized (might be left overnight), the comb was taken away, the apparatus was rinsed with dH<sub>2</sub>O and pre-run for half an hour at 1000 volts. Samples were loaded after being boiled for 3 minutes, and the gel was run at 1600 volts until the dye had migrated almost to the bottom of the gel.

#### **C.II.5 Subcloning of the Full-Length Gene Fragment into pBluescript**

Since the original clone obtained from screening the cDNA library contained a second EcoRI fragment that did not seem to be related to the enolase gene, the original plasmid was completely digested with EcoRI, and then religated to give pBluescript containing the subcloned enolase gene.

3  $\mu$ g of c2a1w (original clone) was digested with EcoRI as described before, and the product was precipitated with ethanol and the pellet was washed. The DNA was redissolved in 8  $\mu$ l of TE (pH8.0) and 1  $\mu$ l of 10x ligation buffer, 10 units of T4 ligase were added and mixed by vortexing briefly. The mixture was incubated overnight at 4°C. 2.5  $\mu$ l of the sample

was used to transform XL1-Blue competent cells, and plated on IPTG/X-Gal plate to identify transformed plasmids with inserts. Plasmid with insert should produce white colonies, plasmids without insert should give blue colonies. EcoRI restriction digestion of the subcloning product DNA was performed to screen for the right subclone (named CA6) with the full-length gene as the insert.

### **C.II.6 Nested Deletion**

Erase-a-Base System from Promega(Cat#E5750) was used to carry out the Nested Deletion of the plasmid CA6 to get the whole sequence of the gene.

The plasmid DNA used for the nested deletion was CA6, the subclone of pBluescript with the insert of the full-length cDNA sequence coding for rabbit  $\beta\beta$  enolase. Two restriction enzymes — SacI and NcoI, were used to produce a 3'overhang and a 5'overhang in the plasmid CA6, respectively. Closed circular DNA (10  $\mu$ g) of CA6 was cut with NcoI enzyme first. The DNA was purified with Phenol:Chloroform(1:1) and precipitated with 95% ethanol before being cut with SacI enzyme, since the reaction buffers for NcoI and SacI are not compatible. Since ExoIII is strongly inhibited by as little as 20mM NaCl, the double cut plasmid DNA had to be extracted with phenol/ chloroform and precipitated with ethanol following restriction digestion.

The deletion procedure was performed following the Instruction Manual

with the Erase-a-Base Kit, with several modifications as follows:

Since the length of the cloned insert was around 1.4 Kb, 7 samples of the digestion mixture were taken at 30 second intervals (Digestion proceeds at about 450 bases/minute at 37°C, and there is a 20-30 second lag for the reaction to begin). 300 units of ExoIII was used. The amounts of other reagents or mixtures used within the procedure were all scaled down proportionally.

Transformation of the nested deletion DNA products into XL1-Blue competent cells was carried out with 200 µl of XL1-Blue competent cells for each time point, following the protocol described before.

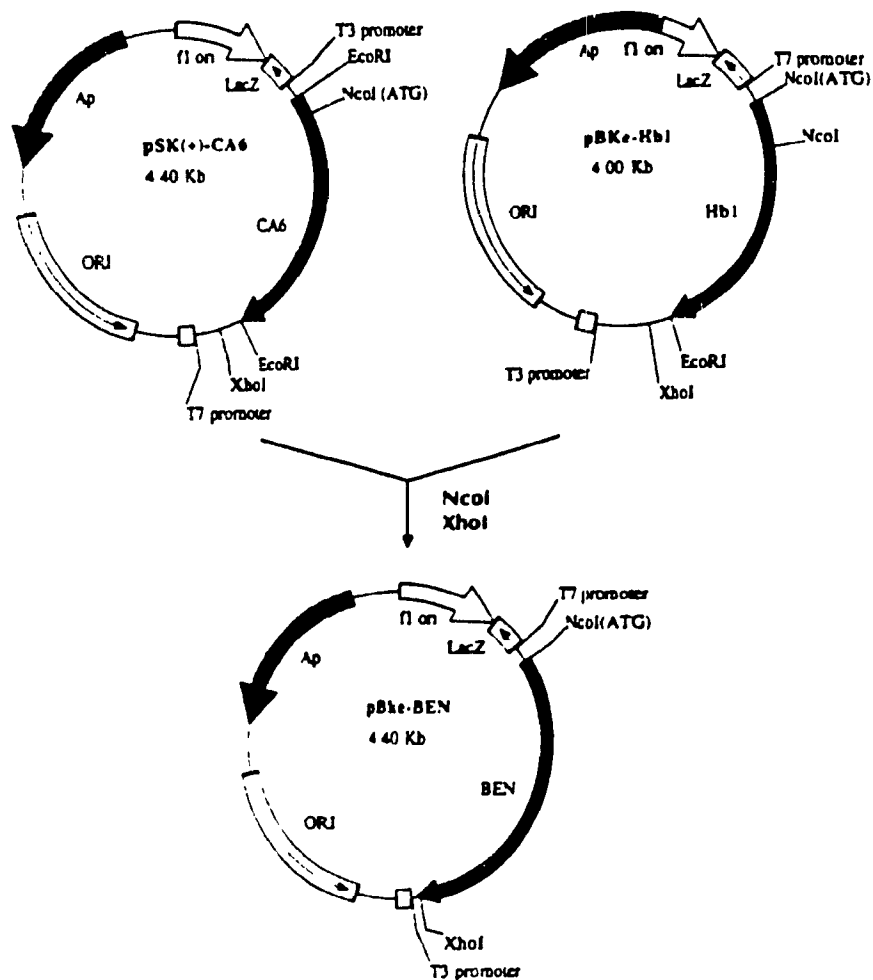
The "rapid screening" procedure described in the Instruction Manual was followed to screen the deletion subclones.

#### **C.II.7 Subcloning of the cDNA Fragment BEN into Expression Vector**

pBKe-BEN was constructed (as described in Figure 4) to express the cDNA encoding rabbit ββ enolase in *E.coli* cells. The insert HB1 in the original expression vector given to us by Dr MacKenzie was removed from pBKe-HB1 by digestion with NcoI and XhoI. Since the rabbit ββ enolase clone obtained from the cDNA library had a unique NcoI site at the start codon of this gene, the subcloning of our gene into the expression vector was convenient. By simply cutting both the pBKe-HB1 plasmid and the pBlue-clone insert plasmid (CA6) with two restriction enzymes — NcoI and XhoI, isolating the linearized NcoI-XhoI expression vector fragment and

the NcoI-XhoI fragment of rabbit  $\beta\beta$  enolase insert, and subsequently using T4 DNA ligase to fuse the insert into the vector, the HbI insert was replaced with the insert BEN. In this case, our enolase gene was in frame downstream of the T7 RNA polymerase promoter, the translational enhancing sequence and the ribosomal binding site. This construct was transformed into the BL21(DE3)pLysS strain of *E.coli*. Enolase activity assays, protein content assays and SDS-PAGE electrophoresis were used to determine the level of expression of  $\beta\beta$  enolase in the lysate.

Figure 4: Construction of the expression plasmid pBKe-BEN.



### **C.II.8 *In vitro* Site-Directed Mutagenesis of the Gene**

*In vitro* site-directed mutagenesis of the BEN gene was performed according to the method previously described (Kunkel et al 1987, Vieira and Messing, 1987, Geisselsoder et al, 1987), following the Bio-Rad MutaGene™ Phagemid *in vitro* Mutagenesis Instruction Manual, Version 2 (Cat.# 170-3581). The template used was pBKe-BEN.

#### **C.II.8.1 Preparation of the Single-Stranded (ss) DNA Template**

Uracil-containing single-stranded DNA template of pBKe-BEN was prepared using CJ236 as the host strain and VCS-M13 (Stratagene, cat#200251, stock titer= $2.0 \times 10^{11}$  pfu/ml) as the helper phage.

First of all, double-stranded DNA of pBKe-BEN was transformed into CJ236 cells, which is a *dut<sup>-</sup>* & *ung<sup>-</sup>* strain; single-stranded DNA of the template for mutagenesis, which is uracil-containing, was synthesized following the Instruction Manual. Helper phage (VCS M13) was added to the culture during log phase at a multiplicity of infection of 20:1 (phage to cells).

ssDNA was visualized on a 1% agarose gel. The VCS M13 and pBluescript with no insert migrate at approximately 6 Kb and 1.6 Kb respectively, when compared to a double stranded marker.

#### **C.II.8.2 Phosphorylation of the Mutagenic Primer**

The 27-base mutagenic primer obtained from Dalton Chemical Laboratories Inc. was phosphorylated in order to get higher frequency of



mutagenesis. The phosphorylation of the primer was carried out following the protocol in the Instruction Manual.

### **C.II.8.3 Annealing of the Oligonucleotide Primer to the Template**

200 ng (0.3 pmol) of single-stranded uracil-containing DNA and 9 pmol of phosphorylated mutagenic oligonucleotide was used for the annealing, according to the protocol.

### **C.II.8.4 Second Strand Synthesis**

Instead of using T4 DNA polymerase as in the old version of the kit, T7 DNA polymerase was used in Version 2. T7 DNA polymerase has several advantages: it does not perform strand displacement, and has a higher rate of polymerization than that of T4 DNA polymerase. Mutagenic efficiencies are comparable with both enzymes.

### **C.II.8.5 Transformation of the Mutagenic Product in MC1066 and XL1-Blue *E.coli* Cells**

5  $\mu$ l of the mutagenic product was used to transform MC1066 strain using the protocol described before. MC1066 is a *dut*<sup>-</sup>, *ung*<sup>+</sup> strain, which has an active uracil N-glycosylase that will inactivate the uracil-containing parental strand and thus will select for the mutant strand as well as allow it to replicate within the cells.

DNA of the mutant plasmid was purified according to the mini-preparation of plasmid DNA protocol and was transformed into XL1-Blue strain, as previously described. Mutant clone was identified by DNA

sequencing.

### **C.II.9 Expression of the Wildtype and Mutant Genes in *E.coli* Cells**

An expression system using T7 RNA polymerase to direct expression of cloned genes was utilized in the work (Studier et al, 1990). The detailed procedure of an expression experiment is as follows:

Cells from a single colony of pBKe-BEN in BL21(DE3)pLysS cells or pBKe-BEN-MUT in BL21(DE3)pLysS were inoculated into 27 ml LB liquid medium in an Erlenmeyers flask, and were incubated at 37°C for around 10 hours till  $OD_{600}=0.8-1$ . IPTG was added at this point to give a final concentration of 0.4 mM, and the cells were incubated for 30 minutes at 37°C. Rifampicin was then added to reduce the level of the background *E.coli* enolase; final concentration of rifampicin was 15 µg/ml. The cells were grown for another 2.5 hours at 37°C before being harvested by centrifugation at 12,000g for 30 minutes at 4°C (Pellets can be stored at -80°C before next step). Pellets were resuspended in 2.5 ml ion-exchange buffer and sonicated for 8x15 seconds at 20KHz intervals of 1 minute on ice. The milky product was centrifuged at 12,000g for 30 minutes at 4°C, and the supernatant was kept as cell extract sample.

### **C.II.10 Separation of $\beta\beta$ Enolase from *E.coli* Enolase by Chromatography on Q-Sepharose**

A 1 ml Q-Sepharose (Pharmacia Biotech. Inc.) column was equilibrated by washing with 20ml ion-exchange buffer, and cell extract sample was

loaded onto the column. The column was washed with another 10 ml of ion-exchange buffer, following by washing with 10 ml of the elution buffer. 1 ml fractions were collected right after the loading. Fractions were assayed immediately for enolase activity. Protein content of the fractions was assayed within 24 hours using Bio-Rad standard protein assay. Fractions with peak activity were pooled and samples were run on SDS-PAGE gel.

#### **C.II.11 Enolase Activity Assays and Protein Assays of the Fractions**

Enolase activity was assayed directly by measuring the changes in PEP concentration spectrophotometrically at 240nm with activity assay buffer (Imidazole-Magnesium Acetate-KCl-EDTA).

Enolase activities are expressed as change of O.D. at 240 nm per minute.

#### **C.II.12 Bio-Rad Protein Standard Assay**

Bio-Rad Protein Assay (Cat#.500-0006) was used for the determination of protein content in the sample by the method of Bradford (Bradford, 1976). The procedure was executed following the Instruction Manual of Bio-Rad Protein Assay.

#### **C.II.13 SDS-PAGE Electrophoresis**

SDS-PAGE of proteins was performed using the system described previously (Laemmli, 1970, Hames et al, 1981).

The apparatus for SDS-PAGE electrophoresis was Mini-PROTEAN II Dual Slab Cell from Bio-Rad (Cat#.165-2940). The gel electrophoresis apparatus

was assembled following the Instruction Manual with the kit.

Protein samples were mixed with 2xloading buffer and boiled for 5 minutes before being loaded into the wells. Protein standards were also loaded on the gel. The gel was run at 100 volts for 45 minutes to 1 hour.

After electrophoresis, the gels were removed from the apparatus and stained in staining solution with Coomassie Blue overnight at room temperature and destained in destaining solution before the gel was dried under vacuum.

## **Chapter D RESULTS AND DISCUSSION**

### **D.1 Design and Logic of the Project**

As described in Chapter A, there are several goals for this study: cloning of the rabbit  $\beta\beta$  enolase gene from a cDNA library, expressing the gene in *E.coli* cells so as to get functional protein, and exploring the effects of modifying intersubunit ion-pair contacts on the behaviour of the enzyme.

#### **(1) First step : to clone the gene.**

A rabbit muscle cDNA expression library was screened for the  $\beta\beta$  enolase gene. The reason  $\beta\beta$  enolase from rabbit was chosen as the target enzyme mainly lies in the fact that purified rabbit  $\beta\beta$  enolase is commercially available, and therefore comparative studies of the properties of the native protein and recombinant protein can be easily effected.

A 641bp murine  $\beta\beta$  cDNA fragment 3'UTRH was utilized as the probe to screen the library. Indeed, this probe is expected to be specific for  $\beta\beta$  enolase, since this probe contains the complete 3'UTR sequence of murine  $\beta\beta$  enolase gene, a region considered to be homologous between  $\beta\beta$  isozymes (Lamande et al, 1989). Furthermore, the coding regions of murine  $\beta\beta$  and rabbit  $\beta\beta$  are presumed to be quite similar, since it has been shown that sequence homology between enolases from the same type of tissues (for example, mouse  $\beta\beta$  versus rabbit  $\beta\beta$ ) is higher than that between enolase from different tissues, but the same species (for example, mouse  $\beta\beta$  versus mouse  $\alpha\alpha$ ).  $\beta\beta$  enolase has been shown to be muscle-specific, hence

using murine  $\beta\beta$  probe to screen the rabbit muscle cDNA library gives a high prospect of acquiring clones of rabbit  $\beta\beta$  enolase. However, since the  $\alpha$  enolase isozyme has been found ubiquitously distributed in different tissues, we can not rule out the possibility that with the  $\beta$  probe, a clone that codes for  $\alpha\alpha$  would have been obtained. To minimize this chance, adult rabbit muscle library was used simply because in adult muscle,  $\beta\beta$  represents a higher percentage of the total amount of enolase than in embryonic tissue.

As for the labelling system for the cDNA probe, a non-radioactive labelling method was chosen. DIG-DNA Labelling and Detection Kit has several privileges over other systems:

- (a) It is much safer for being a non-radioactive system.
- (b) It is highly sensitive. As little as pg levels of DNA can be detected.
- (c) The detection procedure is rapid and handy to fulfil.

(2) Second step: expression of the gene in *E.coli* cells.

An expression vector given to us by Dr. MacKenzie (pBKe-Hb1) was used. This plasmid, which has been constructed to be an excellent expression vector for mammalian genes in *E.coli*, contains the elements that are required for expression, such as a T7 RNA polymerase promoter, a translational enhancer sequence, and a ribosomal binding site upstream of the initiation codon ATG. Another benefit of using this vector was that my cDNA clone (BEN) was easily inserted into this plasmid at the start

codon. This design has several advantages: first of all, it is quite convenient, since the restriction sites NcoI and XhoI are both unique sites for the insert and the vector as well, permitting the complete gene to be subcloned; secondly, the problems caused by expressing a fusion protein were avoided. If a fusion protein was constructed, we would get expression of a protein which is somewhat structurally different from the native protein. In this work, the rabbit  $\beta\beta$  enolase was expressed in *E.coli* system, but the only differences other than when the gene is expressed in rabbit are that: (1) the starting codon ATG gets translated in prokaryote system. (2) there is no acylation of the N-terminal  $\text{NH}_3^+$ . These differences should not affect the tertiary structure and the function of the heterogeneous enzyme expressed in *E.coli*, nor should it affect the subunit interactions.

The *E.coli* strain used for expression was BL21(DE3)pLysS, and the level of  $\beta\beta$  enolase expression was heightened by IPTG induction. The rationale of using this system has been described in the correlated part of Materials and Methods.

(3) Step 3: purification of recombinant  $\beta\beta$  enolase by Q-Sepharose chromatography.

*E.coli* enolase can be separated from some mammalian isozymes with several chromatography procedures. Mouse neuronal-specific enolase( $\gamma\gamma$ ) in an expression plasmid expressed in *E.coli* cells was reported to elute from a DEAE Cellulose Chromatography column at a higher salt

concentration than *E.coli* enolase (Dutta et al, 1990). Previous studies in our lab showed that when a mixture of commercial rabbit muscle-specific ( $\beta\beta$ ) enolase and *E.coli* enolase was passed through a Q-sepharose column,  $\beta\beta$  enolase did not bind to the column while *E.coli* enolase bound and was eluted in the presence of 1M KCl. This phenomenon led to the rationale for our trial of purification of rabbit  $\beta\beta$  recombinant protein from *E.coli* enolase and other proteins.

(3) Step 4: construction of E417L point mutation of the gene.

From the introduction of yeast enolase and enolases from other species, we can see that the primary structures and the catalytic properties of yeast enolase are dramatically similar to those of other enolases. Therefore, we have strong reasons to believe that the 3-dimensional structure of yeast enolase can serve as a good model for enolase isozymes from other species.

Studies of protein subunit association and dissociation reactions provide valuable information, not only on the nature of protein structure but also about the relation between protein structure and function. Differences in the mechanisms of inactivation and dissociation of  $\beta\beta$  and  $\gamma\gamma$  enolase intrigue us to focus on the subunit interactions of the enzyme.

The main goal was to find out if any specific differences in structural change occurring during the procedure of dissociation of this enzyme is the real reason for these different observations.

The ionic bond of Arg8-Glu417 is involved in strengthening subunit



interactions. Arg8 in Strand 9 and Glu417 in Helix H are both in highly conserved regions. By replacing Glu417, which is a polar amino acid, with Leucine, which is a nonpolar residue with a relative small side chain, we hope to get some important information about the effect of this specific mutation on various chemical and physical properties of enolase. In addition, multi-site mutants based on this mutant may be obtained for further study of the subunit interactions of this dimeric enzyme.

Mutating Glu417 to Leucine involved replacing the codon GAG with CTC. This change was designed so that on one hand, the polar amino acid at position 417 Glutamic acid was changed to a neutral amino acid Leucine; on the other hand, an extra restriction site for the enzyme SacI was introduced into the sequence, causing no change in other codon. This mutation provided a useful marker in screening for mutant plasmid DNA — MUT-BEN.

## **D.2 Cloning**

### **D.2.1 cDNA Library Screening**

A 641 bp cDNA fragment, given to us by Dr.M.Lazar, including part of the coding region and the whole 3'UTR region of murine  $\beta\beta$  enolase, was used as a probe to screen a rabbit muscle cDNA expression library. Three strong positive DIG-spots appeared on the membrane, corresponding to 3 clones out of total of 150,000 original plaques (50,000 pfu/plate) on three plates. Secondary, tertiary and quaternary screenings of these three

putative clones were carried out till all three purified cDNA clones showed 100% line-up between the plaques on the plate and the DIG<sup>+</sup> spots on the membrane. Of the three, clone 2 (c2a1w) gave the darkest spots on the membrane. Subsequently, the 3 putative clones were isolated and purified. Recombinant pBluescript plasmids were derived from these clones by *in vivo* excision.

### **D.2.2 Southern Blot Analysis**

In order to confirm that the putative clones do contain a cDNA fragment related to the enolase gene, a Southern blot was performed. An EcoRI restriction enzyme digestion was performed with the DNAs of the three putative clones, the pBluescript plasmid (as negative control), and the 3'UTRH probe as well (as positive control). The agarose gel pattern showed that all three plasmids had their inserts cut out, with the sizes of 1.3Kb, 1.4Kb, and 1.3 Kb, respectively. On the membrane hybridized with DIG-labelled probe, only clone 2 (c2a1w) containing a 1.4 Kb insert showed positive hybridization with the 3'UTRH probe. The signal density of this band was comparable to that of the positive control (the 641bp band of the probe hybridized to the labelled-probe itself), therefore, only clone 2, termed BEN, seemed to be a true positive clone. The other two were proven to be false positive clones.

### **D.2.3 DNA Sequencing of the Clone and Subcloning of the Full-Length Gene into pBluescript.**

Clone 2 was subjected to DNA sequencing. Approximately 150 bases from both the 5' and the 3' ends of the clone (c2a1w) were sequenced. DNA sequence analysis were performed using a computer search of the Genbank data base using the blastn and blastx search program, and PCgene program as well. Database searches revealed that the rabbit  $\beta\beta$  enolase gene had high similarity with previously reported enolases. When a 149-base sequence from the 3' end of clone 2 (c2a1w) was sent for analysis with Genbank blastn program, it was found that this segment of sequence showed an average of 75% sequence identity with human  $\beta\beta$  enolase mRNA, rat  $\beta\beta$  enolase mRNA and mouse  $\beta\beta$  mRNA within the corresponding region. Further analysis of the 5' end sequence led to the discovery that, prior to the enolase gene initiation codon ATG, there was a 113bp-long conjugated fragment of DNA, which seemed to be rRNA sequence. The clone c2a1w was then digested completely with EcoRI enzyme and two DNA fragments were obtained, which were about 100 bp and 1.4 kb respectively. The larger fragment, which turned out to be enolase gene fragment, was purified from agarose gel and subcloned into pBluescript(SK+) vector. DNA sequencing from both ends of the subcloned insert was carried out, and the subclone was identified as a full-length rabbit  $\beta\beta$  enolase gene.

Nested deletion of CA6 (BEN in pBluescript) was successful; sequencing of four different-length nested-deletion product plasmid DNAs ND1, ND2, ND3 and ND4 gave us the complete nucleotide sequence of this rabbit  $\beta\beta$

enolase gene.

DNA nucleotide sequence and published amino acid sequence (Chin et al, 1990) of rabbit  $\beta\beta$  enolase:

```
5' aattcccctagactcagagctccatccaaactccagcgaagacatcccagcc-ATG-
1           5           10           15
GCC-ATG-CAG-AAA-ATC-TTC-GCC-CGG-GAA-ATC-CTG-GAC-TCC-AGG-GGC
ALA MET GLN LYS ILE PHE ALA ARG GLU ILE LEU ASP SER ARG GLY
20           25           30
AAC-CCC-ACG-GTG-GAG-GTG-GAC-CTG-CAC-ACA-GCC-AAG-GGC-CGA-TTC-
ASN PRO THR VAL GLU VAL ASP LEU HIS THR ALA LYS GLY ARG PHE
35           40           45
CGA-GCA-GCG-GTG-CCC-AGC-GGA-GCT-TCC-ACG-GGG-ATC-TAT-GAA-GCT
ARG ALA ALA VAL PRO SER GLY ALA SER THR GLY ILE TYR GLU ALA
50           55           60
CTG-GAG-CTG-AGA-GAT-GGA-GAC-AAA-TCC-CGC-TAC-CTG-GGA-AAG-GGT
LEU GLU LEU ARG ASP GLY ASP LYS SER ARG TYR LEU GLY LYS GLY
65           70           75
GTC-TTG-AAG-GCT-GTG-GAA-CAC-ATC-AAC-AAG-ACC-CTA-GGC-CCT-GCT-
VAL LEU LYS ALA VAL GLU HIS ILE ASN LYS THR LEU GLY PRO ALA
80           85           90
CTG-CTG-GAA-AAG-AAA-CTA-AGC-GTT-GTG-GAT-CAG-GAA-AAG-GTG-GAC-
LEU LEU GLU LYS LYS LEU SER VAL VAL ASP GLN GLU LYS VAL ASP
95           100          105
AAG-TTT-ATG-ATC-GAG-CTG-GAC-GGG-ACC-GAG-AAT-AAG-TCC-AAA-TTC-
LYS PHE MET ILE GLU LEU ASP GLY THR GLU ASN LYS SER LYS PHE
110          115          120
GGG-GCT-AAC-GCC-ATC-CTG-GGG-GTG-TCC-CTG-GCC-GTG-TGT-AAG-GCC-
GLY ALA ASN ALA ILE LEU GLY VAL SER LEU ALA VAL CYS LYS ALA
125          130          135
GGG-GCT-GCA-GAG-AAG-GGG-GTT-CCT-CTG-TAC-CGG-CAC-ATC-GCT-GAT-
GLY ALA ALA GLU LYS GLY VAL PRO LEU TYR ARG HIS ILE ALA ASP
140          145          150
CTG-GCT-GGC-AAC-CAC-GAC-CTA-GTC-***-***-CTG-CCT-GTG-CCT-GCT
LEU ALA GLY ASN HIS ASP LEU VAL *** *** LEU PRO VAL PRO ALA
155          160          165
TTC-AAC-GTG-ATC-AAT-GGG-GGC-TCC-CAC-GCC-GGG-AAC-AAG-CTG-GCC-
PHE ASN VAL ILE ASN GLY GLY SER HIS ALA GLY ASN LYS LEU ALA
170          175          180
ATG-CAG-GAG-TTC-ATG-ATC-CTG-CCC-GTG-GGG-GCC-AGC-TCC-TTC-AGG
MET GLN GLU PHE MET ILE LEU PRO VAL GLY ALA SER SER PHE ARG
185          190          195
GAG-GCC-ATG-CGC-ATC-GGC-GCC-GAG-GTC-TAC-CAC-CAC-CTC-AAG-GGG
GLU ALA MET ARG ILE GLY ALA GLU VAL TYR HIS HIS LEU LYS GLY
200          205          210
GTC-ATC-AAG-GCC-AAG-TAC-GGG-AAG-GAC-GCC-ACC-AAT-GTG-GGG-GAC
VAL ILE LYS ALA LYS TYR GLY LYS ASP ALA THR ASN VAL GLY ASP
```

215	220	225
GAG-GGT-GGC-TTC-GCC-CCA-AAC-ATC-CTG-GAG-AAC-AAT-GAG-GCC-CTG-		
GLU GLY GLY PHE ALA PRO ASN ILE LEU	GLU ASN ASN GLU ALA LEU	
230	235	240
GAG-CTG-CTG-AAG-ACC-GCC-ATC-CAG-GCG-GCG-GGC-TAC-CCA-GAC-AAG-		
GLU LEU LEU LYS THR ALA ILE GLN ALA ALA GLY TYR PRO ASP LYS		
245	250	255
GTG-GTG-ATC-GGC-ATG-GAC-GTG-GCG-GCC-TCC-GAG-TTC-CAC-CGC-AAC-		
VAL VAL ILE GLY MET ASP VAL ALA ALA SER GLU PHE HIS ARG <u>ASN</u>		
260	265	270
GGG-AAG-TAC-GAC-CTG-AC-TTC-AAG-TGG-CCG-GAT-***-GAT-CCG-GCC		
GLY LYS TYR ASP LEU ASP PHE LYS SER PRO ASP *** ASP PRO ALA		
275	280	285
CGG-CAC-ATC-ACT-GGG-CAG-AAG-TTG-GGG-GAG-CTG-TAC-AAG-AGC-TTC-		
ARG HIS ILE THR GLY GLN LYS LEU GLY GLU LEU TYR LYS SER PHE		
290	295	300
ATC-AAG-AAC-TAC-CCT-GTG-GTC-TCC-ATC-GAG-GAC-CCC-TTT-AAC-CAG-		
ILE LYS ASN TYR PRO VAL VAL SER ILE GLU ASP PRO PHE <u>ASN</u> GLN		
305	310	315
GGT-GAC-TGG-GGC-GCC-TGG-AGC-CGC-TTC-CTA-GCC-GGG-GTG-GAC-ATC-		
GLY ASP TRP GLY ALA TRP SER ARG PHE LEU ALA GLY VAL ASP ILE		
320	325	330
CAG-GTG-GTG-GGG-GAT-GAC-CTC-ACG-GTC-ACC-AAC-CCC-AAG-AGG-ATT-		
GLN VAL VAL GLY ASP ASP LEU THR VAL THR ASN PRO LYS ARG ILE		
335	340	345
GCC-CAG-GCC-GTG-GAG-AAG-AAG-GCC-TGC-AAC-TGC-CTG-CTG-CTG-AAG-		
ALA GLN ALA VAL GLU LYS LYS ALA CYS ASN CYS LEU LEU LEU LYS		
350	355	360
GTC-AAC-CAG-ATC-GGC-TCA-GTG-ACG-GAA-TCC-ATC-CAG-GCT-TGC-AAA-		
VAL ASN GLN ILE GLY SER VAL THR GLU SER ILE GLN ALA CYS LYS		
365	370	375
CTG-GCC-CAG-TCT-AAC-GGC-TGG-GGG-GTG-ATG-GTG-AGC-CAC-CGC-TCC-		
LEU ALA GLN SER ASN GLY TRP GLY VAL MET VAL SER HIS ARG SER		
380	385	390
GGA-GAG-ACC-GAG-GAC-ACC-TTC-ATC-GCC-GAC-CTC-GTG-GTG-GGG-CTC-		
GLY GLU THR GLU ASP THR PHE ILE ALA ASP LEU VAL VAL GLY LEU		
395	400	405
TGC-ACG-GGA-CAG-ATC-AAG-ACT-GGC-GCC-CCC-TGT-CGG-TCG-GAG-CGG-		
CYS THR GLY GLN ILE LYS THR GLY ALA PRO CYS ARG SER GLU ARG		
410	415	420
CTG-GCC-AAG-TAC-AAC-CAA-CTC-ATG-AGG-ATC-GAG-GAG-GCT-CTT-GGG		
LEU ALA LYS TYR ASN GLN LEU MET ARG ILE GLU GLU ALA LEU GLY		
425	430	435
GAC-AAG-GCT-GTC-TTC-GCT-GGA-CGC-AAG-TTC-CGT-AAC-CCG-AAG-GCC-		
ASP LYS ALA VAL PHE ALA GLY ARG LYS PHE ARG ASN PRO LYS ALA		

AAG-TGA-gaagcaggaggccctgggactccacttgacacaccctggcctttgagccttt  
 LYS-stop-

tcccctgaaataaacactggtgccagtcacgg-3'

[\* — The numbering system is based on yeast enolase; residues 144, 145 and 267 (\*\*\*) in yeast enolase are missing in rabbit muscle enolase.]

The AAATAAA polyadenylation signal is situated 58 bp downstream from the termination codon of the gene.

The full length gene, designated as BEN, contains 1441 bp totally. It codes for 433 amino acids, and the coding region is flanked with a 52 bp 5'UTR region and an 84 bp 3'UTR region. Comparing the sequences of the corresponding 3'UTR regions of the rabbit  $\beta\beta$  enolase clone and the murine  $\beta\beta$  enolase probe, up to the AAATAAA polyadenylation signal, we find that they share a 64.9% DNA sequence identity. This finding shows that the murine cDNA 3'UTRH serves as a good probe for mammalian  $\beta\beta$  gene cloning. The 3'UTR region also shows high sequence homology (70%-78%) with 3'UTR regions of other mammalian  $\beta\beta$  enolases.

The Open Reading Frame (ORF) region exhibits 87%, 86%, and 87% sequence identity to human  $\beta\beta$ , rat  $\beta\beta$ , and murine  $\beta\beta$  enolases, respectively. In addition, our sequence analysis has revealed that the 5' non-coding regions of these genes are also closely related, 91% sequence identity was found in the 5'UTR region of this rabbit  $\beta\beta$  gene and that of human  $\beta\beta$  gene. These results are in agreement with data demonstrating a high degree of homology between  $\beta\beta$  enolases sequences from different species, indicating that enolase has been remarkably conserved during eukaryotic evolution.

The nucleotide sequence obtained from DNA sequencing coincided with the published amino acid sequence, except in amino acid positions 255 and 299, where the DNA sequencing identified Asparagines, while the automatic sequencing (amino acid) gave Aspartate. The reason for this controversy may be ascribed to the fact that in amino acid sequencing, the strong acid treatment used in the separation of peptides often caused extensive deamination of Asn and Gln containing peptides, therefore the possibility of mistakenly identifying Asn as Asp was considered quite real. The discrepancy in these residues most likely falls in this category.

#### **D.2.4 Conclusions on Cloning**

1 out of 150,000 phage from the cDNA library was found to be the target clone that we were looking for. The clone was subjected to DNA sequencing and found to encode the complete ORF of rabbit  $\beta\beta$  enolase. The Southern Blot experiment and the WT-BEN plasmid DNA sequencing results permitted unambiguous identification of the  $\beta\beta$  enolase clone.

#### **D.3 Subcloning of the Gene into Expression Vector**

NcoI and XhoI enzymes were used to cut the full-length  $\beta\beta$  enolase gene fragment out of the pBluescript cloning vector. The fragment was isolated and purified, and T4 DNA ligase was used to fuse this insert into the expression vector pBKe, digested with the same two enzymes. The subclone pBKe-Hb1 was confirmed by DNA sequencing.

The cDNA clone was found to be inserted into the expression vector with

the start codon under the control of T7 promoter.

#### **D.4 Site-Directed Mutagenesis**

35 colonies were present on the plate of MC1066 transformed with the product of the mutagenesis reaction. Minipreparations of plasmid DNA from these 35 colonies were prepared; each DNA was digested with SacI restriction enzyme, and electrophoresed on 1% agarose gel. Among these 35 digestions, 5 (lanes a5, a7, a18, a24, and b1 in Figure 5 a&b) showed the expected pattern for mutated plasmid DNA, which was expected to contain 2 bands, 1.3 kb and 2.9 kb respectively; the rest showed the pattern expected for wild type DNA, which should contain only one linear band of 4.2 kb. pBke-BEN, the wild type plasmid, contains a unique SacI site in the polylinker region of the vector, upstream of the starting codon ATG of the target gene. pBke-MUTBEN, on the other hand, contains an extra SacI site between codons 416 and 417 of the gene. Therefore, SacI restriction enzyme digestion experiment helped us to distinguish mutated plasmid from wild type plasmid. Following SacI restriction enzyme digestion, the mutated MUT-BEN yields the expected digestion patterns on 1% agarose gel, with two different-size bands, 2.9 kb and 1.3 kb respectively; while the wild type BEN is expected to yield only one linear band of 4.2 kb (Figure 5 & 6).



Figure 5: SacI enzyme digestion of plasmid DNAs isolated from the 35 colonies of transformed mutagenic product.

Figure 5a:

MW a1 a2 a3 .....a24

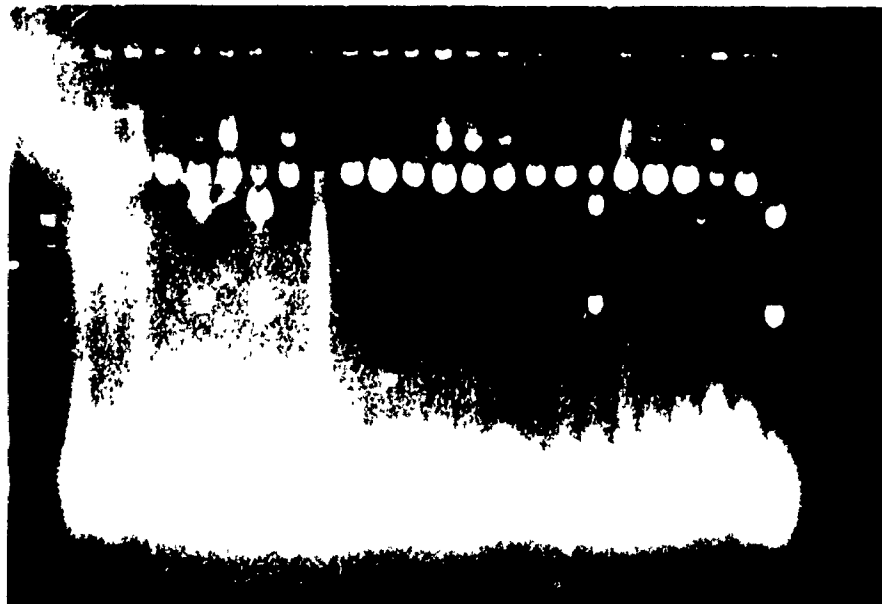


Figure 5b:

b1 b2 b3 b4 b5 b6 b7 b8 b9 b10 b11

21226

5148

4973

4268

3530

2027

1904

1584

1375

947

831

564

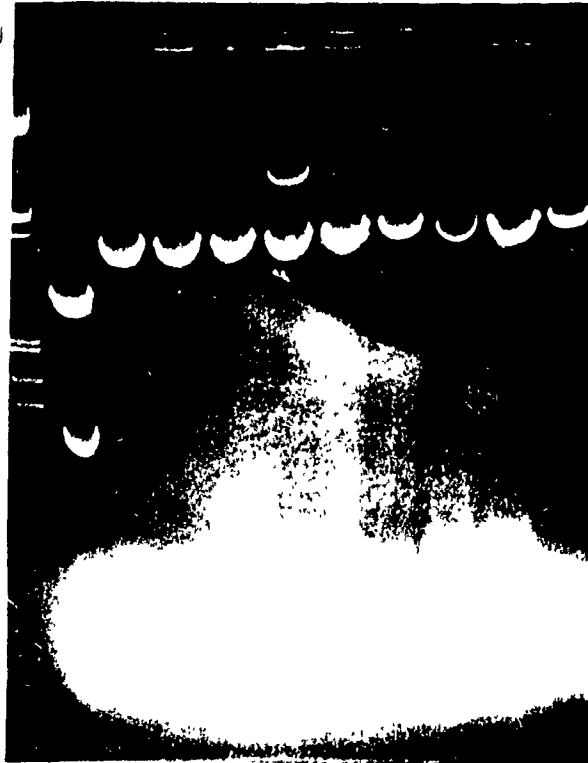
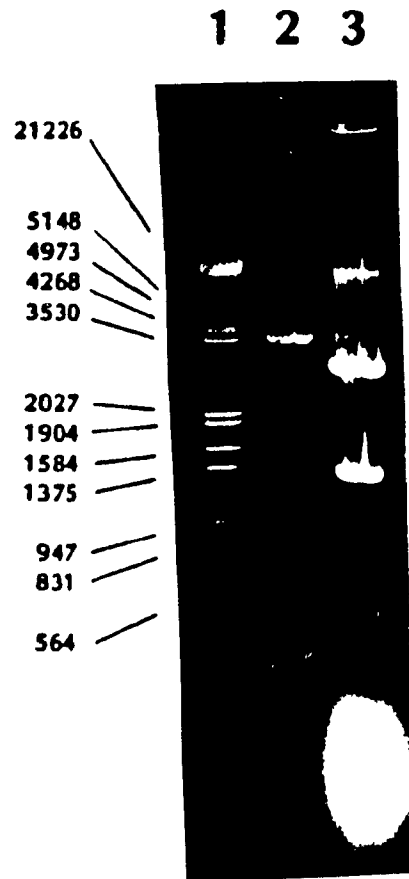


Figure 6: SacI enzyme digestion of pBKe-BEN (WT) and pBKe-MUTBEN (mutant) plasmid DNA (lane 1: Molecular Weight Marker; lane 2: SacI-digested pBKe-BEN; lane3: SacI-digested pBKe-MUTBEN).



DNA sequencing of the region close to the mutated codon confirmed that except for the codon at position 417, which was changed from GAG to CTC, there were no other sequence changes in the vicinity.

Both the WT-BEN and MUT-BEN were transformed into *E.coli* expression system BL21(DE3)pLysS and characterized.

#### **D.5 Expression of the Wild-Type and Mutant Proteins**

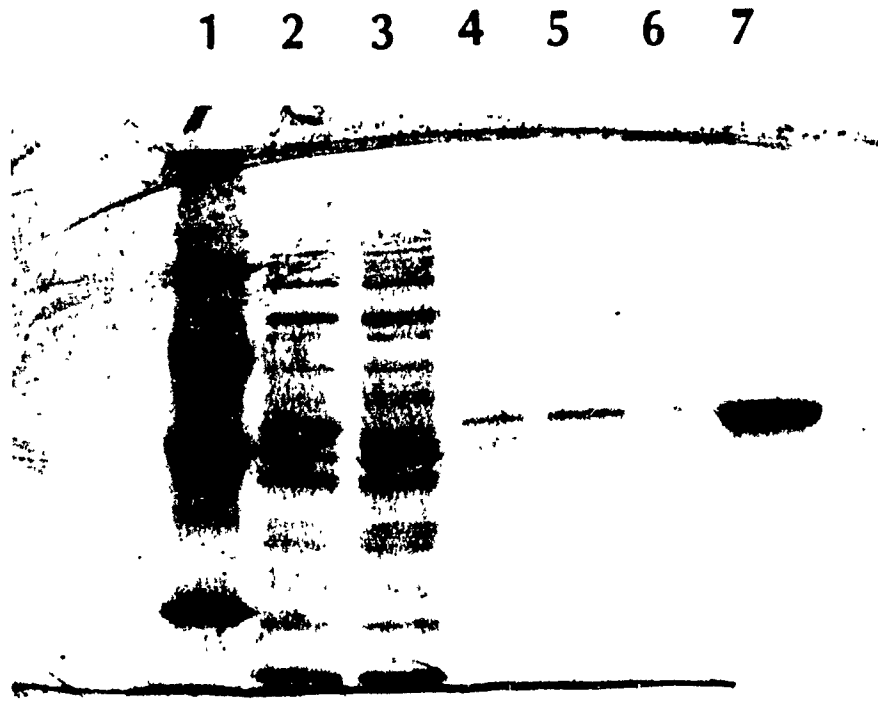
The pBKe expression plasmids harbouring the WT-BEN and MUT-BEN

gene inserts were transformed into BL21(DE3)pLysS *E.coli* strain. Rabbit  $\beta\beta$  enolase wild-type and mutant enzymes were successfully expressed in *E.coli* cells using the phage T7 RNA polymerase-based system (Studier and Moffatt, 1986). Induction at 37°C by the addition of 0.4mM IPTG to exponentially growing cultures results in the production of soluble, active recombinant enzymes. The protein content of the crude extracts was about 800  $\mu\text{g}$  from a 25 ml culture; yields were similar for both *E.coli* expressing WT-BEN and MUT-BEN.

The cell extract samples were collected from the cultures of WT or mutant. All BEN and MUT-BEN enolase activity stays in the supernatant following lysis and centrifugation. 2 ml of the cell extracts were loaded on a 1 ml Q-Sepharose column, recombinant enzymes were eluted from the column with ion-exchange buffer. Enolase activity assays with 1 ml-fractions showed peak activity within the first 3 fractions. This peak represents the activity of recombinant  $\beta\beta$  enolase in the sample, which does not bind to the column. *E.coli* enolase, together with some other proteins in the sample, was eluted from the column with the elution buffer (with 1M KCl), corresponding to another peak activity around fraction 10. Peak fractions were then pooled and the activities of the pooled samples were assayed. Percentage of enolase activity that was shown not binding to the column ( $\beta\beta$  enolase), and percentage of activity that bound to the column at low salt concentration was calculated (data not shown).

SDS-PAGE electrophoresis was carried out with samples of the WT protein of crude cell extract, fractions #1, #2 and #3 (containing partially purified expressed  $\beta\beta$  enolase), fraction #10 (containing *E.coli* enolase and other proteins), and purified rabbit  $\beta\beta$  enolase from Boehringer Mannheim. It can be seen from the gel that the recombinant proteins comigrated with natural rabbit  $\beta\beta$  enolase (Figure 7).

Figure 7: SDS-PAGE electrophoresis of Q-Sepharose fractions of WT expression product (lane 1: molecular weight marker; lane 2: crude cell extract; lane 3: fraction #10; lane 4: fraction #3; lane 5: fraction #2; lane 6: fraction #1; lane 7: native  $\beta\beta$  enolase.)



MUT-BEN expression system gave similar results (data not shown).

Unfortunately, for some unknown reason,  $\beta\beta$  enolase (WT-BEN or MUT-BEN) expressed in the BL21(DE3)pLysS with IPTG induction did not give us reproducible yields.

	BEN			MUT-BEN		
Trial #	1	2	3	1	2	3
% $\beta\beta$ activity	59%	62%	86%	51.13%	23.3%	39%

The gene products of both the WT-BEN and MUT-BEN were confirmed to have enolase activity, and to show the same molecular mass on SDS-PAGE gel as commercial purified rabbit  $\beta\beta$  enolase. Therefore, the cloned gene is considered to code for rabbit  $\beta\beta$  enolase, and the proteins expressed in *E.coli* cells seemed to be functional, which means that both the wildtype and the mutant proteins are synthesized and folded properly in the prokaryote expression system.

Our results showed that a functional mutant enzyme was derived from this mutation, even though this mutation was designed to weaken the contacts between the subunits of the enzyme. Although the SDS-PAGE and the Q-sepharose chromatography results did not show any significant difference between the wild-type and mutant enzyme, apparently the mutant protein is expressed at a lower level than the wild-type protein. On average, mutant  $\beta\beta$  activity recovered from Q-sepharose column was lower

than wild-type  $\beta\beta$  from the same procedure. In addition, the mutant protein appears to be less stable than the WT protein, enolase activity of the cell extract sample of the mutant enzyme dropped 15% after storage at  $-20^{\circ}\text{C}$  for 24 hours, with the WT sample having 99% activity remaining.

#### **D.6 Purification of the wild-type and mutant proteins**

Satisfactory progress has been made in getting relatively pure enzyme by colleagues in the same group, using Q-Sepharose, DEAE and hydrophobic chromatographies. However, due to unknown reasons, the percentages of yields of wild-type or mutant proteins were not constant from different expression batch. In addition, the mutant protein in the cell extract appeared to be relatively unstable. Until the barrier of irreproducible yields is overcome, purification of the enzymes is not feasible.

## CONCLUSION

Data presented here show that a full-length cDNA encoding rabbit  $\beta\beta$  enolase has been cloned. The complete nucleotide sequence of this gene has been obtained by nested deletion and DNA sequencing, and the E417L mutant gene of rabbit  $\beta\beta$  enolase has been constructed. Both the wild-type and mutant gene have been successfully expressed in *E.coli*, using T7 RNA polymerase expression system. The recombinant enzyme products show enolase properties, indicating that both the WT and mutant protein are folded properly and appear to be functional.



## CHAPTER E APPENDIX

### E.1 Media

#### **E.1.1 LURIA-BERTANI (LB) Media**

Bacto-tryptone            10g

Bacto-yeast extract      5g

NaCl                        10g

Dissolve in 1 Litre of distilled water (dH<sub>2</sub>O), divide into 100 ml aliquots in milk dilution bottles, autoclave (20 minutes at 120°C, same below).

#### **E.1.2 LB Medium Plates**

NaCl                        10g

Yeast extract            5g

Bacto-tryptone          10g

Sigma agar                10g

dH<sub>2</sub>O                        1L

Autoclave and then pour into plates (10mm<sup>2</sup>) and allow to cool. Store at 4°C. Add antibiotics when necessary.

#### **E.1.3 LB Soft Top Agar**

NaCl                        5g

Yeast extract            5g

Bacto-tryptone          10g

Agarose                    7g

dH<sub>2</sub>O                        1L

Autoclave and add the following components afterwards:

Maltose: 10.0 ml of 20% stock solution (see Solutions).

MgSO<sub>4</sub>: 25.0ml of 10% stock solution (see Solutions).

#### **E.1.4 LB phage plates**

NaCl                    5g

Yeast Extract        5g

Bacto-tryptone      10g

Bacto-agar            7g

dH<sub>2</sub>O                    1L

After autoclaved, add maltose: 10.0 ml of 20% stock solution,  
and add MgSO<sub>4</sub>: 25.0 ml of 10% stock solution.

#### **E.1.5 2xYT Broth**

NaCl                    10g

Yeast extract        10g

Bacto-tryptone      16g

dH<sub>2</sub>O                    1L

Autoclave prior to use.

#### **E.1.6 IPTG/X-gal plate**

X-gal and IPTG must be added to cooled top agar just before pouring. For 3ml of top agar use: X-gal, 40 µl of a 20 mg/ml solution in dimethyl formamide (make fresh); IPTG, 4 µl of a 200 mg/ml aqueous solution (store at -20°C).

## **E.2 Solutions**

### **E.2.1 10M Sodium Hydroxide (NaOH)**

NaOH pellets      40g

dH<sub>2</sub>O              100ml

Store in plastic container at room temperature.

### **E.2.2 10% SDS (sodium dodecyl sulfate)**

Dissolve 10g SDS in 90ml dH<sub>2</sub>O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding concentrated HCl, followed by adding dH<sub>2</sub>O until the total volume is 100ml. Do not autoclave.

### **E.2.3 Solutions for alkaline lysis extraction of plasmid DNA**

#### **Solution I :**

50mM Glucose

25mM Tris-HCl, pH 8.0

10mM EDTA

Autoclave, store at room temperature.

#### **Solution II :**

0.2N NaOH

1% SDS

Freshly prepare from stock 10N NaOH and 10% SDS before use.

#### **Solution III :**

3M potassium acetate, pH 4.8

Adjust pH to 4.8 with glacial acetic acid, autoclave, store at room

temperature.

#### **E.2.4 CaCl<sub>2</sub> solution for *E.coli* competent cells preparation**

CaCl<sub>2</sub>            60mM

Glycerol            15%

PIPES    10mM, pH7.0

Autoclave.

#### **E.2.5 20% maltose stock solution**

Dissolve 20g of maltose in 100ml dH<sub>2</sub>O, autoclave.

#### **E.2.6 10% MgSO<sub>4</sub> stock solution**

Dissolve 10g MgSO<sub>4</sub> in 100ml dH<sub>2</sub>O, autoclave.

#### **E.2.7 SM buffer**

NaCl                    5.8g

MgSO<sub>4</sub>                2.0g

1M Tris-Cl at pH7.5    50ml

2% Gelatin            5ml

Autoclave before use.

#### **E.2.8 Enzyme activity assay buffer**

Imidazole            50mM

Magnesium acetate,    1mM

Potassium chloride    0.25M

EDTA                 0.1 mM

pH7.1

### **E.2.9 TE (Tris-EDTA) buffer**

10mM Tris-HCl, pH8.0

1mM EDTA

### **E.2.10 Solutions for restriction enzyme digestion**

(1) 10xBSA

(2) 10xenzyme buffer: varies with different enzymes.

Refer to the manual with each enzyme purchased.

### **E.2.11 5xTBE buffer**

Tris Base                    54g

Boric Acid                 27.5g

0.5M EDTA pH8.0 20ml

Add dH<sub>2</sub>O to total volume of 1L.

### **E.2.12 Loading buffers**

(1) agarose gel loading buffer (5x):

25% Glycerol

5% Ficoll

100mM EDTA

0.5% Bromophenol Blue

0.5% Xylene Cyanol

(2) SDS-PAGE loading buffer(5x):

0.25M Tris, pH6.8

40% Glycerol

0.05% Bromophenol Blue

5% SDS

5% (v/v)  $\beta$ -mercaptoethanol

**E.2.13 Ethidium bromide (EtBr) stock solution**

EtBr 10mg/ml in dH<sub>2</sub>O, store in dark.

**E.2.14 Phenol:Chloroform (1:1)**

Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1M Tris-Cl (pH7.6). Store the equilibrated mixture under an equal volume of 0.01 M Tris-Cl (pH7.6) at 4°C in dark glass bottles.

**E.2.15 20xSSC**

NaCl                    175.3g

Sodium citrate    88.2g

dH<sub>2</sub>O                    1L

Adjust the pH to 7.0 with a few drops of 10N NaOH; autoclave.

**E.2.16 Solutions used for transfer of DNA from top agar plates to nylon membranes in screening procedure**

(1) Denaturing solution:

1.5M NaCl,

0.5M NaOH.

(2) Neutralizing solution:

1.5M NaCl,

0.5M Tris-HCl, pH7.2,

0.001M EDTA

**(3) 2xSSC**

Dilute from 20xSSC stock.

**E.2.17 Solutions used in DIG system that are not provided with the kit**

**(1)SSC wash solution I (2xSSC, 0.1% SDS):**

20xSSC: 100ml

10%SDS: 10ml

Add dH<sub>2</sub>O to adjust total volume to 1L.

**(2) SSC wash solution II (0.1xSSC, 0.1%SDS):**

20xSSC: 5ml

10% SDS: 10ml

Add dH<sub>2</sub>O to adjust total volume to 1L.

**(3) Detection buffer 1 (maleate buffer):**

Maleic acid: 0.1M

NaCl: 0.15M

Adjust to pH7.5 with concentrated or solid NaOH, autoclave.

**(4) Detection buffer 2 (1xblocking reagent in maleate buffer):**

10% Blocking Solution (attached with the kit) is dissolved 1:10 to a final concentration of 1% blocking reagent in sterile buffer 1.

**(5) Detection buffer 3:**

Tris-HCl 0.1M, pH9.5

NaCl 0.1M

MgCl<sub>2</sub> 0.05M

**(6) Detection buffer 4:**

Tris-HCl 0.01M, pH8.0

EDTA 0.001M

**E.2.18 Ion-exchange buffer for Q-Sepharose column**

50 mM Tris

1mM Magnesium Acetate, pH7.2

0.1mM EDTA

**E.2.19 Elution buffer for Q-Sepharose column**

50mM Tris

1mM Magnesium Acetate, pH7.2

0.1mM EDTA

1M KCl

**E.2.20 Staining solution for SDS-PAGE gel (100 ml)**

Coomassie Brilliant Blue R250: 0.25g

Methanol: 45ml

dH<sub>2</sub>O: 45ml

Glacial acetic acid: 10ml

**E.2.21 Destaining Solution for SDS-PAGE gel**

30% methanol, 10% acetic acid in dH<sub>2</sub>O

**E.3 Antibiotics**



### **E.3.1 Ampicillin**

The stock solution is 10mg/ml of the sodium salt in water. Sterilize by filtration and store in aliquots at -20°C . The working concentration is 100 µg/ml.

### **E.3.2 Chloramphenicol**

The stock solution is 30 mg/ml in 100% ethanol. Store in aliquots at -20°C. The working concentration is 15 µg/ml.

### **E.3.3 Kanamycin**

The stock solution is 10 mg/ml in water. Sterilize by filtration and store in aliquots at -20°C. The working concentration is 50 µg/ml.

### **E.3.4 Tetracycline**

The stock solution is 30 mg/ml in 90% ethanol, store in aliquots at -20°C. The working concentration is 12.5 µg/ml.

## **E.4 Molecular Weight Markers**

### **E.4.1 MW Marker for DNA**

(1) Phi $\chi$ 174 RF DNA-Hae III Digest, 1,000 µg/ml, New England Biolabs, cat#302-6L.

(2) MW III, Boehinger Mannheim, cat#528552.

### **E.4.2 MW marker for protein**

Low Molecular Marker, Bio-Rad, cat#161-0305.

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