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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 91/057

ICGEB ref. #: CRP/GRE90-03

Project initiation: 07/05/1991

Project termination: 06/05/1994

International Centre for Genetic Engineering and Biotechnology
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TERMINAL EVALUATION REPORT

Part I

Title of Project

Structural and Functional Analysis of Human Glutamate Dehydrogenase

Keywords: Glutamate Dehydrogenase, expression, 3-D model, crystallization.

UNIDO contract # 91/057	**ICGEB ref. #:** CRP/ GRE90-03
Project initiation: 07-5-1991	**Project termination:** 06-5-1994

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Abstract:

Glutamate dehydrogenase (GLUD, E.C. 1.4.1.3) is a mitochondrial enzyme that catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate using NAD or NADP as co-factors. GLUD plays an important role in neuronal transmission mechanisms. Evidence implicating a direct role of the enzyme in the pathogenesis of certain human neurodegenerative disorders of the olivoponto-cerebellar atrophy (OPCA) type has been suggested by a number of studies showing decreased enzyme activity and abnormal glutamate metabolism in patients with neurodegeneration characterized by multisystem atrophy. The goal of the project was to obtain structural and functional data on GLUD protein molecule by using genetic engineering, 3-D modelling and protein crystallization approaches. In compliance with the proposal, we managed to express the human recombinant enzyme in heterologous eucaryotic expression systems in quantities sufficient for enzymological analysis of GLUD, we built up a 3-D model of the NAD(P) binding domain of the enzyme and managed to establish conditions which eventually will lead to the generation of GLUD protein crystals of a size appropriate for crystallographic studies. Our results is expected to be useful in understanding the role of the enzyme in neurotransmission and contribute to the elucidation of the molecular basis of certain neurodegenerative disorders.

Part 2

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

Objectives

1. Expression of human GLUD cDNA in heterologous cell systems. Establishment of an effective cell system would be a useful tool in structure-function analysis of GLUD at the molecular and enzymological level. Designing and construction of appropriate human GLUD cDNA recombinant molecules using various expression vectors. Introduction of the respective constracts into eucaryotic and/or bacterial expression systems. Evaluation of the recombinant products.

2. Studies on the theoretical 3-D structure of human GLUD. Designing of a theoretical model of the protein based on structural comparisons between homologous GLUD protein sequence segments. Information to be derived from various eucaryotic species. Design of mutants to evaluate the success of the design work.

3. GLUD protein crystallization. Testing of the enzyme crystallization conditions by using human GLUD protein preparations. Search for heavy atom derivatives. Collection of crystallographic data.

Methodology

1. *Expression of human GLUD cDNAs in heterologous systems*
Cloning of full-length GLUD cDNA in appropriate eucaryotic and/or procaryotic expression vectors. Transfection of eucaryotic (mammalian and insect) cell lines and/or bacteria. Expression of human GLUD cDNA to be tested by RNA blot hybridization analysis, immunoprecipitation and enzyme assays. Producers of high levels of GLUD mRNA and GLUD protein to be selected.

2. *Design of the GLUD 3-dimensional model*
Use of extensive homology comparisons in GLUD protein sequences through protein sequence databases. Use of additional information on the structural properties of other dehydrogenases. e.g. the existene of specific structural domains such as the Gly-X-Gly-X-X-Gly loop. Programmes in use: FRODO, HYDRA, INSIGHT, GROMOS, etc.

3. *Protein crystallography and physicochemical properties*
Crystallization of the native GLUD protein derived from either human liver or cerebellum (provided by the colaborative laboratory of Prof. A. Plaitakis (Mount Sinai School of Medicine) by using various crystallization conditions and protein solutions. The vapour diffusion method (hanging drops) to be used to test a variety of conditions with ammonium sulfate, polyethylene glycol or organic solvents as precipitants. Several macromolecular crystallization reagent kits to be used.

RESULTS
(compare against the set objectives)

1. Expression of GLUD in heterologous cell systems

Two eucaryotic cell expression systems, i.e. Chinese Hamster Ovary (CHO) and Drosophila Schneider 2 cells, were used for the production of recombinant human GLUD protein. In both cases, the expression levels of recombinant GLUD were found sufficient for *in vivo* assays of the activity of the recombinant enzyme and thus, useful in structure-function studies. However the amounts of GLUD enzyme produced were low for the isolation of pure recombinant protein and thus, inappropriate for crystallization experiments.

A human GLUD cDNA molecule representing the entire coding and part of the 3'UTR sequence (Z1), was used as the initial template in both cases. For the inducible expression in CHO cells, Z1, after appropriate engineering, was subcloned in pMSG expression vector featuring the dexamethasone inducible promoter contained within the MMTV 5' LTR. The selection for stable transfectants was performed by HAT, mycophenolic acid and xanthine. Human GLUD cDNA was expressed upon induction by dexamethasone in 10 out of 30 selected clones as shown by Northern analysis. The level of GLUD enzymatic activity compared to that of untransfected dexamethasone-treated cells was found increased by about 3 to 4 fold in four positive clones.

Aiming to better yields of biologically active GLUD, the *D. melanogaster* Schneider 2 cells in combination with the pMta 120 expression vector (a generous gift of Dr. M. Rosenberg, Smith Cline and French Labs) were used. Z1 DNA cloned in the pMta 120 vector under the D. melanogaster metallothionein promoter, was stably introduced into Schneider 2 cells after co-transfection with pco-hygro plasmid DNA. Selection of transfectants was performed by hygromycin B. The transfection and selection experiment was performed twice and positives were isolated. Human GLUD expression level was determined in 40 positive clones by Northern analysis and enzymatic assays. The respective copy number of GLUD cDNA inserts was estimated by Southern blots. Possibly due to low copy number integration, human GLUD expression at the RNA and enzyme activity level, was lower than expected from the literature but still 1-2 times higher to that achieved by CHO cells.

Both types of experiments indicated that the recombinant human GLUD polypeptide, probably in the form of a homoexamer, is capable of confering GLUD enzymatic activity in heterologous eucaryotic cell expression systems. The level of over-expression was considered sufficient for *in vivo* structure-function analysis of potential GLUD structural mutants. The Schneider 2 cell system was found to be more useful due to its convenience in terms of growth rates, transfection efficiency and growth medium requirements.

2. Modelling the NAD(P) binding domain of human GLUD

Our attempt to built up a 3-dimensional model for human GLUD was based on the analysis of sequence patterns present on molecules of known 3-D structures and on patterns of physicochemical properties spread along its sequence. A 3-D molecular model of the NAD(P)-binding domain of the protein has been designed based on structural and sequence information derived from malate (MDH), lactate (LDH, glyceraldehyde-3-phosphate (GPD) and alcohol (ADH) dehydrogenase molecules of lower and higher eucaryotes of known sequence and structure. Because of the low percentage of amino acid sequence identity between the human GLUD and the above referred molecules, the building of the model was based on the generation of a template from the 3-D structures of dehydrogenases and a consensus aligment of the four different dehydrogenase protein families. This approach determined a 3-D structure with significant similarities to known dinucleotide binding domains and sufficiently revealed the spatial interaction between the protein and the NAD(P) dinucleotide (Fig. 1).

The structure of the NAD(P) domain is a parallel β-sheet constructed of 5-7 strands and covered by α-helices on both sides and is known as the "Rossman" fold.

Procedures for building a model structure by using an already known similar structure as the template, are indeed available but they require a good homology between the template sequence and the one to be modelled. Since there is no detectable homology between the families mentioned above nor between any of them and GLUD, these procedures were not applicable. Thus, we decided to use sequence patterns which have been identified in the past on the dinucleotide binding domains of several proteins, mainly of the oxidation-reduction group.

A central point of our approach was the idea that comparisons of primary protein structures would be made among protein families rather than individual protein sequences. In this context, a FASTA search was effected on the SwissProt databank for 4MDH, 3LDH and 1GPD. Sequences that could be unambigously alinged to the above queries, were selected and aligned. The alignments were converted to the form of position-specific frequency matrices. These matrices have the dimensions length of alignment X 20, so that for each position along the alignment 20 different values are present corresponding to each of the 20 amino acids. To align these profiles to each other, we used the Needlman & Wunsch algorithm with a minor modification. Different approaches were taken for the different parts of the GLUD molecule. Unfortunately we did not have any information about the catalytic domain of human GLUD, not even by homology to the *Clostridium symbiosum* GLUD sequence where an experimentally determined 3-D structure exists. At this region the two sequences have a prohibitive difference in length, about 50 residues, rendering any speculation unreliable. For the GLUD dinucleotide binding domain, we made the assumption that it adopted the tertiary fold of the dinucleotide binding domain of the clostridial GLUD. Given the unavailability of the coordinate set, we aligned the structures of the NAD binding domain from MDH, LDH, GPD and ADH. The averaged structure of their NAD binding domains served as the scaffold on which we built our model. The absence of the alignment between the GLUD sequence and the scaffold was overcome as follows: To align the first half of the scaffold to the human GLUD sequence, a series of polypeptide 27-mers specific for NAD binding were aligned and the profile of their alignment was extracted. These 27-mers include the sequence pattern GlyXGlyXXGly which is known to form the loop of the NAD and other dinucleotide binding β-loop-α motifs. We verified the existence of this motif in MDH and GPD which were not included in the sample of Sheridan and Venkataraghavan; our procedure correctly located the pattern on these molecules. Then we switched to GLUD; the location of the best hit based on the alignment between the human and the clostridial GLUD at this point is GfGnvA a sequence quite similar to the expected GxGxxG. This Gly to Ala amino acid change is also present in other members of the protein family.

The NAD(P) binding domain of the enzyme corresponds to 60 amino acids encoded by part of GLUD exon 6, 7, 8 and part of exon 9. Due to the high degree of sequence conservation, the proposed 3-D model stands also, for all known mammalian and avian GLUD molecules. The validity of the model was tested by a series of mutations that might influence the function of the domain. Our results strongly suggested that certain amino acid residues, e.g. Gly308, Gly310, Val306, Val312, etc., are indispensable for the functional interaction between the enzyme and the dinucleotide co-factor

3. GLUD protein crystallization

Throughout the entire period of the project, we tested, without success, several conditions for human liver GLUD protein crystallization. This was probably because of the instability of the enzyme preparations. Since only limited amounts of highly purified human GLUD protein were available from Prof. A. Plaitakis (Mount Sinai, Dept. of Neurology, Mount Sinai Medical Centre), we decided to continue our efforts using as starting material commercially available purified bovine liver GLUD protein which is highly homologous to the human enzyme as has been shown by protein sequence comparison. We tested various types of bovine GLUD enzyme including preparations stored in $(NH_4)_2SO_4$ and citrate buffer suspension and in glycerol and potassium phosphate solution. Two macromolecular crystallization reagent kits, i.e. Crystal Screen and Gen Screen, were used. The numerous preliminary conditions tested are presented in Table 1A and 1B.

To determine preliminary crystallization conditions, we used the "hanging drop" procedure with diafiltrated GLUD protein preparations dissolved in 10mM MES pH 6.0, 2mM EDTA and 2mM DTT in a protein concentration of less than 20mgr/ml. The A3 condition (10% PEG 6000, 0.1M MES pH 6.0) offered the most encouraging preliminary results. Rod-shaped small GLUD crystalls were obtained in a period of less than two weeks. For further optimization of the crystallization conditions, we used 1-12% PEG 2000, PEG 6000 and PEG 8000, in 0.02-1.2M MES, 0.02-0.08M $(NH_4)_2SO_4$ and 0.2-0.8M citrate in pH ranging between 5.0-6.6. Several of these combinations resulted in GLUD crystallization. However, the size of the crystalls remained small (Fig. 2).

To increase the size and the quality of crystals, we used a "seeding" procedure. Despite our repeated efforts, until now, we have failed to obtain significantly larger crystalls appropriate for crystallographic analysis. This could be due to the tendency of mammalian GLUD to create aggregates/polymers in concentrations higher than 3 mgr/ml (Birktoft et al., J. Biol. Chem., 254:4915, 1979). We believe that further optimization of the "seeding" conditions, e.g. by adding our small crystalls to dilute protein solutions(<3 mg/ml), will eventually lead to enlarged GLUD crystalls of improved quality.

TABLE 1

A. Crystal Screen conditions tested

	Salt	Buffer	Precipitant
1.	0.02 M Ca Chloride	0.1M Na Acetate pH=4.6	30%v/ v 2-methyl-2,4 pentanediol
2.	-----	-----	0.4M K, Na Tartrate
3.	-----	-----	0.4 M NH4 Phosphate
4.	-----	-----	2.0 M NH4 Sulfate
5.	0.2M Na Citrate	0.1 M Na Hepes pH=7.5	30%v/ v 2-methyl-2,4 pentanediol
6.	0.2M Mg Chloride	0.1 M Tris HCl pH=8.5	30%w/ v PEG 4000
7.	-----	0.1 M Na Cacodylate pH=6.5	1.4 M Na Acetate
8.	0.2M Na Citrate	0.1 M Na Cacodylate pH=6.5	30% v/ v 2- propanol
9.	0.2M NH4 Acetate	0.1 M Na Citrate pH=5.6	30%w/ v PEG 4000
10.	0.2M NH4 Acetate	0.1M Na Acetate pH=4.6	30%w/ v PEG 4000
11.	------	0.1 M Na Citrate pH=5.6	1.0 M NH4 Phosphate
12.	0.2M Mg Chloride		0.1 M Na Hepes pH=7.5
	30% v/ v 2- propanol		
13.	0.2M Na Citrate	0.1 M Tris HCl pH=8.5	30%v/ v PEG 400
14.	0.2M Ca Chloride	0.1 M Na Hepes pH=7.5	30%v/ v PEG 400
15.	0.2M NH4 Sulfate	0.1 M Na Cacodylate pH=6.5	30%w/ v PEG 8000
16.	-----	0.1 M Na Hepes pH=7.5	1.5 M Li Sulfate
17.	0.2 M Li Sulfate	0.1 M Tris HCl pH=8.5	30% PEG 4000
18.	0.2M Mg Acetate	0.1 M Na Cacodylate pH=6.5	30%w/ v PEG 8000
19.	0.2M NH4 Acetate	0.1 M Tris HCl pH=8.5	30% v/ v 2- propanol
20.	0.2M NH4 Sulfate	0.1M Na Acetate pH=4.6	25% w/ v PEG 4000
21.	0.2M Mg Acetate	0.1 M Na Cacodylate pH=6.5	30%v/ v 2-methyl-2,4 pentanediol
22.	0.2M Na Acetate	0.1 M Tris HCl pH=8.5	30% w/ v PEG 4000
23.	0.2M Mg Chloride	0.1 M Na Hepes pH=7.5	30%v/ v PEG 400
24.	0.2M Ca Chloride	0.1M Na Acetate pH=4.6	20% v/ v 2- propanol
25.	-----	0.1M Imidazole pH=6.5	1.0M Na Acetate
26.	0.2M NH4 Acetate	0.1M Na Citrate pH=5.6	30%v/ v 2-methyl-2,4 pentanediol
27.	0.2M Na Citrate	0.1 M Na Hepes pH=7.5	20% v/ v 2- propanol
28.	0.2M Na Acetate	0.1 M Na Cacodylate pH=6.5	30% w/ v PEG 4000
29.	-----	0.1 M Na Hepes pH=7.5	0.4M K, Na Tartrate
30.	0.2M NH4 Sulfate	-----	30% w/ v PEG 8000
31.	0.2M NH4 Sulfate	-----	30% w/ v PEG 4000
32.	-----	-----	0.2M NH4 Sulfate
33.	-----	-----	4.0M Na Formate
34.	-----	0.1M Na Acetate pH=4.6	2.0M Na Formate

35.	-----	0.1 M Na Hepes pH=7.5	1.6 M Na, K Phosphate
36.	-----	0.1 M Tris HCl pH=8.5	8% w/ v PEG 8000
37.	-----	0.1 M Na Acetate pH=4.6	8% w/ v PEG 8000
38.	-----	0.1 M Na Hepes pH=7.5	1.4 M Na Citrate
39.	-----	0.1 M Na Hepes pH=7.5	2% v/ v PEG 400 & 2.0M (NH4)2SO4
40.	-----	0.1 M Na Citrate pH= 5.6	20% v/ v 2- propanol & 20% w/ v PEG 4000
41.	-----	0.1 M Na Hepes pH=7.5	10% v/ v 2- propanol & 20% w/ v PEG 4000
42.	0.05 M K Phosphate	-----	20% w/ v PEG 8000
43.	-----	-----	30% w/ v PEG 1450
44.	-----	-----	0.2 M Mg Formate
45.	0.2M Zn Acetate	0.1 M Na Cacodylate pH=6.5	18% w/ v PEG 8000
46.	0.2M Ca Acetate	0.1 M Na Cacodylate pH=6.5	18% w/ v PEG 8000
47.	-----	0.1 M Na Acetate pH=4.6	2.0M NH4 Sulfate
48.	-----	0.1 M Tris HCl pH=8.5	2.0 M NH4 Phposphate
49.	1.0 M Li Sulfate	-----	2% w/ v PEG 8000
50.	0.5 M Li Sulfate	-----	15% w/ v PEG 8000

B. GEN SCREEN conditions tested

A1.	5%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=4.0
A2.	5%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=5.0
A3.	5%	w/ v	PEG 6000	0.1 M MES	PH=6.0
A4.	5%	w/ v	PEG 6000	0.1 M HEPES	PH=7.0
A5.	5%	w/ v	PEG 6000	0.1 M TRIS	PH=8.0
A6.	5%	w/ v	PEG 6000	0.1 M BICINE	PH=9.0
B1.	10%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=4.0
B2.	10%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=5.0
B3.	10%	w/ v	PEG 6000	0.1M MES	PH=6.0
B4.	10%	w/ v	PEG 6000	0.1 M HEPES	PH=7.0
B5.	10%	w/ v	PEG 6000	0.1M TRIS	PH=8.0
B6.	10%	w/ v	PEG 6000	0.1 M BICINE	PH= 9.0
C1.	20%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=4.0
C2.	20%	w/ v	PEG 6000	0.1 M CITRIC ACID	PH=5.0
C3.	20%	w/ v	PEG 6000	0.1 M MES	PH=6.0
C4.	20%	w/ v	PEG 6000	0.1 M HEPES	PH=7.0
C5.	20%	w/ v	PEG 6000	0.1 M TRIS	PH=8.0

C6.	20%	w/v	PEG 6000	O.1M BICINE	PH=9.0
D1.	30%	w/v	PEG 6000	0.1 M CITRIC ACID	PH=4.0
D2.	30%	w/v	PEG 6000	0.1 M CITRIC ACID	PH=5.0
D3.	30%	w/v	PEG 6000	0.1 M MES	PH=6.0
D4.	30%	w/v	PEG 6000	0.1 M HEPES	PH=7.0
D5.	30%	w/v	PEG 6000	0.1 M TRIS	PH=8.0
D6.	30%	w/v	PEG 6000	O.1 M BICINE	PH=9.0

Fig. 1 : The GLUD protein modell at the NADP binding domain. It consists of four β-clones and 2 α-helices in the form of two symmetrical regions (β1-αA-β2 & β1'-αB-β2').

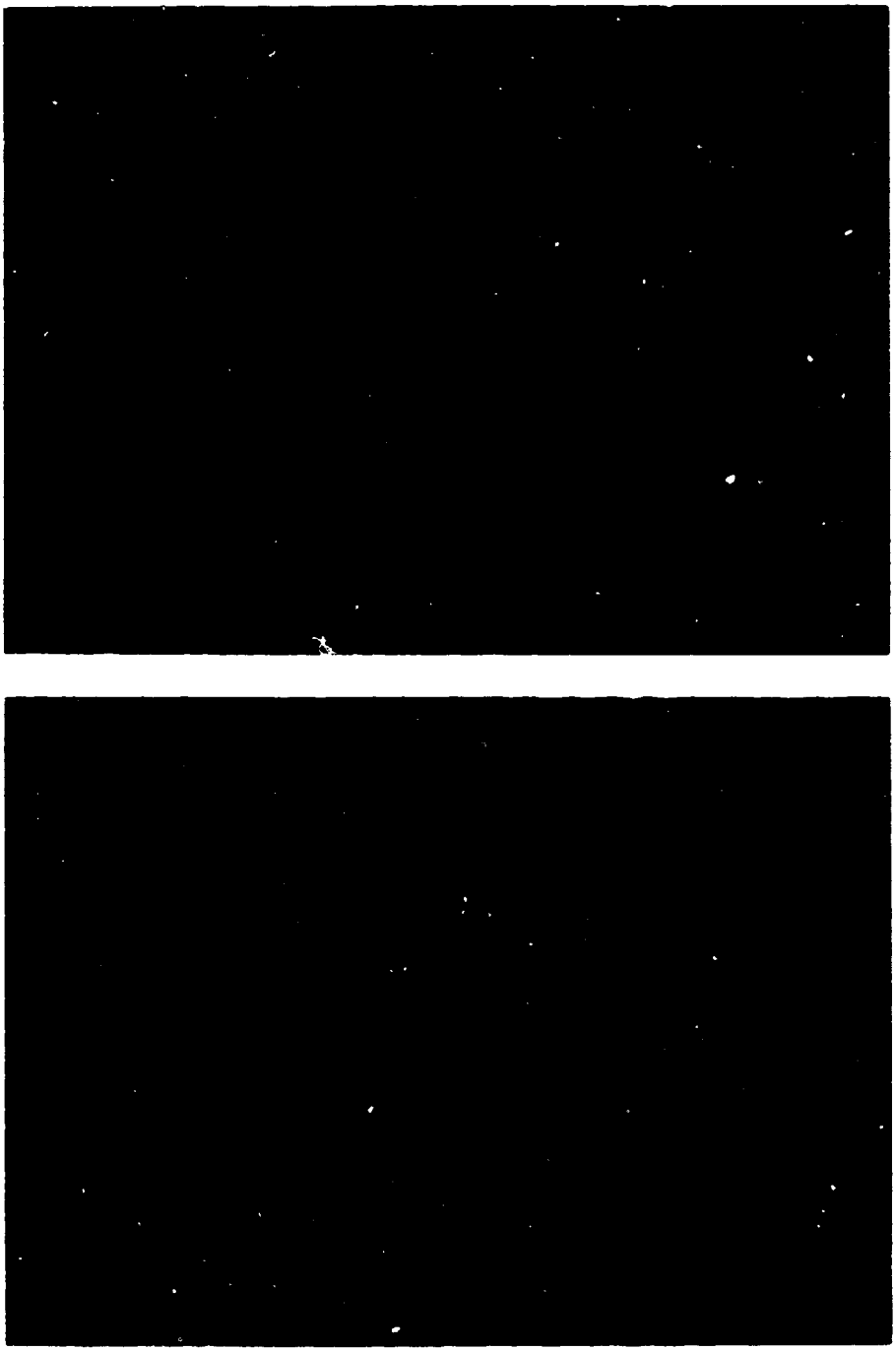


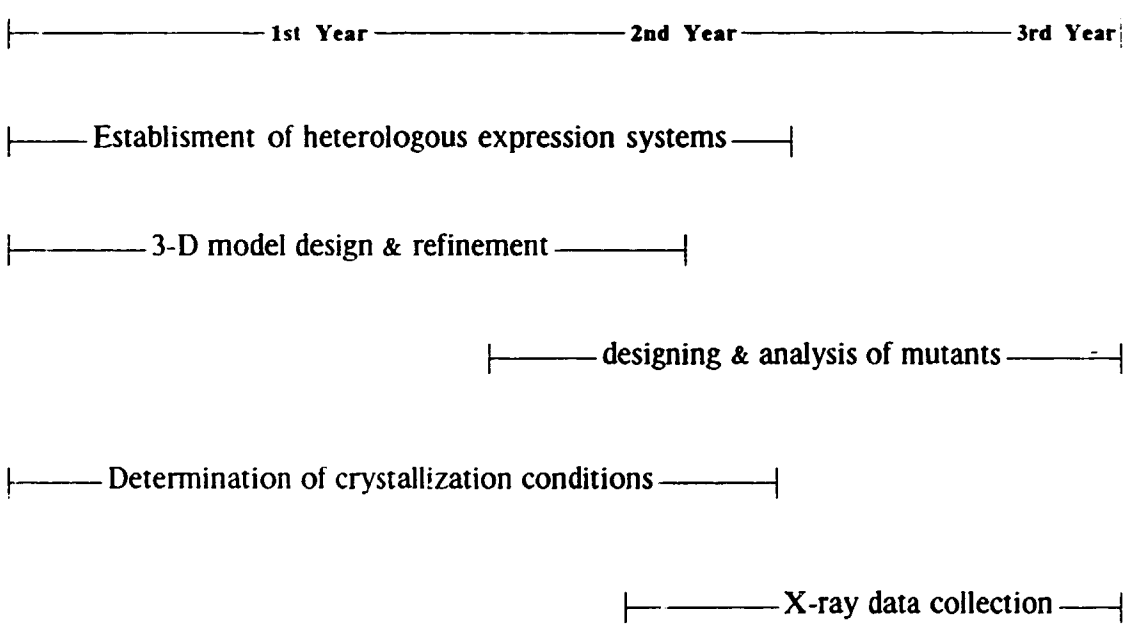
Fig. 2 : Bovine GLUD crystalls obtained in a 12% PEG 6000, 0.06M MES pH 5.8 solution.

Work plan and time schedule
(originally envisaged)

1. Establishment of heterologous expression systems for the recombinant human GLUD protein was planned for the early stages of the project (Years: 1 and 2).

2. Designing of the 3-D model of the protein was planned to be carried out in parallel with recombinant DNA work (Years 1 and 2). Subsequently, hypothetical mutant GLUD sequences would be designed and analysed (Year 3).

3. Determination of crystallization conditions and crystallographic work (X-ray data collection) and solution of the structures were planned to continue throughout the project .



Work plan and time schedule (actual)

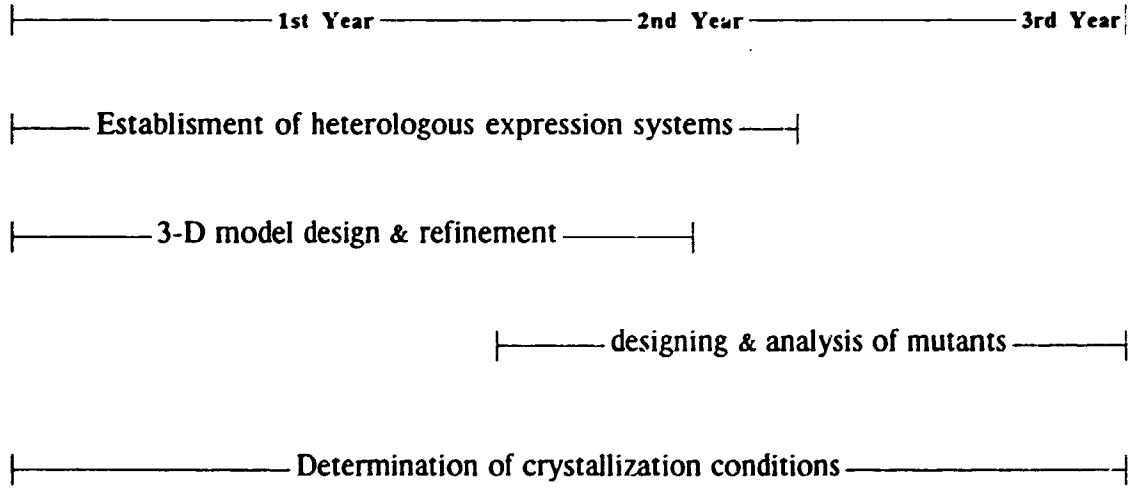
Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

The actual work plan and time schedule was followed, essentially, as originally envisaged.

1. The establishment of two heterologous eucaryotic expression systems (CHO and Schneider 2 cells) was completed within two and a half years. Our work on the expression patterns of recombinant human GLUD will be combined with results from the collaborating laboratory of Prof. A. Plaitakis on the protein product of human GLUD2 gene (a X-linked intronless GLUD gene expressed in particular tissues). A manuscript is in preparation.

2. Due to the limited available structural information, designing of the 3-D model was focused on the dinucleotide (NADP) binding domain of the protein. Several mutants were designed and the model was tested. This work is ready to be submitted.

3. Crystallization conditions for GLUD have been established and small-size crystalls have been obtained. However, despite our continuous efforts, good quality crystalls suitable for X-ray studies are not available, yet.



NETWORKING

Three laboratories collaborated for this project:

Laboratory of Human Molecular Genetics, IMBB-FORTH and Dept. of Biology, U. of Crete, (head: N.K. Moschonas, Assoc. Professor in Molec. Biology)

Laboratory of Protein Structure and Crystallography, IMBB-FORTH and Dept. of Biology, U. of Crete, (head: M. Kokkinidis, Assoc. Professor in Biophysics)

Dept. of Neurology, Mount Sinai Medical Centre, Mount Sinai School of Medicine. (A. Plaitakis, Professor in Neurology)

Information on structure data were exchanged with:

Dr. E. Baker, Sheffield University, Dept. of Molec. Biology, Krebs Institute for Biomolecular Research, UK S10 2TN, Sheffield

PUBLICATIONS

1. Tzimagiorgis G, Chroniary K, Babaratsas M, Leversha M, Ferguson-Smith M & Moschonas NM (1991). Mammalian Glutamate Dehydrogenase gene family: Comparative structural analysis and chromosomal organisation. In "Evolution and Development: Thirty years after the Jacob-Monod paradigm" Conference, Crete, 1991 (poster presentation)

2. Manifava M, Paliakasis K, Kokkinidis M, and Moschonas NK. Molecular modelling of the dinucleotide binding domain of the human glutamate dehydrogenase (in preparation)

3. Tzimagiorgis G, Moschonas NK, Plaitakis A, et al.. Expression patterns of human glutamate dehydrogenase recombinant molecules (in preparation)

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures***	
1) Capital equipment	US$	1) Capital equipment	US$ 13,000
2) consumables	US$	2) consumables	US$ 17,000
3) training	US$	3) training	US$ .....
4) literature	US$	4) literature	US$ .....
5) miscellaneous	US$	5) miscellaneous	US$ .....
TOTAL GRANT	**US$**	**TOTAL**	**US$** 30,000

Please itemize the following budget categories (if applicable)

Capital equipment

PCR-DNA Thermal cycler	US$	12,600.00
Tube rotator	US$	354.00
Thermoblock (2ml tubes)	US$	46.00

Training (provide names, duration of training, host laboratory)

Three graduate students of the Dept. of Biology, U. of Crete, i.e. G. Tzimagiorgis, M. Manifava and C. Paliakasis participated in the above project. These students used the IMBB facilities for their training in various Molecular Biology, Crystallography and Biocomputing methods and procedures. Namely:

G. Tzimagiorgis participated in the initial steps of the project (1991-1992). He worked with the construction of various recombinant molecules containing the GLUD cDNA, the transfer of the recombinants in heterologous expression systems and the evaluation of the expression products (in collaboration with Prof. A. Plaitakis' group). He also studied the homology comparisons in GLUD protein sequences through protein sequence databases. Part of this work has been incorporated in his Ph.D. thesis (Dept. of Biology, U. of Crete, 1992).

M. Manifava in collaboration with C. Paliakasis (a graduate student in Dr. Kokkinidis' laboratory) worked with the construction of the 3-D model of human GLUD (1992-1994). An extensive part of her Master's Thesis (Dept. of Biology, U. of Crete, 1994) is dedicated to this work. In collaboration with J. Papanicolaou (a graduate student in Dr. Kokkinidis' laboratory), Ms. Manifava also worked extensively to establish appropriate conditions for the crystallization of GLUD molecule.
Finally, two technicians, i.e. K. Chroniary asnd A. Babaratsas, were involved in the project, on a part-time basis (May 1991- January 1994).

Literature

*Please do not send invoices, receipts, etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.