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PRINCIPAL INVESTIGATOR: Lawrence J. DeLucas, O.D., Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

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Lawrence J. DeLucas, O.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

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The aim of this project is to identify specific inhibitors of some enzymes of malaria parasite. Inhibitors will be designed based on the high-resolution crystal structure of individual target enzymes. We have already determined the crystal structure of P. falciparum lactate dehydrogenase, a key enzyme in the glycolytic pathway, to high resolution. This structure is currently being used for designing lead inhibitors. We have also purified PPPK-DHPS bifunctional enzyme for structure analysis and are presently screening for crystallization conditions. We have also optimized the purification of P. falciparum cyclophilin. We have also crystallized P. falciparum Rab6 for structure determination. This is the first Rab protein of the malaria parasite to be crystallized.

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Larry R. Lucas 7/15/97

PI - Signature Date

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1. Introduction:

The tropical mosquito-borne disease, malaria, is caused by the *Plasmodium* species. Nearly three million people are infected by these parasites and approximately 1.5 million die each year (1,2). Infection with *P. falciparum,* the most lethal of the four *Plasmodium species* infecting humans, remains a major infectious cause of mortality and morbidity worldwide. Majority of both clinical cases and deaths from malaria are due to infections with *P. falciparum*. *P. falciparum* undergoes three cycles of development in its vertebrate host and elicits stage-specific human responses. This stage specificity of the immune response has made it difficult to isolate antigens that would be useful in developing a vaccine against malaria. Therefore, malaria is currently treated by chemotherapy. Recent increases in the frequency of chloroquine and pyrimethamine-resistant malarial strains (3) have emphasized the urgent need for development of new chemotherapeutic agents. This investigation involves a rational approach to the development of chemotherapeutic agents using structure-based pharmaceutical design. Using this technique, it will be possible to design and identify compounds that act as active site inhibitors of key metabolic enzymes of the parasite. Targeting the active sites reduces the likelihood of eventual parasite resistance due to genetic mutation.

Crystallographic (structure-based) drug design provides unique information that can be exploited to design more effective pharmaceuticals, attacking sites active in the parasitic enzymes. These areas in the protein targets are typically less likely to mutate, thereby making drug resistance more difficult. The use of a crystallographic approach requires that

the protein targets are first purified in large quantities and subsequently crystallized. It is this critical step, the production of high quality crystals for structure analysis, that often slows the overall progress in the structure-aided drug design process. For this reason, our laboratory chose to attempt crystallization of several different critical malarial enzymes so that we would maximize the chance of producing crystals for one or more targets in a relatively short period of time. Our strategy has been successful in that we have now determined the high-resolution structure of lactate dehydrogenase, one of the original protein targets. We are currently using the active site information to design a variety of novel inhibitors. We have also recently produced diffraction quality crystals of Rab6 and we expect to determine its structure this year.

Other targets, PPPK-DHPS and DHFR, have proved more difficult to crystallize although we plan to continue our efforts on PPPK-DHPS while we move forward with the inhibitor design for lactate dehydrogenase.

Generally, with most proteins, crystals can be obtained that diffract to high-resolution. Those that are more difficult require the dedicated efforts of personnel (biochemists and crystal growers) to investigate thousands of crystallization conditions using as many different modified protein forms as is possible. The modified forms may include complexes with substrates and/or cofactors, site-directed mutants that do not alter biological function, complexes with receptors, inhibitors or monoclonal antibodies. Slight changes in the protein can often provide new possibilities for molecular contacts that are conducive to crystal formation. We intend to pursue a variety of approaches to optimize the chances of ultimately obtaining useful crystals for DHPS and cyclophilin.

In this project we initially focused on certain malarial enzymes because of their availability and critical role in metabolic pathways. Targeted enzymes included dihydroorotic acid dehydrogenase (DHOD) of the pyrimidine biosynthetic pathway, the dihydropteroate synthetase (DHPS), dihydrofolate reductase (DHFR), and lactate dehydrogenase (LDH). These enzymes and their metabolic pathways are briefly discussed here.

The sulfone/sulfonamides are a group of antimalarial compounds that act either to inhibit the DHPS or converted by this enzyme to a toxic sulfa analog that inhibits the folate biosynthetic enzyme(s). The metabolism of sulfadoxine by DHPS in *P. falciparum* is decreased in sulfadoxine-resistant parasites suggesting that mutations in this enzyme are involved in the mechanism of resistance to this group of antimalarials (4). The amino acid sequence of wild type DHPS differs from that of sulfonamide resistant (5) *P.falciparum* . The DHPS gene is linked at its N-terminus with the gene for the enzyme dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK). These two enzymes are contiguous enzymes in the folate synthetic pathway, PPPK then DHPS, from the point at which *para*-amino benzoic acid(PABA)is integrated. The PPPK and DHPS enzyme activities in all prokaryotes studied thus far exist as separate enzymes, whereas in *Pneumocystis carinii*, these enzymes exist as part of a trifunctional protein which also includes dihydroneopterin aldolase.

Unlike their hosts, *Plasmodium sp.* is reliant on the *de novo* biosynthetic pathway for its pyrimidine precursors (6). The activities of all six enzymes involved in the synthesis of UMP have been identified in *P. falciparum* (7,8). The first three enzymes of the pathway, carbamyl phosphate synthase II (CPS II), aspartate transcarbamylase (ATCase), and dihydroorotase (DHOase) appear to exist as discrete proteins whereas, in mammalian

systems these activities are found to reside in a single multifunctional protein. Dihydroorotic acid dehydrogenase the fourth enzyme in the pyrimidine biosynthetic pathway, catalyzes the conversion of dihydroorotate to orotate and is presumably linked to the electron transport system in the malarial mitochondrion.

In *P. falciparum*, folate coenzymes are critical for thymidylate biosynthesis and presumably methionine synthesis (9). *P. falciparum* is capable of both *de novo* and salvage biosynthesis of its folate pathway intermediates. This is in contrast to the human host which relies on the uptake of intact folate for its coenzyme factors. Dihydrofolate reductase (DHFR), the best characterized of the folate biosynthetic enzymes, is found as a bifunctional enzyme with thymidylate synthase (TS). Resistance of *P. falciparum* to the DHFR inhibitors, pyrimethamine and cycloquanil has been linked to point mutations in the DHFR-TS locus (10). These mutations are associated with a decreased binding affinity of the respective inhibitors for the DHFR enzyme.

Lactate dehydrogenase (LDH) plays an important role in regulating glycolysis and in balancing the reduced/oxidized state of the malaria parasite. The enzyme is essential for the anaerobic life of the *Plasmodium*. Lactate dehydrogenase inhibitors lethal to the parasite.

The basic steps involved in our approach include genetic overexpression of each target enzyme followed by extensive purification to produce homogeneous protein. The protein is then used to screen thousands of crystallization conditions to find the appropriate pH, ionic strength, buffer type, concentration of protein, precipitant concentration, temperature and crystallization technique that produces crystals suitable for high resolution X-ray structural analysis. The structure is used to identify the active site which is subsequently used to design

organic compounds that will specifically interact with the active site amino acids thereby inhibiting the protein.

Falcipain

Amino acid requirements for protein synthesis in the erythrocytic parasite are fulfilled by degradation of erythrocyte hemoglobin in acidic food vacuole. The cystein protease falcipain has been shown to be necessary for hemoglobin degradation and is therefore, a potential target for antimalarial drug design. Falcipain belongs to papain family of cysteine proteinase. The predicted molecular mass for the mature falcipain is 26.8 kDa.

We have also extended our investigation to some new areas to improve our understanding of the biology of the parasite.

Malarial cyclophilin (peptidyl-prolyl-*cis-trans* isomerase):

Cyclosporin A(CsA), an immunosuppressive drug binds to cyclophillins from several species (20). Cyclophilin catalyzes the cis-trans isomerization of an Xaa-proline peptide bonds and accelerates the rate limiting steps in the refolding of several proteins *in vivo* (21,22,23). Since CsA is known to suppress the humoral and cell-mediated immune response in many mammalians, it is expected that this drug would enhance the susceptibility to infections by *P. falciparum*. In contrast, CsA acts as a potent inhibitor for the growth of rodent malarial parasites (24,25,26). In addition, several non-immunosuppressive analogues and weak immunosuppressive analogues of CsA have significant antimalarial activity against *P.falciparum* (24, 27, 28). Bell et al. (29) observed that CsA inhibits the PPIase(cyclophilin) activity in *P. falciparum*. Recently, Reddy (30) isolated a gene coding *P. falciparum* cyclophilin. In collaboration with Dr. Reddy, we have also expressed the recombinant protein and the activity of this recombinant protein is inhibited by CsA.

Rab6

Malaria parasite spends much of its life cycle inside erythrocytes. Within the erythrocyte the parasite is surrounded by its own plasma membrane, parasitophorous vacuole membrane, and the cytoplasm and plasma membrane of the erythrocyte. Mechanisms by which proteins are trafficed within and beyond the plasma membrane is not clear. Several components of the standard eukaryotic trafficing machinery are known to be present. On the other hand, the trafficking machinery of *Plasmodium* possesses distinctive features as well. Rab proteins are small GTP binding proteins. The cytoplasmic surface of each compartment along the secretory pathway appears to have its own unique Rab proteins. The Rabs alternate between GTP-bound and GDP-bound form. They also alternate between cytosolic and membrane bound forms. They appear to act as timers that regulate the kinetics of transport vesicle docking and fusion with target membranes. Cycling of Rab proteins is regulated, at least in part, by a GDP dissociation inhibitor (GDI) and a GDP/GTP exchange protein (GDS).

2. Experimental Methods, Assumptions, Procedures, Results and Discussion

2.1 PPPK-DHPS:

Expression of PPPK-DHPS, a bifunctional enzyme:

A cDNA clone coding PPPK-DHPS of *P. falciparum* a bifunctional enzyme was obtained from Dr. Alan F. Cowman (11), The Water and Eliza Hall Institute of Medical Research, Melbourne, Australia. The PPPk-DHPS was subcloned into pRSET vector. BL21(DE3) and BL21(DE3)pLysS *E. coli* strains were transformed with the recombinant pRSET-PPPK-DHPS.

The overnight cultures from a single colony of *E. coli* BL21(DE3) or BL21(DE3)pLysS containing pRSET-PPPK-DHPS were used to inoculate SOB medium containing ampicillin (50µg/ml) and grown at 37°C. The culture was induced with IPTG (1mM final concentration) after the $O.D_{550}$ of cultured reached 0.5 and incubated for 4 hours at 30°C. The culture is centrifuged at 5,000 rpm for 10 min at 4° C.

Purification of PPPK-DHPS:

The harvested cells are washed with PBS and resuspended in SP/GP lysis buffer containing 50 mM sodium phosphate pH7.8, 500 mM NaCl, 20mM imidazole, 10mM B-mercaptoethanol, 10% glycerol, 0.5mMPMSF, 0.1mg/ml lysozyme, 0.1mg/ml leupeptin, 0.1mg/ml pepstatinA, 0.1mg DNase, and 0.1mg/ml RNase and incubated on ice for 30 min. The lysate was subjected to three cycles of sonication, flash freezing in dry ice/methanol bath and flash thawing at 37° C. The lysate was centrifuged at 12000g for 15 min and the supernatant is used for further purification. All steps were performed at 4°C. Total protein was estimated by Bradford method.

Affinity purification of PPPK-DHPS:

We utilized the metal chelate affinity chromatography to purify the His-tagged PPPK-DHPS using Ni-resins. Both Ni-agarose and Ni-silica matrices were initially used to optimize the purification conditions. Ni-NTA Super flow (Qiagen) provided the most efficient and rapid affinity system for reproducibly producing purified protein (Fig 2). Moreover, Ni-NTA agarose exhibited a binding capacity of 5-10 mg 6xHis-tagged protein per ml resin volume. Step or linear gradients of imidazole (0-500 mM) prepared in SP/GP buffer (with protease inhibitors) were used for elution of Ni-binding proteins. In pilot experiments

of cell lysates (20 ml/liter culture) were applied to Ni-NTA agarose columns (2 ml) previously equilibrated with SP/GP buffer (pH 7.8). Similarly, for the large-scale preparations (5-15 liter cultures), 10-20 ml packed volume of Ni-NTA columns were used. Before loading, samples were mixed with 10 mM b-mercaptoethanol (BME) and 20 mM of imidazole. This essential step allowed the maximum binding of recombinant protein and also prevented non-specific binding during chromatography.

In this pilot study, we utilized chelating sepharose (Pharmacia) to purify PPPK-DHPS from cell lysate. The recombinant protein migrated as a 86 kDa protein (arrow) and was abundant in eluent fractions. However, due to the absence of peptide inhibitors (leupeptin/ pepstatin A) in running buffers, significant loss or degradation of PPPK-DHPS protein was noted. This was further confirmed by Western blot analysis. We have overcome this problem by including a mixture of protease inhibitors in all sample/column buffers.

Cleared cell lysates were loaded slowly onto the affinity column using a peristaltic pump (0.3 ml/min). Ni-NTA column was washed extensively first with SP/GP buffer (pH 7.8) and then with SP/GP buffer (pH 6.3), both containing 50 mM imidazole (flow rate 1ml/min). Moreover, the completeness of washings was ensured by monitoring the absorbance at 280 nm of the flow-through fractions which should be less than 0.01 and it required 10-15 column volumes of wash buffers. Finally, the Ni-bound proteins were eluted from the column with a 30 or 60 ml gradient of 0 - 500 mM imidazole prepared in SP/GP buffer (pH 6.3). Fractions (5 min/tube) were collected and subjected to both protein determination and SDS-PAGE analysis. Fractions with detectable (> 5 ng/μl) recombinant PPPK-DHPS activity were combined and stored at -80^{o}C for further use/analysis.

We have successfully scaled up and purified PPPK-DHPS in milligram quantities using Ni-SF columns (10-20 ml). The nonspecific binding to the Ni-column was minimized using 60 mM imidazole in sample as well as washing buffers prior to elution. The 86-kDa recombinant protein appears to be unstable upon heating with SDS loading buffer or in the absence of protease inhibitors/glycerol. A 40-45 kDa band was noted in such samples by Western blotting which may represent a cleavage product of the native PPPK-DHPS.

A set of experiments were performed to optimize the conditions (stability and recovery) of PPPK-DHPS during the affinity purification steps. Recombinant protein (86 kDa) that eluted from Ni-silica or Ni-SF column demonstrated much higher purity and stability than other Ni-resins (chelating sepharose or Ni-NTA agarose). Therefore, we selected Ni-SF as an affinity media for large scale preparations. Moreover, this single-step protocol yielded PPPK-DHPS preparations with >70% purity as determined by SDS-PAGE and Western blot analysis.

FPLC purification of PPPK-DHPS:

Using the above described one-step affinity protocol, we successfully obtained the recombinant PPPK-DHPS (86 kDa protein), in milligram quantities, with 65-70% purity index (Table 1). To further purify this protein to electrophoretic homogeneity, we performed FPLC-supported ion exchange chromatography. Both anion exchange (Mono-Q/pH 7.8) and cation exchange (Mono-S/pH 6.3) columns were used in pilot experiments. Affinity-purified (Ni-NTA) samples were dialyzed against 20 mM MES containing 10% glycerol, 0.5 mM PMSF and 1 μg/ml peptide inhibitors. For Mono-Q, protein samples were loaded, washed and eluted at 1ml/min of MES buffer (pH 7.8) using a gradient of 0-300 mM NaCl. On the other hand, a low-pH (6.3) MES buffer was used for the Mono-S ion exchange method.

Fractions were collected and analyzed for PPPK-DHPS levels using Western blotting (Anti-His detection method).

We attempted to purify PPPK-DHPS for crystallographic studies by FPLC-linked ion exchange chromatography. Initial experiments using a strong anion exchange Mono-Q were unsuccessful as the loaded proteins (at pH 7.4 - 8.0) did not adsorb to the resin. However, significant resolution was obtained (at pH 6.3) with Mono-S, a cation exchange resin. Further experiments to scale up and purify DHPS-PPPK in its native functional state are now in progress.

Superdex purification of PPPK-DHPS

To further purify this protein, we concentrated the pooled eluted fractions using YM-30 Ultrafiltration membrane. Concentrated protein was loaded onto Superdex-200 column using FPLC system. The column was washed and eluted with SP/GP buffer containing 0.5 mM EDTA and 0.5 ug/ml leupeptin 0.5 ug pepstatin A and 1 mM PMSF. Fractions 36-43 were pooled and analyzed on 10% SDS-PAGE. This purified protein will be used for crystallization experiments. Fig. 1 and 2 show SDS-PAGE of the purified protein.

To further assist this task, we have chemically synthesized the enzyme substrates (dihydropteroates) in our laboratory which will facilitate the proposed structure-function studies of PPPK-DHPS.

Western Blot analysis of PPPK-DHPS:

The levels and amounts of 6xHis-tagged PPPK-DHPS expressed in whole cell lysate and purified fractions were determined by Western blotting using an Anti-His detection kit (Qiagen). Aliquots (5-20 µl) without heating were resolved on 10% SDS-acrylamide gels under reducing conditions. After electrotransfer, nitrocellulose membrane was blocked with 3% blotto, washed twice with TBST and incubated with anti-His monoclonal antibody (1:2000) for 3-4 hr at room temperature. After washing, the filtrate was incubated for 1 hr with alkaline phosphatase-conjugated secondary antibody (1:1000) and developed with NBT/BCIP staining solution for 5-10 min. The PPPK-DHPS levels were quantitated using densitometry (Table 1).

TABLE 1: Two-Step Purification of PPPK-DHPS

Sample/ method	Total protein (mg/L)	DHPS-PPPK (levels)*	Electrophoretic purity (%)
Cell lysate	60-70	+	-
Ni-NTA	8-12	+++++	65-70%
FPLC-Mono S	0.5-1	+++	85-90%

*based on the densitometric analysis of Western blots (86 kDa band).

Potential problems & Alternative approaches:

1. *Enzyme Stability:* PPPK-DHPS undergoes rapid cleavage or degradation after affinity chromatography, therefore a cocktail of protease inhibitors at all steps of purification will be included. We may also perform gel filtration (FPLC) prior to this final step, to remove or neutralize the endogenous low-MW degrading factors.

2. *Functional assay:* Kinetic studies with both native and recombinant enzyme will be performed to establish structure-function relationships.

3. *Antibodies:* Availability of peptide-specific antibodies to PPPK-DHPS will assist in designing better functional and analytical protocols. This alternative task will be undertaken in the next year of the project via active collaboration with Dr. Triglia and Dr. Cowman's laboratory.

Preparation of the PPPK-DHPS substrate

phosphorylated pteridine:

DHPS catalyzes the following reaction:

[p-aminobenzoate + hydroxymethyldihydropterin pyrophosphate = dihydropteroate]

However, PPPK catalyzes the pterin pyrophosphokinae reaction in the folate salvage pathway before DHPS. To determine the enzyme activity and stability of purified recombinant DHPS-PPPK (86 kDa), we chemically synthesized the substrate in our laboratory, as it is not commercially available. Phosphorylated pteridines were prepared by a method described by Shiota et al. (12). Briefly, 100 mg of 2-aminodihydroxymethyl-pteridine and 10 g of pyrophosphoric acid were mixed and stirred in the dark at 60C for 6-8 hrs. After stopping the reaction with cold water, the mixture was treated with 15% acid-washed Norit A (3:1 v/v) and filtered through a Millipore filter (0.45 μ). The absorbed pteridines were eluted and purified using the following three-step protocol:

i) The charcoal pad was extracted five times with 100 ml 3N ammonium hydroxide-ethanol (1:1). The filtrates were pooled and concentrated to 5 ml via roto-evaporation at 45°C.

ii) The concentrated sample was loaded onto a Dowex 50-H+ column (1.3 x 30 cm) and washed extensively (~2 liters) with water.

iii) All effluent liquid was passed through a DEAE-cellulose column (DE-52, 2.6 x 30 cm) and eluted with a 500 ml gradient of 0.1 - 0.2M LiCl prepared in 20 mM Tris-HCl (pH 7.4).

The absorption profile at 280 nm revealed two major peaks which were pooled and lyophilized. The pteridines were washed several times with absolute ethanol to remove free ions and dried with ether for storage. This procedure yielded 35 mg of pteridine monophosphate and 30 mg of pteridine diphosphate. Dihydropterines were obtained by reduction with $NaBH_4$ (10 mg per mg pterines in 0.1N HCl for 30 min). DHPS-PPPK activity is determined by radioassay using dihydropterines and ^{14}C-PABA as substrates (13) and expressed as units of enzymes per μg protein.

2.2. DHFR:

Expression of DHFR in yeast as secretory protein:

We have transformed the yeast with pPIC 9 containing DHFR cDNA. Approximately 40 transformants were screened to select a DHFR expressing transformant by growing the yeast transformants in 10 ml of yeast minimal medium containing glycerol for 2 days at 30°C. The cells were transferred to minimal medium containing methanol (methanol induces the expression of proteins) and grown at 30°C. After 4 days of incubation the cell-free medium was harvested and analyzed on 10% SDS-polyacrylamide gels. None of the transformants expressed any detectable levels of protein. Hence we opted to express this protein in procaryotic system.

2.3 Lactate dehydrogenase(LDH):

Crystal Structure of LDH:

Crystals of LDH were grown from 2-methyl-2,4-pentanediol by hanging drop vapor diffusion technique. Protein used in crystallization contained NADH. We have the

crystal structure of *Pf* LDH at 2 Å resolution using molecular replacement method. Figure 3 shows our present model.

2.4 Sequestrin

CD36 is a cell surface glycoprotein composed of a single polypeptide chain which interacts with erythrocytes parasitized with *P. falciparum*. Sequestrin is a malarial protein that is involved in the endothelial recognition in P. falciparum. In collaboration of Dr. Christian Ockenhouse of WRAIR, we have expressed and purified large quantity of the CD 36 binding domain of sequestrin for structural study. We have not succeded in producing any crystal of this protein. Figure 4 shows an SDS PAGE of the purified protein.

2.5. Rab6

In collaboration with Dr. Gordon Langsley of Pasteur Institute, Paris we have expressed recombinant Rab6 of *P. falciparum*. The recombinant protein was expressed in *E. coli* with an amino terminal extension that facilitated purification by immobilized metal affinity chromatography. Recombinant protein has been purified and crystallized. Crystals were grown by hanging drop vapor diffusion technique. 2-4 µl of concentrated protein was mixed with equal volume of a reservoir solution containing 20-25% of polyethylene glycol in sodium cacodylate buffer, pH 7.0. Crystals grew overnight at room temperature and continued to grow up to 0.5 mm in dimension (Fig. 5).These crystals belong to tetragonal space group $P4_12_12$ with a = b = 82.1, c = 90.87Å. Presently we are collecting X-ray diffraction data using the native crystals .

These crystals diffract upto 2.7Å resolution. Experiments to form complexes with GDP and nonhydrolyzable GTP analogs are also in progress.

We have also expressed a new construct without any extension. The coding sequence for the entire 208 amino acid polypeptide was amplified using PCR and was cloned into pET vector and the recombinant protein was expressed under the control of T7 promotor in *E. coli* BL21(DE3)plys cells. Figure 6 shows SDS-PAGE pattern of the culture 4 hours after induction with IPTG.

The recombinant protein was purified from the soluble supernatant of the *E. coli* lysate by a combination of chromatographic steps. An SDS-PAGE pattern of the purified protein is shown in Figure 7.

The purified protein crystallized under identical condition as the first construct mentioned above. We are in the process of analyzing the crystals of this new version of protein.

2.6 Falcipain

A number of recombinant constructs of falcipain have been cloned in to yeast expression vector pESP-1 (Stratagen). The resulting plasmids have been transformed in to the yeast strain *Schizosaccharomyces pombe*. Transformants have been grown for overexpression of recombinant protein which is expressed intracellularly as a GST fusion protein. Cell free extracts from a number of clones are being examined at the present time for the expression of active protein in our collaborator Dr. Philip Rosenthal's laboratory at The University of California in San Francisco.

2.7. Cyclophilin

We have received cDNA clones coding malarial cyclophilin with and with out signal peptide from Dr. Reddy College of Veterinary Medicine, Univ. Of Florida. We have cloned the cDNA in a pRSET vector and expressed and purified on Ni-NTA column. We have isolated inclusion bodies and solubilized in 8M urea, 100 mm sodium phosphate, 25 mM Tris.Cl pH 8.8. The buffer of solubilized inclusion bodies is altered to contain 5M urea, 100 mm sodium phosphate, 25 mM Tris HCl, pH 8.8 and loaded on to an affinity column. The column is washed with the buffer containing 5M urea, 100mM sodium phosphate pH8.8, 25mM Tris Cl pH8.8 and buffer containing 5M urea,100mM sodium phosphate pH 6.5 , 25mM Tris.Cl pH 6.5. Malarial cyclophilin is eluted with a gradient of 0-650 mM imidazole in buffer containing 5M urea, 100mM sodium phosphate pH 6.5, 25 mM Tris

HCl, pH6.5. This protein will be further purified by ion exchange chromatography. Figure 8 shows SDS-PAGE of purified recombinant cyclophilin.

3. Conclusions:

We have expressed soluble recombinant bifunctional PPPK-DHPS and standardized the purification protocol for purification of bifunctional PPPK-DHPS. The substrate was synthesized for determination of activity of recombinant PPPK-DHPS.

The PPPK-DHPS is a promising candidate crystal structure determination. Recently crystal structure of *E. coli* DHPS has been determined to high resolution. This structure can greatly assist our structure determination and model building steps during structure analysis of the malarial enzyme.

The DHFR project has been frustrating. Although large quantities of purified, homogenous and active protein have produced, the protein aggregates into nonresoluable structures rather crystalline arrays. New constructs and protein will be analyzed to determine if they help circumvent this problem.

We have also expressed soluble malarial recombinant DHOD with transmembrane region. The DHOD is in early stages of production and will be further pursued.

Recently, we and others have determined the three dimensional structure of *P. falciparum* LDH at high resolution. In the structure of the ternary complex determined by Dunn *et a.l* (32) the substrate specificity loop is in a closed conformation. The overall structure of the *P. falciparum* LDH is similar to other LDH structures but there are two regions where the malarial enzyme structure differs from the mammalian enzyme. Of these two areas, the one that is of great interest for structure based drug design is the substrate specificity loop. In the malarial enzyme insertion of five additional residues in this region results in the creation of a distinct cavity in the protein surface adjacent to the catalytic region. Superposition of various structures also revealed an altered positioning of the NADH

cofactor in the malarial enzyme. Three features in the catalytic cavity of the enzyme have been identified that could lead to the change in the cofactor association in *Pf* LDH. In other LDH structures, the nicotinamide ring packs against a flat hydrophobic surface formed by the side chain of Ile 250. In *Pf* LDH the Ile residue is substituted by a Pro which protrudes into the catalytic cavity. As a result, to relieve the steric hindrance the cofactor is moved about an A away. Residue 246 in *Pf* LDH is a Pro which is a Thr in other LDH's. This substitution results in the placement of Og of the Serine 247, which is hydrogen bonded to the substrate analog, in a position very similar to that occupied by a conserved water molecule in similar structures. Unlike other LDH's, malarial LDH is not inhibited by excess pyruvate. This distinctive feature of malarial LDH may be a result of the displacement of the cofactor in malarial; LDH with respect to enzymes from other sources. The most interesting feature observed in *Pf* LDH structure is a distinctive cleft formed alongside the extended substrate specificity loop. This cleft, which is about 10A X 5A X 8A, is formed in part by the nicotinamide portion of the NADH binding pocket contrasts sharply with the shallow groove in the mammalian enzyme. This site is considered as an attractive target site for designing specific inhibitor of the malarial enzyme. Currently we are in the process of designing compounds that fit in this cleft.

Structure based inhibitor design is an interactive process that utilizes computer graphics, computational chemistry, X-ray crystallography, synthetic chemistry and biology. For structure based inhibitor design we need to visualize the structure of the target enzyme and the enzyme-inhibitor complexes. Detailed knowledge of the active site geometry and a clear understanding of the possible conformational changes that can be induced by the inhibitor are essential.

The protein data bank contains close to 400 independent protein structures. The Cambridge Crystallographic Database contains coordinates for more than 70,000 small molecule structures. We also have access to the Chemical Database from MDL that contains names, structures and suppliers for 50,000 compounds. Combined, these data bases contain a wealth of information about different motifs of chemical structures and about the interactions that can stabilize protein-inhibitor chemical contacts. The general approach we will take to identify small molecules that will fit the enzyme active site is:

1) The available space at the active site, described in terms of van der Waals surface is calculated using the concept developed by Conolly (33); 2) Systematic search of the Cambridge Crystallographic Database will be done to identify small molecules that will fit the active site. Constraints with respect to steric and chemical nature of atoms contributing to the accessible surface area in and around the active site will be applied. Each 'DOCK'ed small molecule will be given a score based on its chemical complementarity within the active site. 3) In parallel, molecular probes (such as OH, CH_3, Cl^-, etc) will be used to identify regions for the highest preference having most favorable interactions. Regions of active site where hydrogen bond donors/acceptors would prefer to gather will be identified. Active site residues are scored according to their propensity towards these hydrophobic and non-hydrophobic interactions. 4) Small molecules with highest scores in each type of organic molecules will be examined for structural compatibility with results obtained in step 3. Finally, ease of synthesis and biochemical characteristic of the compounds will be considered for visual scoring of the compound. Using this approach we have identified inhibitor of the human complement protein Factor D. There were structural similarities among the compounds with highest scores. Chemical database was used to locate these compunds and

the similar ones from the vendors. One of the three compounds that were purchased had an IC50 value in micromolar range. Using structure based design we have developed inhibitors with IC50 value in 250 nanomolar range.

4. Plans for next year:

a. Crystallization and structure determination of malarial PPPK-DHPS.

b. Identification of LDH inhibitors.

c. Crystallization and structure determination of cyclophilin.

d. Structure determination of Rab6.

e. Identification of yeast clones producing recombinant falcipain and optimization of expression.

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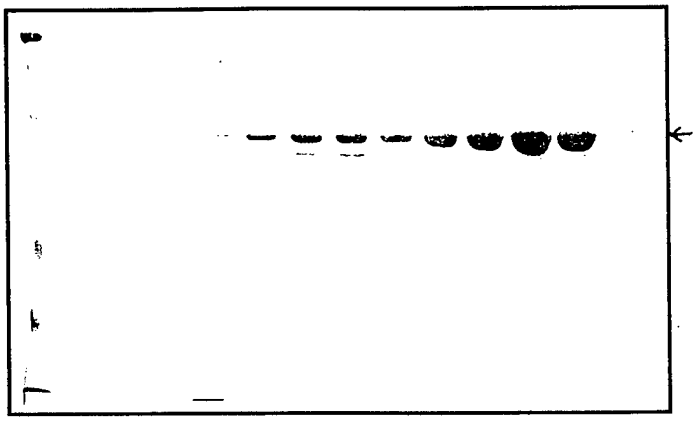
Fig. 1

SDS-PAGE analysis of Superdex-fractions

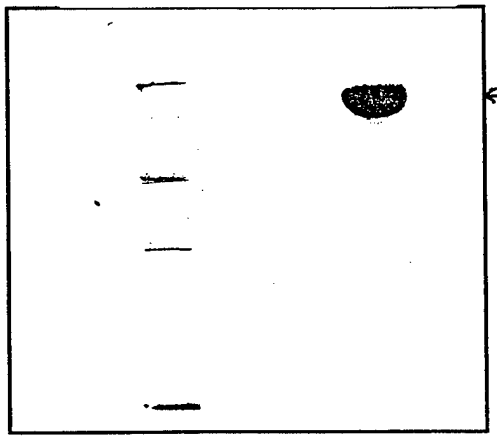


mw 9 14 26 28 34 35 36 37 38 39 40 41 42 47
std

Fraction numbers

Fig. 2

Purified PPPK-DHPS



Lane: 1 2

Fig. 3: Ribbon diagram of
Pf LDH Structure

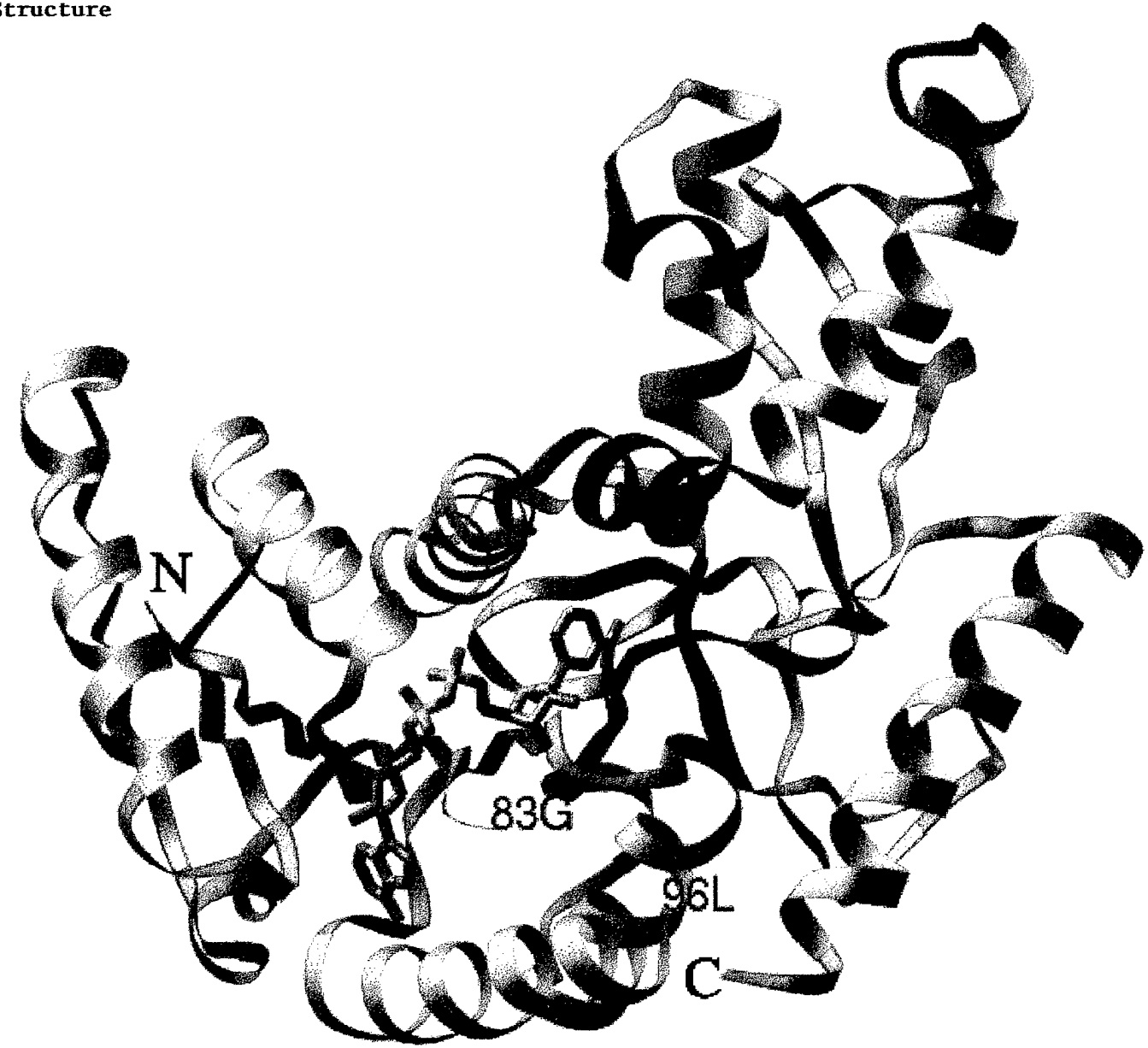


Fig. 3:

Note:

The numbering system of pfLDH is based on *Bacillus stearothermophilus* LDH. The loop region starting from residue 84 ending at residue 95 is missing from the density. Also missing from the density is the nicotinamide ring of NADH or APAD. The R-factor is 27.4%, R-free is 21.5% refining from 6Å to 2Å.

The color scheme:

Blue: α Helix.

Green: β strand.

Brown: random coil.

Deep blue: 3_{10} helix.

In NADH or APAD:

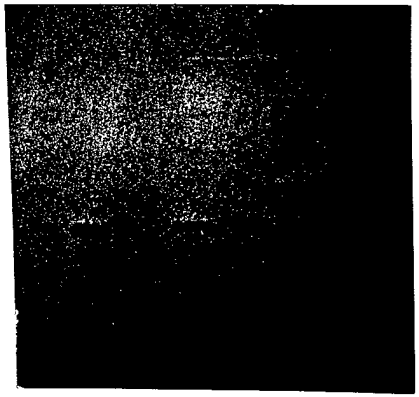
Green: C.

Blue: N.

Red: O.

Magenta: P.

Fig. 4



1 2 3

SDS-PAGE of purified sequestrin

Lanes 1 & 2: 10 & 5 microgram of purified sequestrin
Lane 3: Molecular weight markers (97, 67, 45, 31, 21 & 14 KDa)

Fig. 5: Crystals of recombinant Pf rab6

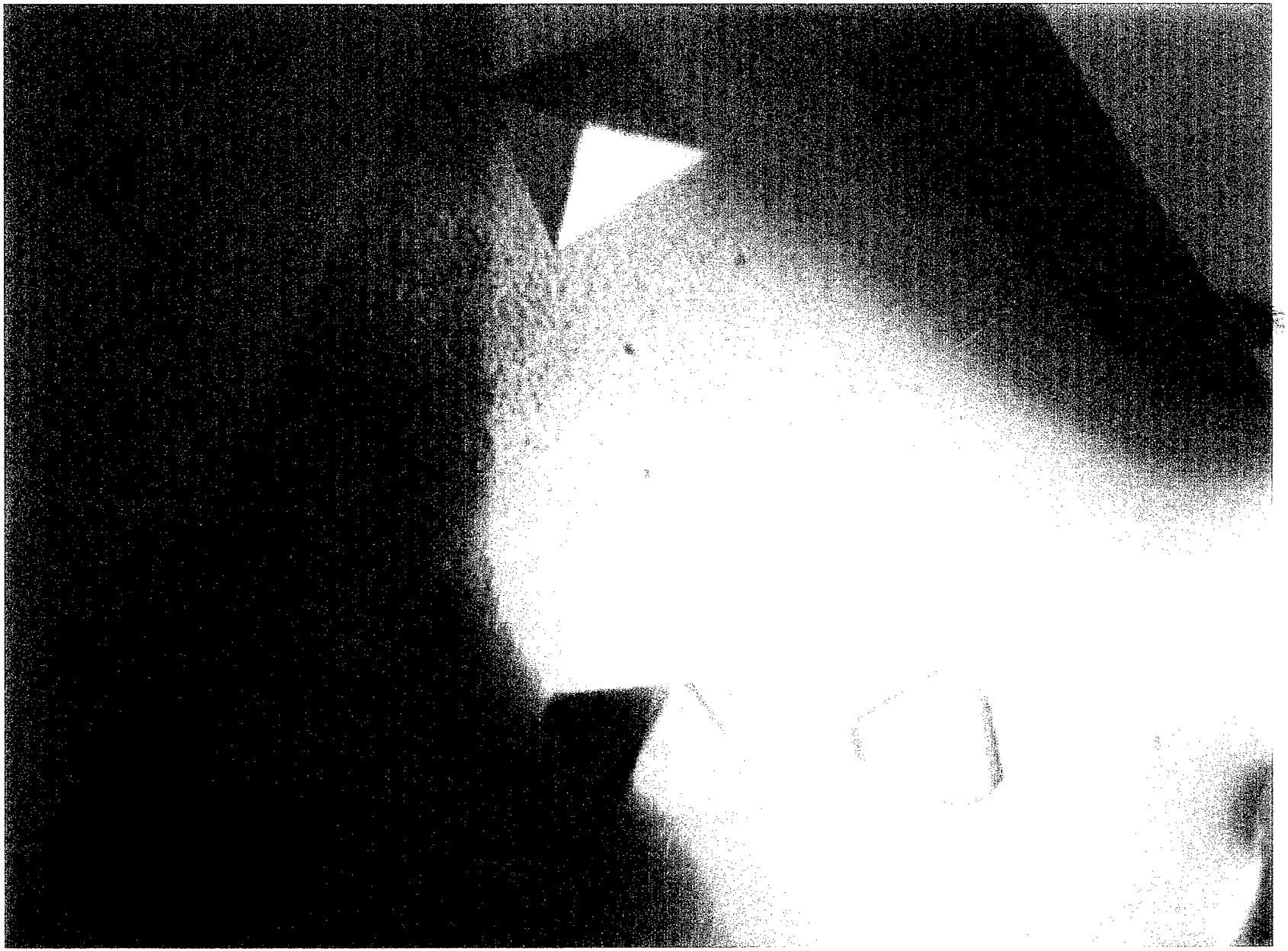
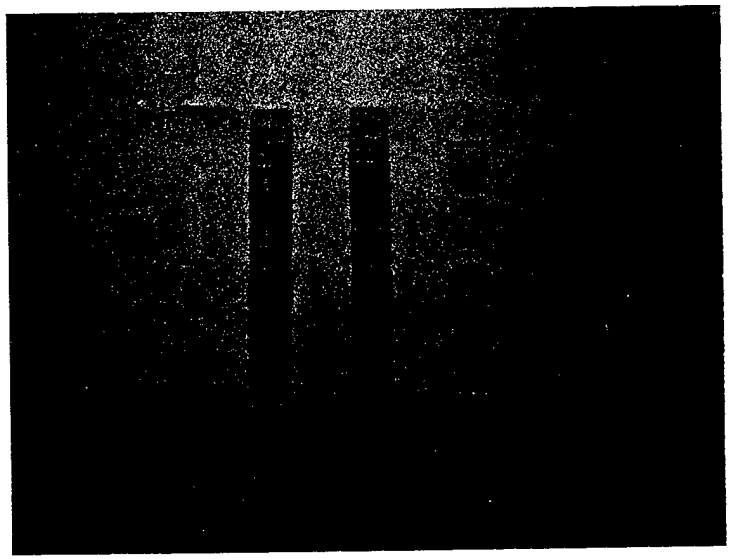


Fig. 6

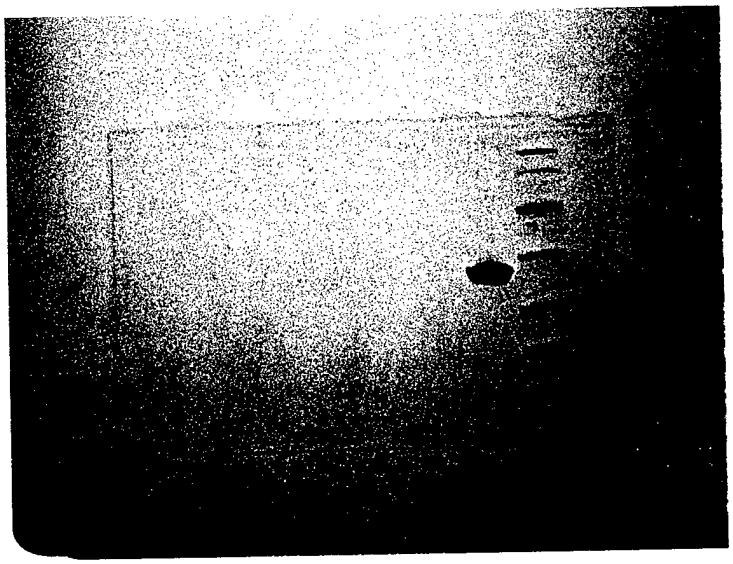


1 2 3

Expression of Pf Rab6 in E.coli

Lane 1: E.coli cell pellet expressing Rab6
Lane 2: Negative control E.coli cell pellet containing vector only
Lane 3: Molecular weight markers. (From top to bottom: 97, 67, 45, 31, 21, & 14 KDa)

Fig. 7

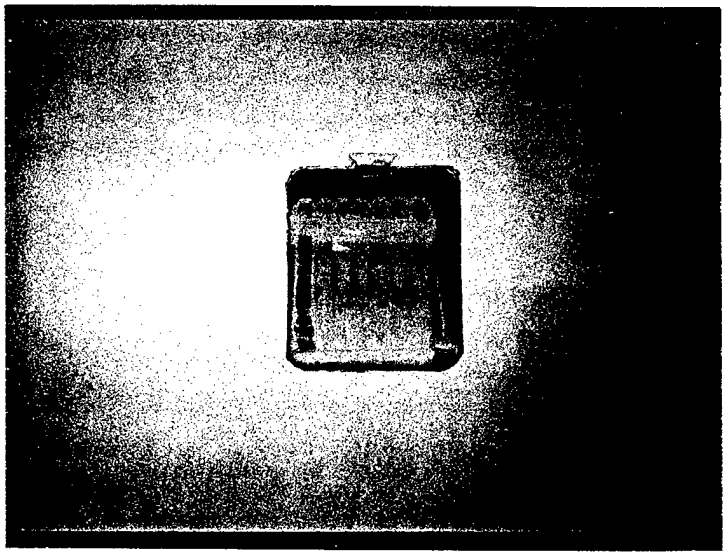


1 2

Purified Pf Rab6

Lane 1: Purified protein
Lane 2: Molecular weight markers

Fig. 8



SDS-PAGE of purified recombinant cyclophilin

6. Personnel Received pay from this contract

i) Narra S. Reddy Ph.D.

Ii) Mamoun Eltahir

iii) Gilberto Ramos

iv) Nasser Iranikhah