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The goal of this project is to design selective anti-malarial compounds using a structure-based inhibitor design approach. Several potent inhibitors of Plasmodium falciparum lactate dehydrogenase have been identified. We are in the process of crystal structure analysis of the enzyme-inhibitor complexes. Attempts to determine the structure of the catalytic domain of Rab6 is in progress. Enzymatic characterization of monofunctional dihydropteroate synthetase will be done before crystallization effort is initiated.

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FOREWORD

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NA [redacted] In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Larry J. DeLucas 7/13/95

PI - Signature Date

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1. Introduction:

Infection with malaria parasite still remains the most deadly infection causing approximately 1.5 million deaths worldlwide (1). The disease is caused by the parasites of *Plasmodium* species and is transmitted by certain strains of mosquito. Majority of the deaths and clinical complications of malaria are due to infection with *P. falciparum*, the most lethal of the four *Plasmodium* species. Although intense effort to produce a useful vaccine against malaria still continues, the results so far has not been rewarding. Malaria is currently treated by chemotherapy. The usefulness of available chemotherapeutic agents is seriously compromised by the development of drug resistant parasite strains (2). There is urgent need for developing new and potent anti-malarials (3). We have undertaken a rational approach for the develpment of anti-malarial agent using structure-based drug design. This technique will enable us to identify active site inhibitors of several key enzymes of the parasite. Targeting the active site of important metabolic enzymes reduces the likelihood of eventual resistance due to genetic mutation. Also, attacking several key enzymes in a combination therapy and simultaneous development of new generation of inhibitors will be useful in long term treatment. Lactate dehydrogenase (LDH) enzyme is essential for the life cyle of the malaria parasite and compounds that inhibit LDH will also kill the parasite (4). In collaboration with scientists at WRAIR we have identified some lead inhibitors of the *Pf* LDH. In a structure-based drug design project high resolution three domensional structure of the enzyme - inhibitor complex provides the basis for further modifications of the inhibitor in order to optimize the favorable interactons with the protein molecule (5, 6). We have begun structure analysis of the LDH-inhibitor complexes.

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

Lactate Dehydrogenase

Characterization of potent inhibitors, crystal structure analysis of enzyme-inhibitor complexes.

We have determined the structure of the *P. falciparum* LDH at 2.0A resolution using molecular replacement method. We have been working closely with the scientists at WRAIR to identify and characterize *pf* LDH inhibitors using the available chemical library at WRAIR. This approach has been very successful and our collaborators (Capt. Karl Weborvitz and others) have identified several *Pf* LDH inhibitors with IC_{50} in the micromolar range. One of these compounds inhibited the growth of erythrocyte stage *P. falciparum* with an IC_{50} of about 15 μg/ml.

We have now intesified our effort on LDH. A post doctoral fellow (Dr. D. Prahadeewaran) has been recently recruited to work full-time on the structure determiantion of LDH-inhibitor complexes under the supervision of Dr. Debasish Chattopadhyay. Once the condtions for preparing the crystals of the enzyme-inhibitor complex is established, structure determination can be completed relatively rapidly since the crystal structure of the native enzyme is already known. The recombinant protein has already been crystallized in the presence of the WRAIR inhibitor BK19981. To prepare the complex the protein was incubated with 2 mM BK19981 and 3.2 mM NADH at 4°C for 3 hrs. The resulting mixture was crystallized at 4°C using hanging drop vapor diffusion technique. The precipitant used was polyethylene glycol 1000. Crystals large enough for X-ray diffraction analysis grows overnight. Crystals of *Pf* LDH - oxamate complex grown under similar conditions diffracted to about 1.7A. Diffraction data for the putative BK19981 complex crystals are currently being collected at -170°C. These crystals also diffract to 1.8A resolution. The crystals are isomorphous with the crystals of the oxamate complex. The unit cell parameters are 79.61, 86.01 and 91.06A and the crystal space group is I222.

Sequestrin:

Two new constructs of sequestrin were prepared by Dr. Chris Ockenhouse of WRAIR. We have purified the recombinant protein to homogeneity and used the purified protein for screening crystallization conditions. This effort was not successful.

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.

Dr. Chattopadhyay laboratory worked closely with Dr. Phil Rosenthal's group at the University of California at San Francisco on this project. While Dr. Chattopadhyay's laboratory focussed on the preparation of expression constructs of falcipain in yeast system, Dr. Rosenthal's group participated in characterization of the clones. A large number of clones in several yeast expression vectors were isolated, transformed into respective yeast hosts and transformants were grown in suitable culture medium for expression. The culture media (for secretory constructs) or the cell free extract (for intracellular constructs) were tested for overexpression of falcipain. Overall result of this effort has been unsucessful.

We have recently known that Dr. Virendra Chowhan at New Delhi, India has been successful in expressing an active falcipain in *E. coli.* This has created new enthusiasm in this project and Dr. Chattopadhyay and Dr. Rosenthal are in the process of initiating this new collaboration.

Rab6:

Malaria parasite spends much of its life cycle indside 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 mechinery 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 Rab's 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).

Recombinant Rab6 was expressed with an amino terminal extension peptide which was designed to facilitate purification of the recombinant protein from the crude bacterial extract using immobilized metal affinity chromatography. The purified protein was crystallized at room temperature. These crystals belong to the space group $P4_12_12$ with a = b = 82.1, c = 90.87A. Complete native data were collected at a resolution of 2.7A (at -170°C). Attempts to determine the structure using human ras, Rap2A and rac1 structures as search model were not successful.

We have then expressed and purified a new construct of the protein without any extension peptide. This new construct crystallized in the space group $P4_122$, with unit cell parameters a = b = 79.99, c = 88.89 A. Native data to about 2.4A resolution (99% complete) were collected. Attempts to solve this structure by molecular replacement were also unsuccessful. We are now concentrating our effort to determine the structure of this construct using multiple isomorphous replacement method. This will involve soaking native crystals into solutions of various heavy atom reagents and collecting diffraction data in order to identify useful heavy atom derivatives. At least two independent heavy atom derivatives are required for determining the phase accurately by using this technique. Table 1 summarizes the statistics of the intensity data.

We have also expressed the putative GTPase domain (amino acids 1 - 175) of Rab6. This domain has been purified and crystallized. These crystals also belong the tetragonal space group $P4_122$ with unit cell parameters a = b = 81.21, c = 90.18 A. We have attempting to solve the structure of this domain by molecular replacement. Several GTPase structures will be used as search model. A summary of statistics of the intensity data is given in Table 2. Attempts to prepare the complex of Rab6 with non-hydrolyzable GTP analog, GTPγS, is also underway.

See Tables 1 and 2.

Dihydropterorate synthetase:

DHPS fragment of PPPK-DHPS cloned into pQE30 vector, resulting in 9 additional amino acids at the amino terminus (underlined). This corresponds to aa 379 to 706 of the PPPK-DHPS molecule (6).

```
mrgshhhhhh kdrisylkek tnivgilnvn ydsfsdggif vepkravqrm    50
feminegasv idiggessap fvipnpkise rdlvvpvlql fqkewndikn   100
kivkcdakpi isidtinynv fkecvdndlv dilndisact nnpeiikllk   150
kknkfysvvl mhkrgnphtm dkltnydnlv ydiknyleqr lnflvlngip   200
ryrilfdigl gfakkhdqsi kllqnihvyd eyplfigysr krfiahcmnd   250
qnvvintqqk lhdeqqnenk nivdkshnwm fqmnymrkdk dqllyqknic   300
gglaiasysy ykkvdlirvh dvletksvld vltkidqv                338
```

Vector: pQE30

Host: XL-1 Blue

Cloning Sites: Bam H- Hind III

Insert Size: 1047bp

Internal six base cutters. Bgl II @nt#900

Calculated molecular weight: 39.4

Migrates as: 42-45 kDA protein

Growth conditions:

Starting with a single colony, grow 1 Lt over night culture of LB at 37° C. No induction is necessary. After 14-16 hr growth, harvest the cells by centrifugation at 3000xg for 10 min, and resuspend the bacteria in 50 ml of phosphate buffer (50 mM, pH 8.0).

Protein extraction and purification:

Break the cells by french press or sonication, and centrifuge the resultant solution at 20,000 rpm for 10 min. Discard the supernatant. Scoop the pellets into a beaker, add 50 ml of 8 M urea in phosphate buffer. Stir at room temperature for 1 hr at low speed. Centrifuge at 20,000 rpm for 30 min, collect the supernatant. Load the supernatant on a 20 ml Ni NTA column (Qiagen) equilibrated with phosphate buffer containing 8 M urea. Collect and save the flow through (to determine unbound protein). Wash with 100 ml of 50 mM phosphate buffer with 8 M urea first at pH 8.0 and then at pH 6.4. Elute with 100 ml phosphate buffer 50 mM, with 8 M urea at pH 4.5. All these steps can be carried out at room temperature.

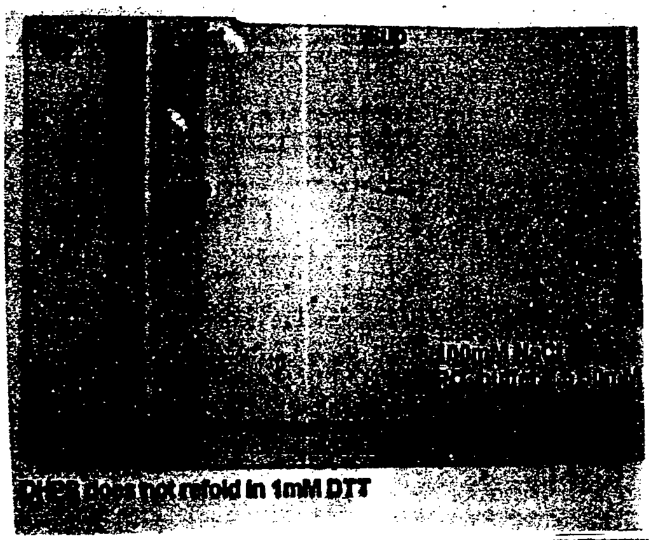
Protein refolding:

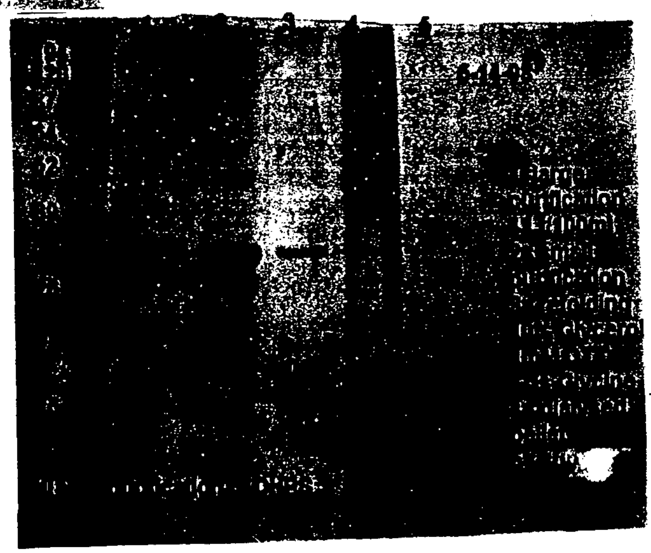
Add β mercaptoethanol to the eluted protein to make it 10 mM final concentration. Dialyze the 100 ml against 4 Lt of 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, 10 mM β mercaptoethanol, 2 M urea and 10% glycerol in cold. After overnight dialysis, dilute the protein to a concentration of 10 μg/ml. Extensively dialyze against same buffer without urea.

Centrifuge the dialysate at 20,000 RPM for 10 min. Collect the pellet and resuspend in 50 mM phosphate pH 7.5, 100 mM NACL, 10 mM mercaptoethanol.

The total yield is 10-12 mg/L.

A photomicrograph of a gel showing the purity follows below.





3. Conclusions:

We have entered the most exciting stage of a structure-based drug design project with *Pf* LDH. Often, finding the first lead inhibitor is a major bottleneck. Fortunately, we have already identified several inhibitors with IC_{50} in the micromolar range. One of these inhibitors also showed inhibited the growth of the erythrocyte-stage *P. falciparum*.. We have already started cocrystallization of these inhibitors with recombinant enzyme. At this stage, crystal structure of the LDH in complex with lead inhibitors will be determined. Information gathered from these structures will be used to chemically modify the compounds so as to optimize the binding with the enzyme. We have already crystallized the full length and the GTPase domain of *Pf* Rab6. Attempts are underway to determine the structure of the GTPase domain using molecular replacement technique. If this approach does not produce useful solution we will concentrate on traditional multiple isomorphous replacement method. We have designed a protocol for purification and refolding of monofunctional DHPS. The refolded protein will be assayed for enzymatic activity. If this construct is found active, we will proceed with crystallization of the protein.

4. Plans for next year:

a. Crystal structure analysis of LDH - inhibitor complexes. Exchange the results with scientists at WRAIR, coordinate directed library search for identifying more potent inhibitors. Prepare and supply active enzyme for conducting inhibitor screens.

b. Determine and refine structure of *Pf* Rab6.

c. Assay enzymatic activity of recombinant DHPS and ,if active, crystallize the protein for structure analysis.

Table 1

I/Sigma in resolution shells:

No. of reflections with I / Sigma less than

shell Lower limit	Upper limit	0	1	2	3	5	10	20	>20	total
99.00	4.87	13	30	43	61	92	146	358	1153	1511
4.87	3.86	12	27	48	61	95	157	350	1092	1442
3.86	3.37	10	28	53	78	131	277	589	829	1418
3.37	3.07	34	77	152	211	302	494	861	536	1397
3.07	2.85	36	102	197	285	456	782	1185	207	1392
2.85	2.68	70	196	360	490	697	1043	1320	80	1400
2.68	2.54	101	258	452	620	872	1216	1387	8	1395
2.54	2.43	132	331	552	745	993	1274	1357	4	1361
2.43	2.34	192	425	704	910	1141	1277	1303	1	1304
2.34	2.26	156	386	569	685	787	825	835	0	835
All hkl		756	1860	3130	4146	5566	7491	9545	3910	13455

I/Sigma in resolution shells:

% of reflections with I / Sigma less than

Shell Lower limit	Upper limit	0	1	2	3	5	10	20	>20	total
99.00	4.87	0.8	1.9	2.7	3.9	5.9	9.3	22.8	73.6	96.4
4.87	3.86	0.8	1.9	3.3	4.2	6.5	10.8	24.1	75.2	99.3
3.86	3.37	0.7	2.0	3.7	5.5	9.2	19.4	41.3	58.1	99.4
3.37	3.07	2.4	5.5	10.8	15.0	21.5	35.2	61.3	38.2	99.5
3.07	2.85	2.6	7.3	14.1	20.4	32.6	56.0	84.8	14.8	99.6
2.85	2.68	5.0	14.0	25.6	34.9	49.6	74.3	94.0	5.7	99.7
2.68	2.54	7.2	18.4	32.3	44.3	62.2	86.8	99.0	0.6	99.6
2.54	2.43	9.6	24.1	40.1	54.2	72.2	92.7	98.7	0.3	99.0
2.43	2.34	13.9	30.7	50.8	65.7	82.4	92.2	94.1	0.1	94.2
2.34	2.26	11.4	28.1	41.4	49.9	57.3	60.0	60.8	0.0	60.8
All hkl		5.3	13.1	22.1	29.2	39.2	52.8	67.3	27.6	94.8

Summary of reflections intensities and R-factors by shells

R linear = SUM (ABS(I - <I>)) / SUM (I)

R square = SUM ((I - <I>) ** 2) / SUM (I ** 2)

Chi**2 = SUM ((I - <I>) ** 2) / (Error ** 2 * N / (N-1)))

In all sums single measurements are excluded

Shell Lower limit	Upper Angstrom	Average I	Average error	stat.	Norm. Chi**2	Linear R-fac	Square R-fac
99.00	4.87	10423.6	333.3	272.0	0.725	0.032	0.035
4.87	3.86	10209.3	314.9	262.8	0.866	0.041	0.046
3.86	3.37	5934.8	234.5	190.7	0.951	0.056	0.059
3.37	3.07	3068.8	168.0	150.3	1.006	0.081	0.079
3.07	2.85	1551.7	140.9	131.5	0.972	0.131	0.126
2.85	2.68	918.7	129.2	124.8	1.010	0.211	0.186
2.68	2.54	581.5	122.7	118.0	1.021	0.311	0.282
2.54	2.43	442.9	126.9	123.5	1.086	0.388	0.348
2.43	2.34	292.8	142.9	140.9	1.151	0.507	0.466
2.34	2.26	234.8	172.2	170.3	1.056	0.523	0.457
All reflections		3613.0	191.4	169.9	0.967	0.066	0.050

Table 2

I/Sigma in resolution shells:
No. of reflections with I / Sigma less than

Shell Lower limit	Upper limit	0	1	2	3	5	10	20	>20	total
99.00	5.16	17	27	41	55	84	149	288	1046	1334
5.16	4.09	11	26	38	47	71	127	235	1025	1260
4.09	3.58	15	29	48	65	98	169	321	912	1233
3.58	3.25	15	42	77	106	160	270	496	730	1226
3.25	3.02	27	62	99	140	228	414	701	529	1230
3.02	2.84	41	101	161	220	328	574	925	313	1238
2.84	2.70	54	125	224	308	451	739	1052	155	1207
2.70	2.58	66	187	311	399	568	863	1155	69	1224
2.58	2.48	73	190	321	443	644	947	1142	54	1196
2.48	2.39	105	250	399	552	734	1006	1142	22	1164
All hkl		424	1039	1719	2335	3366	5258	7457	4855	12312

I/Sigma in resolution shells:
% of reflections with I / Sigma less than

Shell Lower limit	Upper limit	0	1	2	3	5	10	20	>20	total
99.00	5.16	1.2	1.9	3.0	4.0	6.1	10.8	20.8	75.5	96.3
5.16	4.09	0.9	2.0	3.0	3.7	5.6	10.0	18.5	80.5	99.0
4.09	3.58	1.2	2.3	3.9	5.2	7.9	13.6	25.8	73.3	99.1
3.58	3.25	1.2	3.4	6.3	8.6	13.0	22.0	40.3	59.3	99.7
3.25	3.02	2.2	5.0	8.0	11.4	18.5	33.6	56.9	42.9	99.8
3.02	2.84	3.3	8.1	13.0	17.7	26.5	46.3	74.6	25.2	99.8
2.84	2.70	4.5	10.4	18.6	25.5	37.4	61.2	87.2	12.8	100.0
2.70	2.58	5.4	15.3	25.4	32.6	46.4	70.5	94.4	5.6	100.0
2.58	2.48	6.1	15.9	26.8	37.0	53.8	79.2	95.5	4.5	100.0
2.48	2.39	8.7	20.6	32.9	45.6	60.6	83.1	94.3	1.8	96.1
All hkl		3.4	8.4	13.8	18.8	27.1	42.3	59.9	39.0	99.0

Summary of reflections intensities and R-factors by shells

R linear = SUM (ABS(I - <I>)) / SUM (I)
R square = SUM ((I - <I>) ** 2) / SUM (I ** 2)
Chi**2 = SUM ((I - <I>) ** 2) / (Error ** 2 * N / (N-1)))
In all sums single measurements are excluded

Shell Lower limit	Upper Angstrom	Average I	Average error	stat.	Norm. Chi**2	Linear R-fac	Square R-fac
99.00	5.16	3195.0	96.5	34.7	0.782	0.031	0.028
5.16	4.09	3070.0	86.4	35.3	0.874	0.041	0.043
4.09	3.58	2129.1	63.5	31.6	1.075	0.052	0.054
3.58	3.25	1318.2	46.2	29.0	1.164	0.069	0.067
3.25	3.02	701.0	34.0	26.7	1.312	0.108	0.103
3.02	2.84	424.6	29.1	25.6	1.209	0.158	0.149
2.84	2.70	272.3	26.7	24.9	1.208	0.235	0.218
2.70	2.58	195.6	25.8	24.6	1.253	0.317	0.311
2.58	2.48	164.1	26.0	25.2	1.181	0.369	0.360
2.48	2.39	132.9	27.1	26.5	1.179	0.449	0.404
All reflections		1192.2	46.9	28.5	1.122	0.070	0.049

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