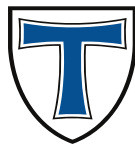


JUSTUS-LIEBIG-



UNIVERSITÄT
GIESSEN

***Plasmodium falciparum* Glucose 6-Phosphate Dehydrogenase-6-
Phosphogluconolactonase. Characterisation of Redox-Related
Networks as Contribution to the Development of Novel
Intervention Strategies**

by

Mailu Boniface Mwongela

from

Mombasa, Kenya

**A thesis submitted to the Faculty of Biology and Chemistry
(FB 08) in partial fulfilment for the requirements of the
Doctor of Science Degree of Justus-Liebig-University
Giessen, Germany**

September 2008

Dedication

This thesis is dedicated to my daughter Lynn Ndimu Mwongela, my son Liam Musyimi Jnr
IIIrd Mwongela, my wife Grace C. Mwangome and the entire Musyimi family.

You all inspire my life to live the dream.

Berichte aus der Biochemie

Boniface Mwongela Mailu

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I Have a Dream.....
(Martin Luther King Jnr, August 28th, 1963)

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Mailu B M, Rahlfs S, Austin C J D, Hunt N H, Becker K. (2006). Optimisation of the heterologous overexpression of mouse indoleamine 2, 3-dioxygenase. *Second joint Ph.D Students Meeting of the Collaborative Research Centres SFB 544 Heidelberg and SFB 630 Würzburg*, Heidelberg, November 2006.

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Summary

Plasmodium parasites are developing unacceptable levels of resistance to one drug after another and many insecticides are no longer useful against mosquitoes transmitting the disease. Years of vaccine research have produced few hopeful candidates and although scientists are redoubling the search, an effective vaccine is at best years away. Therefore there is need for identification of new drug targets and alternative antimalarial regimes. In response to this dire situation the study aimed at evaluating the pentose phosphate pathway of the malaria parasite *P. falciparum* in particular the bifunctional enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase, understanding the kynurenine pathway of tryptophan metabolism in particular the enzymes indoleamine 2,3-dioxygenase (1 and 2) and unravelling more knowledge about the thioredoxin system networks in search for a new potential drug target and new drug alternatives.

The first two steps of the pentose phosphate pathway in *Plasmodium falciparum* are catalysed by the enzyme glucose 6-phosphate dehydrogenase-6-phosphogluconolactonase (*Pf*GluPho) which is a unique bifunctional enzyme exclusively found in the genus *Plasmodium*. In spite of the importance of the role this enzyme plays in the parasite's pentose phosphate pathway as well as in overcoming oxidative stress, the characteristics of *Pf*GluPho are still a mystery. For the first time *Pf*GluPho has been successfully cloned, heterologously overexpressed and purified to homogeneity. The recombinant enzyme was found to be a hexamer which exhibits lower K_m values that favour substrate turnover by the parasite enzyme when compared to the human homologue. The steady state kinetics of *Pf*GluPho's glucose-6-phosphate dehydrogenase (*Pf*GluPho's G6PD) demonstrates that the enzyme follows an ordered sequential mechanism with $NADP^+$ being the leading substrate. Three novel inhibitors of *Pf*GluPho's G6PD which are active at the lower micromolar range were identified and found to be non-competitive with respect to glucose-6-phosphate and $NADP^+$. The study offers the first clear documentation of the cloning, heterologous overexpression, biochemical as well as kinetic characterisation, crystallisation and the first novel inhibitors of *Pf*GluPho.

For 30 years, the established dogma regarding tryptophan catabolism was that the first step of the kynurenine pathway, the cleavage of the 2,3-double bond of the indole ring of tryptophan was performed by two enzymes, indoleamine 2,3-dioxygenase-1 (IDO-1) and tryptophan 2,3-dioxygenase (TDO). Recently, indoleamine 2,3-dioxygenase-2 (IDO-2) a third enzyme capable of performing this reaction has been discovered. Reported here is a study of the kinetic activity, pH stability, oligomeric structure as well as secondary structural features of recombinant mouse IDO-2 in direct comparison with mouse IDO-1. A screen for new more potent inhibitors of IDO-1 which lack the indole core and avoid the liability arising from the use of indole derivatives which have been reported to be neuroactive gave rise to compound 55D11 (K_i 0.05 μM) which is more potent than the already existing IDO inhibitors. A structure activity study was done using various derivatives of compound 55D11 to determine

the elements that could be modified to increase potency. The study clearly demonstrates that IDO-1 and IDO-2 differ significantly in terms of their affinity for substrates as well as structure.

The malarial parasite *Plasmodium falciparum* possesses a functional glutathione and thioredoxin system comprising the redox-active proteins thioredoxin (Trx), glutaredoxin (Grx), and plasmoredoxin (Plrx) which all belong to the thioredoxin superfamily and share the active site motif Cys-X-X-Cys. A better understanding of the role of these members of the thioredoxin superfamily in *P. falciparum* as well as other systems could be achieved if more was known about their target proteins. Using thioredoxin affinity chromatography prepared by immobilising mutants of the redoxins lacking the resolving cysteine at the active site on CNBr⁻ activated sepharose, target proteins of *P. falciparum* cell extract were trapped. The covalently linked proteins were eluted with dithiothreitol and analyzed by matrix assisted laser desorption ionization time of flight (MALDI-TOF). Twenty one potential targets were identified for plasmoredoxin. Besides confirming known interacting proteins, potential target candidates involved in processes such as; protein biosynthesis, energy metabolism and signal transduction were identified. Further confirmations of the interaction of plasmoredoxin and the target proteins were done using BIAcore surface plasmon resonance experiments.

Zusammenfassung

Der Malaria-Parasit *Plasmodium* entwickelt bemerkenswert hohe Resistenzen gegenüber einem Medikament nach dem anderen. Außerdem verlieren viele Insektizide, die gegen die Überträger-Moskitos eingesetzt werden, an Wirkung. Jahrelange Forschung an Impfstoffen gegen Malaria hat bisher nur wenige hoffnungsvolle Kandidaten erbracht und obwohl Wissenschaftler Ihre Bemühungen verstärken, ist eine effektive Impfung bestenfalls immer noch Jahre entfernt. Deshalb sind dringend neue Zielmoleküle für die Medikamentenentwicklung zu identifizieren, die zu alternativen Behandlungsmethoden führen können. Diese Situation vor Augen, waren es Ziele dieser Arbeit, i) das bifunktionelle Enzym Glukose-6-Phosphatdehydrogenase-6-Phosphogluconolactonase aus dem Pentosephosphatweg des Parasiten als potentiell wirkungsziel zu bestätigen, ii) den Kynurenin-Stoffwechselweg, insbesondere die Enzyme Indolamin 2,3-Dioxygenase (1 und 2) der Maus als Modell näher zu charakterisieren und iii) mehr über das Redox-Netzwerk des Malariaparasiten zu erfahren, um neue mögliche Zielmoleküle aufzuzeigen.

Die ersten zwei Schritte des Pentosephosphatweges werden in *Plasmodium falciparum* durch das Enzym Glukose-6-Phosphatdehydrogenase-6-Phosphogluconolactonase (*PfGluPho*) katalysiert. Dieses ist ein einzigartiges bifunktionelles Enzym, das bisher nur in *Plasmodien* gefunden wurde. Obwohl diesem Enzym im Pentosephosphatweg des Parasiten und damit auch in der Bekämpfung oxidativen Stresses eine enorme Bedeutung zukommt, ist das Enzym aus *P. falciparum* nicht besonders gut charakterisiert, da es bisher nicht kloniert werden konnte. Zum ersten Mal konnte *PfGluPho* jetzt kloniert und überexprimiert werden und das Genprodukt konnte bis zur Reinheit gebracht werden. Es wurde gezeigt, dass das rekombinante Enzym als Hexamer vorliegt, welches niedrigere K_m -Werte im Vergleich zu seinen humanen Orthologen aufweist, die einen bevorzugten Substratumsatz durch das parasitäre Enzym aufzeigen. Kinetische Untersuchungen zeigen, dass der Glukose-6-Phosphatdehydrogenase (G6PD)-Teil von *PfGluPho* einem geordneten Mechanismus folgt, bei dem NADP^+ das erste Substrat ist. Drei neue Inhibitoren, die den G6PD-Teil des Enzym im unteren mikromolaren Konzentrationsbereich hemmen, konnten gefunden werden und zeigten sich gegenüber Glukose-6-Phosphat und NADP^+ als nicht-kompetitiv. Somit zeigt diese Arbeit die Klonierung, heterologe Expression, biochemische und kinetische Charakterisierung von *PfGluPho* auf, sowie darüberhinaus die Kristallisation und erste, neue Inhibitoren.

Seit 30 Jahren ist es ein etabliertes Dogma, dass im Tryptophan-Katabolismus der erste Schritt des Kynurenin-Stoffwechselweges die 2,3-Doppelbindung des Indolrings des Tryptophans durch zwei Enzyme gespalten werden kann: einerseits durch Indolamin 2,3-Dioxygenase (IDO), andererseits durch Tryptophan 2,3-Dioxygenase (TDO). Kürzlich konnte mit IDO-2 ein drittes Enzym in der Maus und im Menschen entdeckt werden, das in der Lage ist, diese Reaktion zu vollziehen. In dieser Arbeit sind Daten zur Kinetik, pH-Stabilität, zu oligomeren Strukturen, sowie Besonderheiten der Sekundärstruktur von rekombinanter IDO-2

der Maus im direkten Vergleich mit rekombinanter IDO-1 der Maus erhoben worden. Die Suche nach neuen, effektiveren Inhibitoren für IDO-1, die den Indolkern nicht mehr besitzen und somit weniger neurotoxisch sein könnten, führte zu 55D11 (K_i 0,05 μ M), einer Substanz aus einer öffentlichen Sammlung von Naturstoffen. Zahlreiche Derivate von 55D11 wurden untersucht, um Molekülreste zu zeigen, die verändert werden können und die Aktivität noch erhöhen. Die Untersuchungen zeigen, dass sich IDO-1 und IDO-2 eindeutig hinsichtlich ihrer Affinität zu Substraten, aber auch in ihrer Struktur unterscheiden.

Der Malaria-Parasit *Plasmodium falciparum* besitzt ein funktionelles Glutathion-, sowie ein Thioredoxin-System, die unter anderem aus den redox-aktiven Proteinen Thioredoxin (Trx), Glutaredoxin (Grx) und Plasmoredoxin (Plrx) bestehen, die alle zur sogenannten Thioredoxin-Superfamilie gehören und das Motiv Cys-X-X-Cys im aktiven Zentrum besitzen. Wenn mehr Interaktionspartner dieser Redoxproteine bekannt wären, könnte die jeweilige Rolle der Redoxine in ihrem Netzwerk besser verstanden werden. Deshalb wurden Thioredoxin-Affinitätschromatographien an CNBr-aktivierter Sepharose durchgeführt. Hierzu wurden immobilisierte Mutanten der Redoxine, denen das sogenannte „*resolving cysteine*“ aus ihrem aktiven Zentrum fehlt, mit Zellextrakt aus *P. falciparum* versetzt. Kovalent an die Fängerproteine (Redoxine) gebundene Reaktanden wurden mit Dithiothreitol eluiert und mittels (Maldi-ToF) analysiert. Einundzwanzig potentielle Zielproteine wurden für Plasmoredoxin als mögliche Interaktionspartner identifiziert. Neben bekannten Interaktionspartnern waren darunter auch Kandidaten, die eine mögliche Redoxregulierung der Proteinbiosynthese, des Energiemetabolismus sowie der Signaltransduktion in *Plasmodium* vermuten lassen können. Weitere Untersuchungen, um diese Wechselwirkungen zu bestätigen, wurden mit Plasmoredoxin und einigen seiner potentiellen Interaktionspartner mithilfe von BIACORE Oberflächen-Resonanzexperimenten durchgeführt.

Table of Contents

Dedication	ii
Acknowledgements.....	v
List of Publications.....	vi
Summary.....	vii
Zusammenfassung.....	ix
Table of Contents	xi
List of Figures.....	xv
List of Tables	xvii
List of Abbreviations.....	xviii
 1 Introduction	 1
1.1 Malaria.....	1
1.1.1 Life Cycle of <i>Plasmodium</i> Parasite	3
1.1.2 Oxidative Stress and Antioxidant Defense in Malaria Parasites	5
1.2 Rationale of the Study.....	6
1.2.1 Glucose-6-Phosphate Dehydrogenase	6
1.2.1.1 Glucose-6-Phosphate Dehydrogenase and the Pentose Phosphate Pathway	7
1.2.1.2 G6PD Deficiency and Malaria	8
1.2.1.3 Glucose-6-Phosphate Dehydrogenase-6-Phosphogluconolactonase from <i>P. falciparum</i>	12
1.2.2 Tryptophan Metabolism: Kynurenine Pathway.....	14
1.2.3 Thioredoxin Networks: Glutaredoxin, Thioredoxin and Plasmoredoxin	17
1.3 General Objective.....	18
1.3.1 Specific Objectives	18
1.3.1.1 Glucose-6-phosphate Dehydrogenase-6-Phosphogluconolactonase from <i>P. falciparum</i>	18
1.3.1.2 Tryptophan Metabolism	19
1.3.1.3 Thioredoxin Networks: Glutaredoxin, Thioredoxin and Plasmoredoxin	19
 2 Materials	 20
2.1 Chemicals	20
2.2 Antibodies	21
2.3 Enzymes	21
2.4 Antibiotics Concentrations.....	21
2.5 Kits.....	22
2.6 Biological Materials	22
2.6.1 Plasmids.....	22
2.6.2 <i>Escherichia coli</i> Cells.....	22

2.6.3	<i>Plasmodium falciparum</i> Strains	22
2.7	Buffers and Solutions	23
2.7.1	Buffers for Determination of Enzyme Activity	23
2.7.2	Buffers and Solutions for DNA Electrophoresis	23
2.7.3	Buffer for Extraction of <i>P. falciparum</i> Parasites from Red Blood Cells	23
2.8	Protease Inhibitors	24
2.9	Medium for <i>E. coli</i> Culture	24
2.10	Instruments	24
3	Methods	25
3.1	General Methods	25
3.1.1	Preparation of Competent <i>E. coli</i> Cells	25
3.1.2	Preparation of Competent <i>E. coli</i> Cells Containing pRARE or pRIG Plasmid	25
3.1.3	Transformation of Competent <i>E. coli</i> Cells	25
3.1.4	SDS-Polyacrylamide Gel Electrophoresis	25
3.1.5	Western Blot	26
3.1.6	Gel Filtration	28
3.1.7	Protein Crystallisation	28
3.2	<i>PfGluPho</i> Methods	29
3.2.1	Cloning of <i>PfGluPho</i>	29
3.2.2	Mutagenesis PCR	31
3.2.3	Heterologous Overexpression of <i>PfGluPho</i>	34
3.2.4	Optimisation of the Heterologous Overexpression of <i>PfGluPho</i>	34
3.2.5	Purification of <i>PfGluPho</i>	35
3.2.6	Optimisation of the Purification of <i>PfGluPho</i>	35
3.2.7	Gel Filtration of <i>PfGluPho</i>	35
3.2.8	Western Blot Using <i>PfGluPho</i> Specific Antibody and <i>P. falciparum</i> Proteins	35
3.2.9	<i>PfGluPho</i> 's G6PD Assays	35
3.2.9.1	Determination of K_m Values for G6P, NADP ⁺ and Steady State Kinetics	35
3.2.9.2	<i>PfGluPho</i> 's G6PD Assays with the Substrate Analogue 2-Deoxyglucose-6-Phosphate	37
3.2.9.3	<i>PfGluPho</i> 's G6PD Inhibition with NADPH, Glucoseamine-6-Phosphate and ATP-Ribose	37
3.2.9.4	<i>PfGluPho</i> 's G6PD Assays with GSSG, GSH and <i>PfGR</i>	37
3.2.9.5	<i>PfGluPho</i> 's G6PD pH, Buffers and Salt Profile	37
3.2.9.6	Test for G6PD Activity in Full Blood	37
3.2.10	<i>PfGluPho</i> 's 6-PGL Assays	38
3.2.10.1	Determination of the K_m for 6-PGL	38
3.2.10.2	<i>PfGluPho</i> 's 6-PGL Assays in the Presence of GSSG and GSH	38
3.2.11	<i>PfGluPho</i> Crystal Screening	39

3.2.13	<i>Pf</i> GluPho Inhibitor Screening	39
3.3	Human 6-PGL Methods	40
3.3.1	Heterologous Overexpression of Human 6-PGL.....	40
3.3.2	Purification of Heterologously Overexpressed Human 6-PGL	40
3.3.3	Human 6-PGL Assay.....	40
3.4	MouseIDO-1 and MouseIDO-2 Methods	40
3.4.1	Heterologous Overexpression of Recombinant Mouse IDO-2	40
3.4.2	Optimisation of Rhamnose for Heterologous Overexpression of Mouse IDO-2 ..	40
3.4.3	Purification of Heterologously Overexpressed Mouse IDO-2	41
3.4.4	Heterologous Overexpression of Recombinant Mouse IDO-1	41
3.4.5	IDO Assay Methylene Blue/ Ascorbic Acid Method	41
3.4.6	IDO Assay Cytochrome b ₅ / Cytochrome P450 Method	41
3.4.7	Determination of pH and Salt Profiles.....	42
3.4.8	Gel Filtration of Heterologously Overexpressed Mouse IDO-1 and IDO-2.....	42
3.4.9	Protein Modelling of Mouse IDO-2	42
3.4.10	Inhibitor Screening for Mouse IDO-1	43
3.5	Thioredoxin Networks Methods	44
3.5.1	Site Directed Mutagenesis of <i>Pf</i> Plasmoredoxin	44
3.5.2	Heterologous Overexpression and Purification of <i>Pf</i> Plasmoredoxin	44
3.5.3	Preparation of <i>Pf</i> Plasmoredoxin-Immobilised Resin	44
3.5.4	Collection of Target Proteins by Immobilised <i>Pf</i> Plrx ^{C63S} Mutant	45
3.5.5	Protein Bands Excision, Digestion and Identification	45
3.5.6	Biomolecular Interaction Analysis using Biacore ® X System.....	46
3.5.6.1	Biacore ® X System	46
3.6	<i>P. falciparum</i> Cell Culture Methods	47
3.6.1	Maintainance of <i>P. falciparum</i> Parasites in Cell Culture	47
3.6.2	Synchronisation	47
3.6.3	Drug Sensitivity Assays.....	47
3.6.4	Preparation of <i>P. falciparum</i> Proteins from Cell Culture	49
4	Results	50
4.1	<i>Pf</i>GluPho.....	50
4.1.1	Heterologous Overexpression and Purification of Recombinant <i>Pf</i> GluPho.....	50
4.1.2	<i>Pf</i> GluPho Oligomerisation Studies.....	51
4.1.3	<i>Pf</i> GluPho's G6PD Kinetic Studies	54
4.1.4	<i>Pf</i> GluPho's G6PD Alternative Substrate Studies	54
4.1.5	<i>Pf</i> GluPho's G6PD Glucoseamine 6-Phosphate Inhibition	55
4.1.6	<i>Pf</i> GluPho's G6PD ATP-Ribose Inhibition.....	55
4.1.7	<i>Pf</i> GluPho's G6PD NADPH Inhibition	58
4.1.8	<i>Pf</i> GluPho's G6PD Assay with GSSG, GSH and <i>Pf</i> GR.....	59

4.1.9	<i>Pf</i> GluPho's G6PD Buffers, pH and Salt.....	59
4.1.10	<i>Pf</i> GluPho's 6-PGL Kinetic Analysis.....	59
4.1.11	<i>Pf</i> GluPho's 6-PGL Assay in the Presence of GSSG and GSH.....	61
4.1.12	<i>Pf</i> GluPho Crystal Screening.....	61
4.1.13	<i>Pf</i> GluPho Structure Prediction	61
4.1.13.1	Structure Prediction: Model of <i>Pf</i> GluPho's G6PD.....	61
4.1.13.2	Structure Prediction: Model of <i>Pf</i> GluPho's 6-PGL.....	63
4.1.14	Screening for <i>Pf</i> GluPho's G6PD Inhibitors	64
4.2	Mouse IDO-1 and Mouse IDO-2	66
4.2.1	Heterologous Overexpression of Mouse IDO-2	66
4.2.2	Optimisation of Heterologous Overexpression of Mouse IDO-1	66
4.2.3	Kinetic Assays for Mouse IDO-1 and Mouse IDO-2	67
4.2.4	Test for Buffers, pH and Salt for Mouse IDO-1 and Mouse IDO-2.....	68
4.2.5	Spectral Characteristics of Mouse IDO-1 and Mouse IDO-2.....	69
4.2.6	Oligomerisation Studies of Mouse IDO-1 and Mouse IDO-2.....	69
4.2.7	Crystal Screening of Mouse IDO-1 and Mouse IDO-2	70
4.2.8	Structure Prediction of Mouse IDO-2.....	70
4.2.9	Mouse IDO-1 and Mouse IDO-2 Inhibition Using Substrate Analogues.....	72
4.2.10	Screening for Mouse IDO-1 Inhibitors.....	72
4.3	Thioredoxin Networks	76
5	Discussion.....	79
5.1	<i>Pf</i>GluPho's G6PD.....	79
5.1.2	<i>Pf</i> GluPho's 6-PGL.....	82
5.2	Mouse IDO-1 and Mouse IDO-2	84
5.3	Thioredoxin Networks	86
	References	88