CHAPTER 8

Summary, general discussion, and future prospects
The clinical hallmark of the patients described in this thesis is chronic hemolysis. Regardless of the kind of underlying inherited enzymopathy, the here described deficiencies of hexokinase (HK), pyruvate kinase (PK), and glucose-6-phosphate dehydrogenase (G6PD) ultimately resulted, albeit to a various extent, in shortened red blood cell survival because “metabolically defective red blood cells are old before their time, and suffer from metabolic progeria” (Valentine W.N. and Paglia D.E. Blood Cells. 1980; 6: 819-829). All three mentioned enzymes are key enzymes of the Embden-Meyerhof Pathway (HK and PK) and the Hexose Monophosphate Shunt (G6PD). The underlying mechanisms by which these deficiencies cause hemolysis, however, are distinct. The main cause of hemolysis in either HK or PK deficiency is a depletion of the cellular ATP content whereas the G6PD-deficient erythrocyte is more susceptible to oxidative damage. To induce a clinically significant hemolysis, red blood cell enzyme function must be significantly impaired under physiological conditions. Thus, in compound heterozygous patients overt hemolysis assures that, at least, one gene product is severely defective. The focus of this thesis was to identify the molecular mechanisms by which inherited enzymopathies lead to impaired enzyme function. This constitutes the primary basis of the associated hemolytic disease. The molecular defects underlying the above-mentioned enzyme deficiencies were found to affect correct human gene expression (chapter 1) either at the level of transcriptional regulation (chapter 2), pre-mRNA processing (chapter 3), or protein structure (chapters 4 and 5) and function (chapters 6 and 7).

Enzymopathy due to defects at the level of transcriptional regulation
In chapter 2 we described a severely PK-deficient patient. In reticulocyte RNA from this patient we detected a monoallelic pattern of gene expression. This led to the identification of a novel transcriptional regulatory element in the erythroid-specific promoter of PKLR. RNA from the patient demonstrated sole expression of the paternal c.1529G>A missense PKLR allele. On the DNA level, however, the boy was heterozygous for this missense mutation. Moreover, 3 novel in cis DNA sequence variations were identified on the maternal allele in the erythroid-specific promoter of PKLR: −324T>A, −248delT, and −83G>C. We established the functional consequences of each individual mutation using in vitro transfection assays in K562 cell line. Hence, we demonstrated that −324T>A was a non-functional mutation whereas −248delT represented a non-functional polymorphism. This polymorphism had previously been associated with PK deficiency by others because it disrupts a binding site for the erythroid-specific transcription factor GATA-1. We clearly demonstrated, however, that the concerning GATA-1 binding site was non-functional. The −83G>C mutation caused effective downregulation of the PKLR promoter, thereby explaining the pattern of
monoallelic gene expression of the \textit{in trans} allele. Systematic mutagenesis of the region surrounding nt –83 disclosed the presence of a novel \textit{cis}-regulatory element, which we designated PKR-RE1. To confirm that this novel element was actively involved in DNA-protein interaction we performed electrophoretic mobility shift assay using K562 nuclear extract. These experiments demonstrated \textit{in vitro} DNA-protein interaction at the core of PKR-RE1, involving an as-yet-unidentified \textit{trans}-acting factor. Because no known protein is known to recognize the PKR-RE1 motif, we concluded that PKR-RE1 may be involved in a novel mechanism of erythroid-specific \textit{trans} activation, mediating the effects of factors necessary for regulation of PK gene expression during red cell differentiation and maturation. The monoallelic expression of the 1529A allele rendered this particular patient ‘pseudo-homozygous’ for c.1529G>A, a missense mutation that predicts the substitution of arginine by glutamine at residue 510. It was, therefore, reasonable to assume that the PK-deficient phenotype, \textit{i.e.} severe, transfusion-dependent hemolytic anemia, of the patient described in \textbf{Chapter 2} could be attributed to the sole synthesis of the Arg510Gln PK-R variant. As demonstrated in \textbf{Chapter 5}, the introduction of a glutamine residue at position 510 in the A/C domain interface causes reduced conformational freedom between these two domains. This may explain the very low activity and striking instability that is usually associated with this common PK-deficient variant. It is of interest that the severe clinical picture of the ‘pseudo-homozygous’ patient in \textbf{Chapter 2} was remarkably different from that of patient MB86, described in \textbf{Chapter 4}. The latter, mildly affected patient was homozygous for the c.1529G>A mutation and had never been transfusion-dependent. This may indicate that in spite of the evoked structural perturbation, some residual enzymatic function is still maintained in the Arg510Gln PK-R variant. Thus, the amount of residual enzymatic activity would be higher in the homozygous patient because about twice the amount of aberrant protein is synthesized from the two defective alleles whereas in the ‘pseudo-homozygous’ patient only one allele is actively involved in protein synthesis. 

\textbf{Chapter 2} emphasizes the need for an as-complete-as-possible molecular characterization in general. Molecular diagnostic approaches that are confined to the protein coding region of the gene or use only cDNA transcribed from a patient’s RNA, in this case would either have missed the transcriptional mutant or misdiagnosed the patient as homozygous for the c.1529G>A missense mutation.

\textbf{Enzymopathy due to defects at the level of pre-mRNA processing} 

The synthesis of only one species of mutant PK, in spite of a compound heterozygous genotype, was also demonstrated in another patient with severe PK deficiency. In this case the production of mutant Arg479His PK-R was due to aberrant processing of \textit{PKLR} pre-
mRNA. This patient, described in *chapter 3*, was compound heterozygous for two mutations which were both likely to affect correct splicing. One novel mutation abolished the invariant dinucleotide of intron 5 (IVS5+1G>A) whereas the other mutation (c.1436G>A) altered the exonic consensus sequence of IVS10 and, in addition, encoded an arginine to histidine substitution at residue 479. A novel approach was employed to study the effects of both mutations on pre-mRNA processing. The main feature of this approach constituted the use of *ex vivo* produced nucleated erythroid cells from the patient as a source of RNA. This approach allowed for a more profound analysis of the effects on pre-mRNA processing, as demonstrated by the detection of multiple aberrant transcripts, associated with both mutations but also, for the first time, in wild-type PKLR pre-mRNA. As anticipated, one of the predominant transcripts associated with the IVS5+1G>A mutation lacked the upstream exon 5. Unexpected, however, was the identification of the second major transcript associated with this mutation. This nonsense transcript lacked both exons 5 and 6 (see below). No functional protein was expected nor detected (by Western blotting) from either transcript, which rendered the IVS5+1A allele *de facto* a null allele. The c.1436G>A mutation in this patient was mainly associated with a strong reduction in transcript levels although the majority of these transcripts were normally spliced. Such a strong reduction of transcripts may be the reflection of increased instability of the major part of mutant transcripts derived from the 1436A allele. However, because the processes of transcriptional regulation and pre-mRNA processing are intimately related, an additional effect of the c.1436G>A mutation on transcription cannot be excluded. Altogether, the PK-deficient phenotype in this patient could mainly be attributed to the presence of strongly reduced amounts of Arg479His PK-R. We confirmed this by Western blot. The sole presence of Arg479His PK-R in this patient enabled a direct comparison with the crystal structure and enzymatic properties of the same recombinant PK variant as expressed in *Escherichia coli*. The structural data were in agreement with our own observations but a striking difference was noted with regard to the heat stability of both mutant PK variants. We had demonstrated that the patient’s PK showed reduced thermal stability *in vitro*. This was in agreement with a remarkable sensitivity to heat displayed by the patient during febrile episodes or even prolonged exposure to sunlight. These two observations suggested a decreased stability of the Arg479His PK tetramer. The recombinant Arg479His PK variant, however, showed nearly unaffected thermal stability. This discrepancy resembles a similar contradiction between enzymatic properties of mutant human enzyme and its recombinant analog observed in a case of hexokinase deficiency (*chapter 7*) and will be further discussed below (*Enzymopathy due to defects at the protein level – protein function*). It emphasized, however, that knowledge obtained from recombinant (mutant) enzymes should be interpreted.
with care when applied to their human analogs.

As mentioned above, the composition of one transcript identified in the patient described in chapter 3 was quite unusual. During pre-mRNA processing, two consecutive exons (exons 5 and 6) had been simultaneously skipped from the pre-mRNA transcribed from the allele with the IVS5+1G>A mutation. These two events were likely related and led us to propose a mechanism in which correct processing of exon 6 in wild-type PKLR pre-mRNA depends on the presence of exon 5 in the primary transcript. In other words, in wild type PKLR pre-mRNA, exon 5 contains regulatory splicing elements necessary for proper processing of exon 6. It is obvious that due to a mutation at the intron 5 donor splice site and the subsequent removal of the entire exon 5, the putative regulatory elements were automatically skipped from the pre-mRNA, leading to inefficient inclusion of the sixth exon. The presence of a splice enhancer element in PKLR exon 5 automatically implies that other base changes in this exon, for example the missense mutations as described in chapter 4, may be capable of disturbing correct pre-mRNA processing. For further discussion, see Future prospects.

**Enzymopathy due to defects at the protein level – protein structure**

The major molecular defect underlying the hereditary enzymopathies are single-base changes that predict the substitution of a structurally and functionally important amino acid. This is especially true in case of PK and G6PD deficiency (chapter 1) and our results obtained from a large group of patients with PK deficiency, described in chapter 4, are in agreement with this observation of missense mutations being predominant. The 28 patients studied and reported in chapter 4 originated from 26 different families. We identified 24 different alleles associated with PK deficiency and 14 of these mutations were novel. Of the 24 mutant alleles, 20 carried a missense mutation (83%), including 12 novel ones. This is slightly higher than the global distribution of missense mutations which is approximately 70% (chapter 1). The four remaining mutations were nonsense mutations (two mutations, including one novel) or predicted to affect pre-mRNA processing (two mutations, including one novel). The frequently detected c.331G>A mutation was of particular interest. This mutation predicts the unfavorable substitution of glycine by arginine at residue 111 (PK Utrecht) and was identified in five unrelated Dutch families. This meant that the novel c.331G>A mutation constituted a major cause of PK deficiency in The Netherlands. More specific, together with the common mutations c.1456C>T and c.1529G>A, c.331G>A accounted for nearly 50% of mutant PKLR alleles in The Netherlands. When applied to diagnostic procedures, we recommend that an efficient initial screening for PKLR mutations in Dutch PK-deficient patients should include these 3 mutations.

Ideally, the effects of missense mutations on protein structure and function should be
examined using purified mutant PK from the patient. However, apart from the practical limitations, a mutant enzyme purified from red blood cells of the patient may be unstable or lack enzymatic activity, thereby hampering further biochemical characterization. The variant G6PD Utrecht described in chapter 6 is an illustration of the latter. Furthermore, in case of PK deficiency most patients are compound heterozygous. Hence, when both genes are actively producing a mutant monomer, five different tetrameric forms can be assembled. This limits the value of biochemical characterization considerably. An alternative approach comprises the expression of the recombinant mutant enzyme. In that case, especially with regard to the evaluation of enzymatic properties, chapters 3 and 7 have demonstrated that significant differences in enzymatic behaviour may be observed when comparing recombinant human enzymes with enzymes obtained from the patient. An initial approach to elucidate the mechanism by which amino acid substitutions may lead to impaired enzymatic function involves the evaluation of the concerning residue and the interactions in which it is involved in the three-dimensional model of the crystal structure. The tetrameric crystal structure of human erythrocyte R-state PK has recently been elucidated and we used this model in chapter 4 to study the consequences of the novel amino acid changes on structure and function of PK. By doing so we obtained additional structural support to affirm the association of the respective mutations with PK deficiency. The effects of some mutations were quite clear because they concerned the substitution of amino acids buried in the hydrophobic core of the protein. Others altered the conformation of the B domain near the active site or directly affected the ATP binding site. Provided that stable protein is synthesized, these mutations may significantly alter PK enzymatic activity. Because the allosteric regulation of PK is considered to be regulated by interdomain and intersubunit interactions (chapter 1), a group of mutations that affected those regions were of particular interest. One of these amino acid changes altered the C/C’ subunit interface, Arg569Gln, whereas three mutations predicted a disruption of the A/A’ subunit interface: Arg337Trp, Gly358Glu, and Arg385Lys. More specific, the mutations regarding residues Arg337 and Arg385 may disrupt the formation of salt bridges, crucial for the T- to R-state allosteric transition of the tetrameric enzym. Again, provided that stable PK monomers are synthesized. The Gly358Glu substitution was the only mutation that could be directly correlated to the patient’s phenotype because of homozygosity of this patient for the causative c.1073G>A mutation. The patient was severely affected and transfusion-dependent, thereby confirming the detrimental effects of this mutation at the A/A’ subunit interface. Unfortunately, further biochemical characterization of enzymatic properties of this mutant PK was not possible because the, Syrian, patient was not available for further study. Our investigations reported in chapter 4 confirmed the association of the common
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c.1456C>T mutation with, in general, mild clinical expression of PK deficiency. This mutation encodes a PK-R variant characterized by an arginine to tryptophan substitution at residue 486 in the A/C subdomain interface. In this critical region of PK, replacing Arg486 with tryptophan is an amino acid change with potentially dramatic consequences. Interestingly, in the three-dimensional model, a neighbouring residue of Arg486 is Gly364. While the drastic Arg486Trp change causes little structural perturbation and, consequently, leaves the enzymatic properties nearly unaffected, a similar drastic amino acid substitution at residue 364 (Gly364Asp) caused severe PK deficiency and ultimately led to death in a homozygous patient (chapter 5). This illustrates that the impairment of enzymatic function is strongly dependent not only on the location of the replaced amino acid but also on the amino acid with which it is replaced. A similar example is described in chapter 6 and concerns two different G6PD variants that are both characterized by a perturbation of the same critical region of the dimeric enzyme (see below). In chapter 5, the above-mentioned mutation that predicted the Gly364Asp substitution concerned a novel c.1091G>A mutation, identified in the homozygous state in the deceased patient. Because, at that time, the human crystal structure had not yet been elucidated, the predicted structure of human R-state PK-R was developed based on the homologous crystal structures of R-like muscle PK from cat and rabbit. A detailed structural analysis was made of the effects of this mutation by molecular modeling of aspartate at residue 364 of the PK monomer. We were able to demonstrate that, located at the A/C domain interface, aspartate at residue 364 in the A domain interacts with the backbone carbonyl of Gln482 of the C domain and introduces an alternative inter-domain salt bridge with Arg488. As a result, the critical conformation of the A/C interface is disrupted. Our results regarding the inter-domain interactions at the A/C interface being critical for stability of the protein as well as the deleterious effect of the Gly364Asp substitution were recently confirmed by the elucidation of the crystal structure of human Gly364Asp PK-R (Valentini G. et al. Journal of Biological Chemistry. 2002; 17: 23807-23814). The homozygous patient described above was the first child from the mother’s second marriage. The mother had the misfortune to re-marry a second husband who was, again, heterozygous for PK deficiency. Her first husband was also heterozygous and the child born from this first marriage was compound heterozygous for the c.1091G>A and the c.1529G>A mutations. This child was severely affected and required a blood transfusion every three months at the time this study was conducted. He showed clinical symptoms that were comparable with the clinical symptoms of two other patients from a second family who were also compound heterozygous for the same combination of mutations in PKLR (chapter 5). A comparison of these PK deficiency-associated phenotypes may lead to the conclusion that the co-inheritance of the 1529A allele confers some residual enzyme stability or
function that is lost upon inheritance of two 1091A alleles. This is in agreement with the beneficial ‘additive effect’ of the Arg510Gln PK-R variant as proposed above.

**Enzymopathy due to defects at the protein level – protein function**

Dramatic changes in the enzyme’s structure brought about by significant structural perturbation are obviously also likely to result in altered enzymatic activity, most frequently in a negative manner. The location and interactions of the affected amino acid, as well as the nature of the introduced substitution are important determinants for the resulting molecular perturbation and its impact on protein function. This has been mentioned above with regard to two drastic substitutions in PK (Gly364Asp and Arg486Trp) that led to differently impaired enzyme function, and is further illustrated by the study described in chapter 6. In this study, two patients with severe G6PD deficiency were investigated at the molecular level. One patient suffered from severe chronic hemolytic anemia whereas in the other patient only low level, subclinical, chronic hemolysis could be detected. Molecular analysis revealed that both patients carried a variant G6PD gene on their X-chromosomes. The respective mutations had arisen de novo, either in early embryonic development or in the maternal germline. The amino acid substitutions Pro409Ser (G6PD Utrecht) and Val431Gly, predicted by the mutations, both affected the G6PD dimer interface. Formation of the G6PD dimer is crucial for G6PD enzymatic activity and nearly all mutations that affect the dimer interface cause the most severe form of G6PD deficiency, the one that is associated with chronic nonspherocytic hemolytic anemia (class I G6PD deficiency) (chapter 1). We studied the two mutations in the three-dimensional model of human G6PD. This revealed that Pro409 and Val431 were also located in the dimer interface and moreover, being located on different subunits, interacted directly across the subunit interface. It was shown that Pro409 makes inter-subunit contacts with Tyr424, Tyr428, and Val431, and that mutation to serine would disrupt some of these contacts. Likewise, inter-subunit contacts of Val431 with Pro409 were likely to be disrupted upon mutation of Val431 to glycine. Moreover, the Pro409Ser and Val431Gly mutations both introduced a residue that allows for greater main chain flexibility, which could destabilize subunit interactions. We concluded that both the Pro409Ser and Val431Gly substitutions could weaken dimer interactions, thereby compromising the stability and enzymatic activity of G6PD. On one hand, these observations, when combined with the different phenotypes of both patients, pointed out the importance of both the position as well as the nature of amino acid replacement with regard to the extent of chronic hemolysis. On the other hand, however, they also clearly demonstrated that the ultimate outcome of identical amino acid substitutions with regard to the clinical phenotype is not solely dependent on the amino acid substitution itself because the phenotype of the patient...
with the Val431Gly substitution described in chapter 6, differed markedly from the only other patient described to date that carried this G6PD variant. This other patient displayed persistent chronic jaundice since birth, splenomegaly, mild chronic anemia, and reticulocytosis. In contrast, the patient we described showed no neonatal jaundice and had been clinically normal until he presented with favism at age 29 months. Therefore, like the observed differences in expression of the Arg510Gln PK-R variant (chapters 2 and 4), we concluded that the clinical picture also depended on other, genetic, factors.

Chapter 6 demonstrates that differences in chronic hemolysis exist among patients with different class I G6PD variants and even among patients with the same G6PD variant. This underscores the need for a re-evaluation of the G6PD deficiency classification system (chapter 1) which is based on residual G6PD enzymatic activity and clinical features. The homozygous PK-deficient patient in chapter 5 died of severely impaired enzyme function caused by a dramatic perturbation of a critical inter-domain interface. The G6PD-deficient patients described in chapter 6 displayed different phenotypes upon a drastic molecular perturbation of the G6PD subunit interface. In chapter 7 we showed that also subtle alterations in crucial areas of the enzyme can cause a severe impairment of enzyme function in vivo, leading to chronic hemolysis. The patient described in chapter 7 had been diagnosed with severe HK deficiency 20 years ago and we recently elucidated the molecular basis underlying this rare case of HK deficiency. Previously, the enzymatic properties of purified mutant HK from the patient had been biochemically characterized and had revealed a two-fold decrease in affinity for Mg\(^2\)+-ATP and a markedly (3- to 9-fold) decreased affinity for inhibitor glucose-1,6,-diphosphate. We could easily correlate these features to the now established molecular defect in the homozygous patient. We detected a novel c.2039C>G missense mutation which predicted the substitution of active site residue Thr680 by serine (HK Utrecht). Although structural changes due to this conservative substitution were likely to be limited, structural analysis using the three-dimensional model of human HK1 displayed a central position of the absolutely conserved Thr680 in the active site. In the active site, Thr680 interacts with phosphate moieties of ADP/ATP and glucose-6-phosphate. Hence, the removal of a methyl group upon mutation from threonine to serine could cause small conformational changes, resulting in altered binding of phosphate-containing ligands and illustrating the delicate balance of interactions that enzymes typically require for optimal activity. The only 2-fold higher \(K_m\) for Mg\(^2\)+-ATP reflected the relatively modest effect of the Thr680Ser substitution on ATP binding to the active site. We anticipated a more significant effect in vivo, however, because of the severe clinical picture of the patient. A plausible explanation was provided by intracellular Mg\(^2\)+-ATP concentrations of the patient, as measured in previous studies. These concentrations were much lower in vivo, and close to
the $K_m$, than the excess Mg$^{2+}$-ATP concentrations used for HK activity measurements \textit{in vitro}. We postulated that at physiological concentrations of Mg$^{2+}$-ATP, the two-fold increase in $K_m$ for Mg$^{2+}$-ATP results in significantly decreased HK activity in the patient’s red blood cells.

Strikingly, again the enzymatic properties of the human red blood cell HK contradicted with published kinetic data obtained from a recombinant human brain HK (HK-I) that harboured the same mutation. The recombinant mutant HK was characterized solely by a slightly higher affinity for glucose, whereas the $K_m$(ATP), and $K_i$(G6P) were unaffected. These unexpected discrepancies in kinetic behaviour between human mutant red blood cell HK and recombinant mutant HK-I may be attributed to the different sources of the respective enzyme, \textit{i.e. Escherichia coli versus} human red blood cells. An alternative explanation, however, may be provided by the differences in N-terminal ends of red blood cell HK and HK-I. Human red blood cell HK and HK-I are transcribed from the same gene and both proteins are identical except for their N-terminal ends. Similarly, human PK-R and PK-L are transcribed from the same gene and both proteins are identical except for a unique peptide that is only present at the N-terminal end of PK-R. The differences in enzymatic properties between the two HK mutants is reminiscent of similar discrepancies between two Arg479His PK-R mutants as observed in \textit{chapter 3}. In this case, to avoid partial proteolysis of the recombinant enzyme, a truncated mutant PK-R was produced. This protein lacked 49 amino acids from its N-terminal end, including the erythroid-specific peptide. Although \textit{in vitro} enzymatic activity was essentially unaffected in case of the truncated PK-R (\textit{de facto} PK-L), the observed discrepancies between two otherwise identical proteins might point at a functional role for the erythroid-specific N-terminal peptides of HK and PK \textit{in vivo}.

It has been suggested that in PK, structure and function are so finely tuned that even moderate molecular alterations may significantly perturb cell metabolism. Among the single amino acid changes described in \textit{chapter 4}, one was postulated to cause little, if any, structural perturbation. This concerned a conservative leucine to valine substitution at the surface of the protein. The underlying mutation, c.814C>G (Leu272Val), was detected by chance in an asymptomatic individual who, moreover, displayed normal PK enzymatic activity. Enzymatic activities as determined under artificial conditions in the laboratory, may not always truely reflect the behaviour of aberrant enzymes \textit{in vivo}, and the Leu272Val PK-R variant may cause a very mild form of PK deficiency. The individual carrying this variant originated from an African country, Ghana, and in this area of the world, (severe) PK deficiency is very rare. Interestingly, very recently it was reported that PK deficiency in mice protects against malaria (Min-Oo, G. \textit{et al.} Nature Genetics. 2003; 35: 357-362). The defense mechanism itself remains unclear but the PK-deficient mice displayed splenomegaly,
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constitutive reticulocytosis, and were resistant against *Plasmodium chabaudi* infection. Thus, PK deficiency contributes to the complex genetic control mechanism in humans that controls the susceptibility of infection with *Plasmodium* species as well as the ultimate outcome of the disease. It is therefore tempting to speculate that the Leu272Val substitution, as identified in the Ghanese individual, represents a PK-R variant which is characterized by only slightly modified enzymatic properties, such that cellular survival is ensured but infection with *Plasmodium* species is withstood. Perhaps this is achieved in conjunction with other alleles that alter red blood cell metabolism and that have been retained by positive selection in areas of the world where malaria is an endemic disease. These other alleles include sickle-cell anemia, β-thalassemia, and G6PD deficiency.

It is worth noting that the PK deficiency itself in the malaria-resistant mice could be attributed to a single Ile90Asn amino acid substitution, which was encoded by the c.269T>A missense mutation. Coincidentally, we had identified the same missense mutation in two, related, compound heterozygous patients with severe PK deficiency, described in chapter 4.

**Future prospects**

Several aspects of the studies conducted in this thesis may be the subject of future research. Of considerable interest is the identification of PKR-RE1, a novel transcriptional regulatory element in the erythroid-specific promoter of *PKLR*, described in chapter 2. Electrophoretic mobility shift assay has demonstrated the binding of a *trans*-acting factor to this element and transfection experiments have demonstrated its functional importance. Obviously, a major aim for further study will be the identification of the putative transcription factor. Thus far, the core binding motif resembles no known transcription factor binding elements. This could imply that a novel *trans*-acting factor is involved or, alternatively, a known factor with yet unrecognized binding properties. The putative transcription factor has been demonstrated to play a crucial role in *PKLR* erythroid-specific transcription but may do so also in other, erythroid, genes. Future research should, therefore, consider a broad perspective and include other, erythroid-specific, promoters.

Discussing the putative aberrant effects of enzyme function is based upon the assumption that the respective protein is actually synthesized and stable enough *in vivo* to allow dimer (G6PD) or tetramer (PK) assembly. However, numerous examples are available in the literature that show that mutants proteins can be unstable. For a comprehensive understanding of the effect of a mutation, the quantitative determination of (mutant) protein would be of surplus value. Another important factor to consider in this respect is the, additional, effect that mutations may have on pre-mRNA processing. Such putative effects on RNA splicing have always been a major focus in our research and, if possible, the
appropriate experiments have always been conducted to exclude any such additional effect (chapters 5 and 7). Regarding the results of the RNA experiments conducted in chapter 3, we hypothesized the presence of an exonic splice enhancer element (ESE) in PKLR exon 5, required for the efficient inclusion of exon 6 in wild-type PKLR pre-mRNA. Future experiments using recombinant constructs and in vitro splicing assays may aim to confirm and identify the putative ESE(s) in PKLR exon 5, and the exact sequence motif(s) it comprises. The importance of considering additional effects on pre-mRNA processing is underscored by the recent general appreciation of the role of ESEs in constitutive splicing. Because ESEs constitute degenerate sequence motifs that may be spread throughout the exon, their important functional role implies that every other mutation that does not directly disrupt any splice site consensus sequence may still be a candidate mutation to affect correct pre-mRNA processing. In fact, the number of missense and synonymous mutations that do so is increasing. Because RNA was unavailable from most patients described in chapter 7, additional effects on pre-mRNA processing have not been taken into account. Especially with regard to amino acid substitutions Val134Asp, Ala154Thr, and Gly165Val which are predicted by missense mutations in PKLR exon 5, it should be excluded that the identified mutations do not cause additional effects on pre-mRNA processing. Considering the fact that ESEs may be present in many, if not all, human exons, a comprehensive approach to investigate the mechanism of disease should include RNA analysis.

The sophisticated allosteric mode of regulation of PK complicates the evaluation of the effect of molecular perturbation on the enzyme’s structure and function. A limitation in this respect is the often-mentioned fact that no T-state and R-state crystal structures are available from the same species. The latter would enable a more profound analysis of the effects of the appropriate mutations in both the T- and R-state enzymes, like for instance the amino acid substitutions Arg337Trp and Arg385Lys described in chapter 4. The recent elucidation of the crystal structure of R-state human erythrocyte PK has significantly contributed to a better understanding of the structure and function of PK. It would be of even greater benefit to the scientific community if both human crystal structures would become available.

In Africa, (severe) PK deficiency is very rare but malaria is an endemic disease. The recent report that PK deficiency in mice protects against malaria combined with the identification of a novel, putatively mild, Leu272Val PK-R variant in an individual from Ghana (chapter 4) may indicate that the two are connected. The c.814C>G mutation (Leu272Val) was not detected in a Caucasian normal control population and it will be of interest to screen a representative African control population. This may resolve the question whether the African PKLR 814G allele is either very rare, or a polymorphic variant that has remained undetected because of only subtle effects on enzymatic activity.
Twice, discrepancies were observed between the enzymatic properties of mutant enzymes (PK and HK) obtained either from the patient’s enzyme or from a recombinant enzyme (chapters 3 and 7). These differences may be attributed to the different sources of the enzyme but it is intriguing that both recombinant enzymes lacked the erythroid-specific N-terminal peptide. This may indicate that the N-terminal peptide has a functional role \textit{in vivo} and future studies may be directed towards the elucidation of this putative role.

The only cure available to patients with an inherited enzymopathy is bone marrow transplantation. This is only rarely performed because of its negative side effects. Instead, blood transfusion is the therapy of choice to alleviate the anemia and to protect patients from fatal hemolysis. With regard to PK, the PK-M2 isozyme is gradually replaced by the red blood cell-specific PK-R isozyme during erythroid differentiation and maturation (chapter 1). Persistent PK-M2 expression throughout erythroid maturation might be able to compensate, in part, for a defective PK-R isozyme and thereby ameliorate the hemolysis. Patients with sickle-cell anemia display a similar amelioration of clinical symptoms due to persistent expression of fetal hemoglobin (HbF). In certain PK-deficient patients, a persistent expression of PK-M2 has been detected but the beneficial effect is unclear. Also, the reason why this ‘compensation’ occurs in some patients but not in others is unknown. From a therapeutical point of view it might be worth to first resolve these questions, followed, if appropriate, by a detailed analysis of the transcriptional control mechanisms that drives PK-M2 expression throughout erythroid differentiation and maturation. Eventually, this may lead to clues regarding the means to upregulate PK-M2 expression in patients with severe PK deficiency.

\textit{Concluding remarks}

The variety of clinical features associated with the various enzymopathies, regardless of the underlying molecular mechanism, have unequivocally made clear that the phenotype of hereditary red blood cell enzymopathies, and perhaps genetic disease in general, is not solely dependent on the molecular properties of mutant proteins but rather reflects a complex interplay between physiological, environmental and other (genetic) factors. Putative phenotypic modifiers include differences in genetic background, concomitant functional polymorphisms of other glycolytic enzymes (many enzymes are regulated by their product or other metabolites), post-translational modification, ineffective erythropoiesis, and different splenic function.

It was the focus of this thesis, as a first step towards a better understanding of the correlation between genotype and phenotype, to identify the molecular basis of disease in hereditary human red blood cell enzymopathies. In summary, the investigated mutations cause disease...
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by means of a variety of mechanisms and they affect several aspects of gene expression. They may either abolish gene expression completely (chapter 2), or lead to aberrant RNA splicing events (chapter 3). Once a mutant enzyme is synthesized it may be unstable because of significant structural perturbation (chapters 4 and 5), or unable to assemble its proper active conformation (chapter 6). Even subtle changes may still significantly impair an enzyme’s function, especially at physiological conditions (chapter 7). It is not even excluded and far from unlikely that some mutations exert their effect via multiple of these molecular mechanisms. With regard to the patients described in this thesis we are far from understanding the factors that contribute to their complex phenotypes but, hopefully, we have shed that much light on the underlying molecular mechanisms of disease so that we can state: “That is why the patient is ill”.