ELSEVIER

Biophysical Chemistry 66 (1997) 269–284

Biophysical Chemistry

Erythrocyte Pyruvate Kinase- and Glucose Phosphate Isomerase Deficiency:

Perturbation of Glycolysis by structural defects and functional alterations of defective enzymes and its relation to the clinical severity of chronic hemolytic anemia.

Max Lakomek and Heinz Winkler
Universitäts-Kinderklinik und Poliklinik and Max Planck Institut für biophysikalische Chemie Göttingen, Germany

Dedicated to Prof.Dr.Manfred Eigen on the occasion of his 70th birthday

Received 10 April 1997; accepted 17 April 1997

Abstract

The pathogenesis of two metabolic disorders caused by enzyme defects in the red blood cell leading to hemolytic anemia, and in some cases of glucose phosphate isomerase (GPI) deficiency additionally to neurological impairment was investigated. Rheological studies were performed to determine the influence of a shortage of energy on the deformability of the erythrocytes. The *functions of the enzymes* were determined by studying the enzyme kinetics, the temperature dependence of the enzyme activity and the migration of the proteins in an electric field. A detailed molecular genetic analysis of the gene encoding for the given protein allowed the detection of mutations involving amino acid exchanges which cause *alterations of the protein structure*. For both enzyme deficiencies, a good correlation was found between the structural changes (usually caused by single point mutations in the gene), the altered function of the enzymes and the severity of the clinical picture. The exchange of amino acids close to either the active site or the regulatory domain results in a decreased turnover as well as an alteration of the regulatory properties of the enzymes; this usually leads to an increased severity of the disease. Increased concentrations of glucose-6-phosphate (G-6-P), found in all red blood cells of patients suffering from hemolytic anemia caused by pyruvate kinase (PK) and GPI deficiency, correlate well with the severity of the clinical picture, apparently reflecting the degree of the *perturbation of glycolysis*. This results in a lack of the energy donor adenosine triphosphate (ATP); this leads then to a destabilization of the red cell membrane which causes *earlier lysis of the red blood cell*, which in turn gives rise to hemolytic anemia of variable degrees. One patient with neurological symptoms has been studied so far biochemically and at the molecular genetic level. The point mutations found in this patient's GPI gene support the idea that GPI may have a *neurological function* in addition to its role in the carbohydrate metabolism; this is due to the presence of a monomeric sequence analogue called neuroleukin (NLK). The mutations apparently lead to the incorrect folding of this neurotrophic factor, and thus destroy the neurological activity. © 1997 Published by Elsevier Science B.V.

Keywords: Pyruvate kinase deficiency; glucose phosphate isomerase deficiency; neuroleukin; hemolytic anemia; neurological impairment; enzyme regulation; energy rich phosphates; molecular genetic analysis

PII S0301-4622(97) 00057-4

1. Introduction

Red blood cell formation in vertebrates is a complex process; it starts in the bone marrow with a nucleus containing proerythroblast. During the different steps of maturation the cells loose their nuclei and organelles, such as ribosomes and mitochondria. The erythrocyte, natures most effective transporter of oxygen and carbondioxid is the final product of this maturation. Its metabolism is designed for simply maintaining the cell structure, a specific composition of organic compounds, the cation flux between the red blood cell and the blood plasma, and to keep the hemoglobin functional. Therefore, only few metabolic pathways are necessary, such as glycolysis, the hexose monophosphate shunt, nucleotide metabolism and glutathione synthesis. The metabolic energy is supplied anaerobically with glucose as the exclusive nutrient; the key product for a proper function of the erythrocyte is adenosine triphosphate (ATP), which is produced during the course of glycolysis.

Pyruvate kinase (PK) deficiency and glucose phosphate isomerase (GPI) deficiency of the erythrocyte belong to a category of autosomal recessive hereditary disorders that affect the main energy production machinery of the red blood cell-glycolysis, thus causing earlier destruction of the erythrocyte (hemolysis). The life span of the erythrocyte becomes in general shortened from normally 100-120 days to below 15 days and therefore often transfusions are necessary. Together with glucose-6-phosphate dehydrogenase deficiency which belongs to the pentosephosphate cycle, these deficiencies are the most frequent enzyme defects occuring inside the red blood cell. In Europe, the USA, Canada and Japan the frequency of inherited hemolytic anemias caused by these enzyme defects is between 1:20.000 to 1:40.000. The patients suffer from typical signs of hemolytic anemia, such as general weakness, jaundice, cholelithiasis or splenomegaly, and in many cases frequent transfusions are required [1]. In some cases of GPI deficiency mental retardation, muscular weakness and ataxia accompany the disease; even immediate neonatal death has been reported in cases of severe GPI deficiency [2, 3, 4]. As those metabolic disorders seem to be a suitable model, considering the correlation between lack of energy and earlier death of the cell the first aim of our research project was to determine the mechanism of hemolysis that is caused by these enzymopathies of the red blood cell metabolism, and to search for possible correlations between altered protein structures, perturbed functions and the severity of the clinical aspects of the disease. First, the biochemical properties (altered enzyme activity, enzyme regulation, nucleotide specifity, temperature dependence of the defective enzymes and electrophoretic mobility) were investigated, and then the mutations that cause the alterations in the protein structures were characterized by a detailed molecular genetic analysis. In our laboratory 62 patients with pyruvate kinase deficiency and 12 cases with glucose phosphate isomerase deficiency have been studied so far, in many cases together with their parents and siblings. In this presentation, as representative examples the only families that were subjected to a genetic analysis were those, where the enzyme variants exhibited unusual features during the biochemical characterization which indicated significant structural alterations at the catalytic site, the regulatory site or the intersubunit binding domains.

Additionally the research project deals with neurological impairments that have been found worldwide in 5 cases of GPI deficiency; one of them was studied the first time on a molecular genetic level in our laboratory. Surprisingly, it was found that the GPI monomer shows a sequence identity with neuroleukin (NLK), a protein that promotes the survival of neurons in cell culture [5, 6, 7]. It also shares homology with the differentiation and maturation mediator for

human myeloid leukemia cells [8] and with parts of the gp120 protein of HIV-1 [9]. It is known that patients suffering from these diseases exhibit also neurological impairment. All these findings suggest that GPI/NLK might play a role outside of the carbohydrate metabolism as well. Therefore, by sequencing the GPI gene and making a detailed biochemical characterization of these enzyme variants, we will probably gain more information on the pathogenesis of this second type of disease caused by GPI/NLK deficiency which is completely different.

2. Methods

Nomenclature

The enzyme variants were named following the recommendations of the International Committee for Standardisation in Haematology [10]; they were slightly modified by us taking into account the results of the molecular genetic studies. A true homozygote (both parents carry the identical defect on one allele) was designated by using the geographic title of the patient's place of birth, i.e. PK "Beirut". The heterozygote parents (one allele normal, one allele defective) were called for example, PK "Beirut /normal". In the cases of compound heterozygotes we add indices to designate a given allele (haploid entity), for example, if $Bukarest_1$ and $Bukarest_2$ are the names of each haploid entity, then $Bukarest_{1,2}$ is the name of the compound heterozygote phenotype (diploid entity). Consequently, the heterozygote parents would be designated PK "$Bukarest_1$/normal" and PK "$Bukarest_2$/normal" [11].

Hematological and biochemical characterization of the variant enzymes

Routine hematological studies were performed according to Dacie and Lewis [12]. *Hemolysate* for the biochemical investigations was prepared according to Beutler [13]. *Protein* was determined following the procedure of Lowry et al. [14] taking bovine serum albumine as a standard. *Enzyme activity* was measured using computerized spectrophotometers, according to optimized assay procedures [15]. *Enzyme kinetic studies*, yielding Vmax, $K_{0.5S}$, and the Hill coefficient nH according to the procedures of Lineweaver-Burke [16], Eadie-Hofstee [17], Scatchard [18] and Hill [19] were evaluated with a computer program developed in our laboratory [20].

Correction of the measured activity in the presence of high reticulocyte counts

During our studies of the biochemical properties of normal and defective enzymes, a significant influence of the reticulocyte count on the enzyme activity, as well as on the enzyme kinetic properties was found. The quotient AR/AE represents the decline of enzyme activity from the reticulocyte (AR) to the erythrocyte stage (AE) and is 16.2 for pyruvate kinase, 9.4 for hexokinase, 4.3 for glucose-6-phosphate dehydrogenase, 4.3 for phosphofructokinase and 2.9 for glucose phosphate isomerase. These values are almost identical for normal controls and for patients who suffer from hemolytic anemia due to enzymopathies. For the diagnosis of enzyme deficiencies this correction method is of great importance, because for many patients rather high reticulocyte counts were found, sometimes in postsplenectomy state up to 90 %, which complicate a correct diagnosis, due to the measurement of falsely high enzyme activities. Therefore, the measured activity had to be corrected according to a procedure described elsewhere [15].

Rheological studies

The deformabilty of erythrocytes was determined by cell filtration through polycarbonate sieves. For measuring the red cell deformation the filtration method of Schmid-Schönbein [21] was used, where the aspirated autologous plasma is passed through cellulose-ester filters. The flow rate

of the suspension was calculated by measuring the complete passage of 1 ml erythrocyte suspension through the filter; each value represents the mean of three determinations. Details were published in 1993 [22].

Molecular genetic analysis

Molecular genetic studies were published recently [11], where the preparation of the total genomic DNA from peripheral blood and the amplification of the target sequences by the polymerase chain reaction have been described in detail. The PCR products were subcloned into pUC 18/19, and they were sequenced using the dideoxy chain termination method with sequenase [11], or the direct sequencing method according to Innis et al. [23] with minor modifications. More recently, sequencing was performed by cycle sequencing using the Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

3. Results and Discussion

Deformability of the erythrocytes and elevated concentrations of energy rich phosphates as a measure for a perturbed energy metabolism

The survival of human erythrocytes in the microcirculation network depends primarily on their deformability. Decreased deformability, as observed in patients with defective glycolytic enzymes, leads to echinocyte formation and thus to an earlier sequestration of the red blood cells in the spleen, and hence is closely related to the degree of hemolysis. From the clinical as well as from the biochemical point of view a very interesting correlation was found between the degree of hemolysis, caused by enzyme deficiencies and elevated concentrations of the energy rich phosphates glucose-6-phosphate(G-6-P) and 2.3-diphosphoglycerate (2.3-DPG) in the erythrocytes of PK or GPI deficient patients. As shown in Table 1 increased G-6-P, and additionally increased 2.3 diphosphoglycerate (2.3-DPG) values as well as decreased flow rates in the rheological studies seem to be a sensitive parameter for demonstrating the perturbation of glycolysis within the red blood cells [24].

Impairment of glycolysis apparently causes a reduction of ATP which in turn leads to an increased mechanical rigidity of the red cell membrane. For normal cells Allen et al. [25] could show that an artificially induced decrease of ATP causes a reduced deformability of the red blood cells that is comparable to that of PK-deficient erythrocytes. We could experimentally confirm these findings for normal controls and for patients suffering from PK deficiency by performing rheological measurements. The deformability of erythrocytes of 30 healthy controls and 22 patients was determined by

Table 1: Deformability of the red blood cell and concentrations of metabolic phosphates in case of erythrocyte enzyme deficiencies

	G-6-PD-deficiency	GPI-deficiency	PK-deficiency
Flow rates of the Erythrocytes	normal	up to 80 % decreased	between 50 - 80 % decreased
2.3-DPG	normal to 2 fold increased	normal to 1.5 fold increased	normal to 2,5 fold increased
Gluc-6-P	1.5 - 2.5 fold increased	1.5 - 2.5 fold increased	normal to 9 fold increased
ATP	normal	decreased	decreased

cell filtration through polycarbonate sieves. As can be seen from **Figure 1**, the deformability of red blood cells, with measured flow rates of 16-20 microliters/sec from patients with a mild clinical course was markedly decreased compared to the controls (54±4 microliter/sec). Red blood cell suspensions from patients with a severe degree of hemolysis showed even lower flow rates (4-8 microliters/sec) [22].

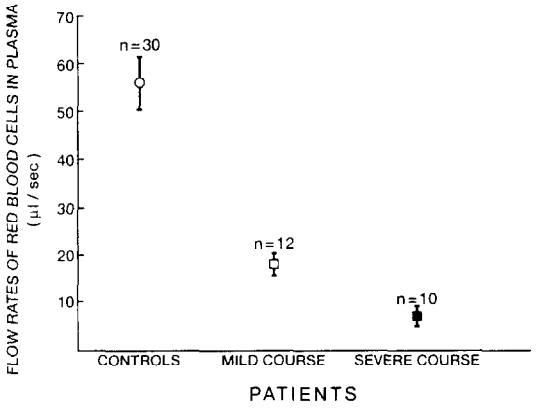

Figure 1: Flow rates of whole red blood cell populations of patients with PK deficiency. Flow rates are expressed as mean values,+/- S.D.

Pyruvate kinase deficiency

Pyruvate kinase (EC2.7.1.40; ATP: pyruvate 2-O-phosphotransferase; PK) is found in all cells and tissues, where it plays an important role in controlling the flux of glycolysis from fructose-1.6-diphosphate to pyruvate. PK has been isolated and characterized from many organisms and is usually a tetramer with a molecular weight of about 240.000 dalton. It catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate by addition of a proton and the loss of a phosphorous group, which is transferred to ADP, yielding the energy donor ATP. In mammals PK exists in four distinct isoenzymic forms, which are expressed in a tissue-specific manner to meet the different metabolic requirements of the cells. M-1 is predominant in skeletal muscle and M-2 is mainly found in leukocytes, platelets, spleen and kidney; L and R isoenzymes are found in the liver and in erythrocytes, respectively. All these isoenzymes display sigmoidal reaction kinetics with respect to PEP. The three-dimensional structure of the cat muscle M-1 isoenzyme has been solved by Muirhead et al. at a resoluion of 0.26 nm [26], demonstrating that each subunit consists of four domains, N, A, B and C. The active site is located in a cleft between the domains A and B, whereas a putative effector site is situated between A and C. The L and R isoenzyme types are transcribed from the same structural gene, which is located on chromosome 1, band q21 where two different tissue specific promotors are used [27]. In red blood cells the specific isoenzyme composition changes during cell maturation: in the proerythroblast M-2 is predominant, whereas in the mature erythrocyte only the R-type is present [28].

Erythrocyte pyruvate kinase deficiency, a disorder associated with hereditary nonspherocytic hemolytic anemia,was first described by Valentine, Tanaka and Miwa in 1961 [29]. More than 380 cases have been described so far by different laboratories [30]. Apparently in most cases PK deficiency is caused by the production of mutant enzymes with abnormal biochemical characteristics and the first mutations in the R-PK gene were described in 1991, two of them in our laboratory [31, 32]. In the absence of consanguinity of the parents, most PK variants are considered to be compound heterozygous for two different mutant enzymes, i.e. one allele carries the paternal and the second allele the maternal mutation. In the case of true homozygotes both alleles carry the same mutation. Usually heterozygotes are clinically and hematologically unaffected, whereas compound heterozygotes and true homozygotes show hemolytic anemia with different degrees of severity [33, 34].

Molecular genetic analysis was performed in order to approach the structural aspect of PK deficiency. Besides exhibiting altered biochemical properties, PK variants also show differences from the normal enzyme kinetic pattern, concerning mainly the enzyme regulation. This implies that the putative gene defects probably involve parts of the enzyme close to or within either the active site or the regulatory domains. Between 1991 and 1994 five different point mutations in the coding sequence of the R-type PK gene were found in 11 different unrelated patients homozygous for PK deficiency causing single amino acid substitutions. In 1993 Baronciani and Beutler, as well as Kanno et al., described the first gene defects in 9 compound heterozygotes [35, 36] and in 1994 Lenzner et al. [37] presented mutations in the R-PK gene of two homozygotes and 10 compound heterozygotes. In 1996, Beutler and Baronciani summarized 58 mutations found in PK- deficient patients, most of them occuring in exon 5, 7, 8, 9, 10 and 11 [38]. However, compared to the total number of PK deficiencies that have been described only a fraction of these enzyme variants has been analyzed on a molecular genetic level.

Relationship between altered structure, perturbed function of the mutant enzymes and clinical course

During our investigations enzyme variants from patients suffering from PK deficiency - with alterations of their regulatory properties, with remarkably reduced activity or thermolability - were preferentially selected for the molecular genetic studies. This was done because one could expect considerable alterations of the structure. One goal of our studies was to learn more about the relationship between altered structure, perturbed function and clinical severity. To illustrate this goal better, the following three enzyme variants PK

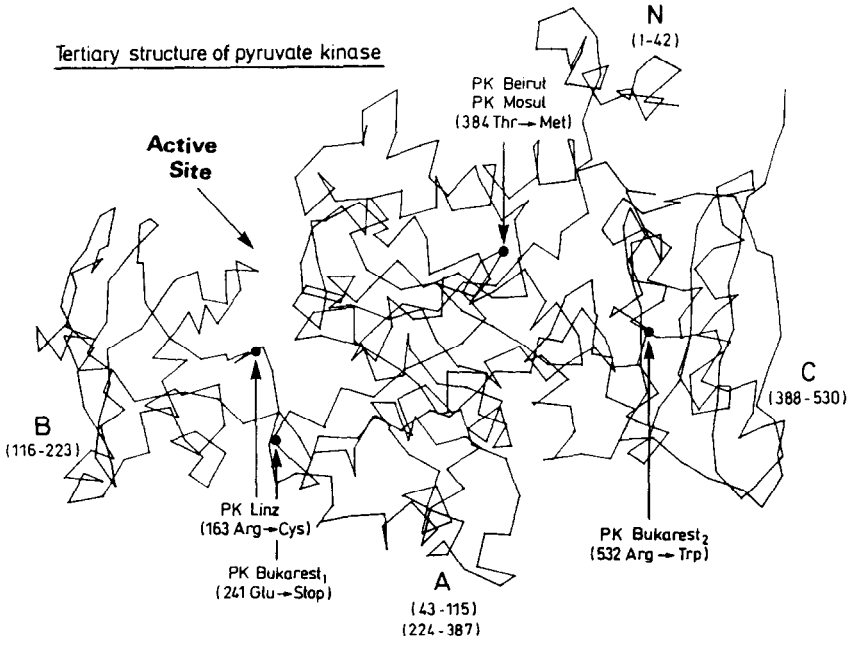

Figure 2: Three dimensional structure of cat muscle PK, given by Muirhead et al., EMBO J. 1986; 5: 475-481. The mutations discussed in more detail in this article are marked with arrows indicating the locus of a given amino acid exchange.

"Beirut", PK "Linz" and PK "Bukarest", - each of them showing a given type of enzyme cooperativity - will be discussed in more detail. The structural discussion is based on the three-dimensional structure of cat muscle PK (**Figure 2**) published by Muirhead et al. 1986 [26]; the amino acid exchanges found in this enzyme variants are also illustrated in this figure.

.Positive cooperative PEP binding

The mutant enzyme *PK "Beirut"* [31], where the patient is homozygous for PK deficiency, exhibits a c1151 C→T substitution leading to an amino acid exchange Thr 384 to Met. Thr 384 is located in the A α 7 domain without a closer connection to the active site or to the intersubunit binding sites. Thus exchanging Thr 384 to Met only leads to a decreased thermostabiliy and a reduced enzyme activity, probably due to a somewhat weaker co-substrate binding as indicated by a slightly increased $K_{0.5ADP}$; but this amino acid exchange does not influence the normally occuring positively cooperative PEP binding, where $K_{0.5\ PEP}$ remains in the normal range (upper part of **Figure 3**). The G-6-P concentration was slightly elevated which is characteristic for a moderate perturbation of glycolysis. Correspondingly this patient showed a rather mild clinical course of the disease. An identical mutation, leading to the same biochemical and clinical properties was found in another patient carrying the variant PK "Mosul".

Negative cooperative PEP binding

The patient bearing the variant *PK "Linz"* [31, 11] is also homozygous for the disease with a mutation c487 C→T, and the amino acid exchange Arg 163 to Cys found in this patient is located in domain B which neighbors the active center of the enzyme. Due to the basicity of its side chain, Arg 163 interacts electrostatically with the phosphate

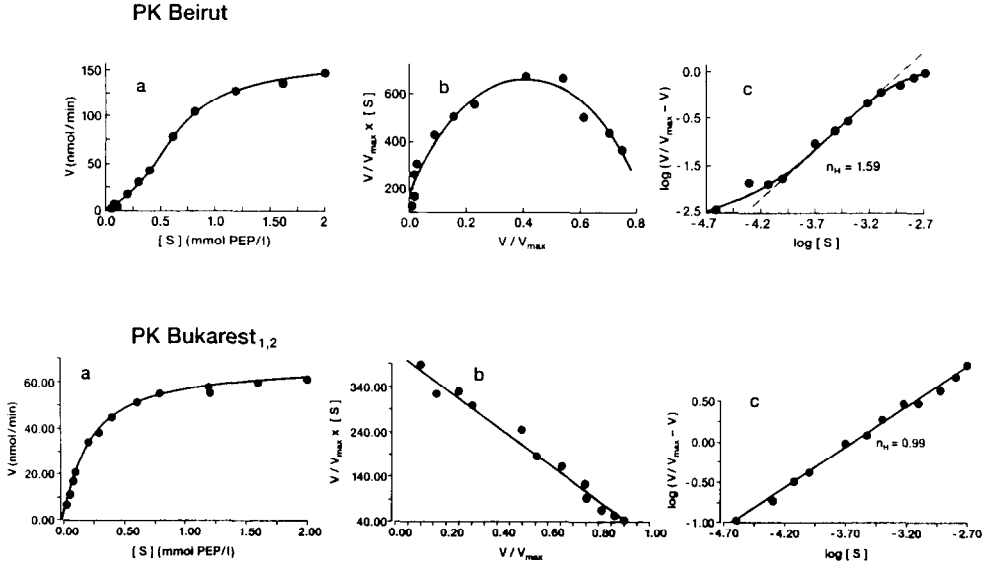

Figure 3: Upper part: Positive cooperative substrate binding of PK "Beirut".
Lower part: Non-cooperative substrate binding of PK "Bukarest$_{1,2}$".
From left to the right the Michaelis Menten-, the Scatchard- and the Hill plot are given.

group of the co-substrate ADP; therefore, it is one of the important functional amino acids in the active center of PK. The substituting amino acid, Cys 163, is a neutral amino acid, that lacks positively charged side chains, and thus cannot interact with the phosphate group of ADP. This becomes noticeable in a remarkably decreased affinity for ADP, where the $K_{0.5ADP}$ is increased by a factor of 4.5. As described in a subsequent section ("Predictions deduced from enzyme kinetic studies") the co-substrate ADP acts as a positive allosteric effector and increases the Hill coefficient of the PEP binding process. In the normal case this results in positively cooperative substrate binding. However, decreased levels of ADP at the co-substrate binding site as found in the case of PK "Linz", apparently alter the cooperativity of the PEP binding from positively to negatively cooperative behavior (cf. upper part of **Figure 4**). This negative cooperativity has a side effect, due to the steeply increasing biphasic hyperbola of the PEP binding curve, with half saturation occuring at much lower PEP concentrations than in the normal case; this indicates stronger PEP binding already at lower substrate levels and, therefore, could result in a decreased turnover. Experimentally, this could be verified in enzyme kinetic studies, where a decreased $K_{0.5PEP}$ and reduced activity (20 % of normal) was found. As evidenced in the lower part of **Figure 4**, the parental enzymes that bear the Arg 163 to Cys exchange as well as the wild type enzyme, exhibit mixed cooperative PEP binding. The patient carrying PK "Linz" suffers from a severe clinical course of the hemolytic anemia, requiring frequent transfusions.

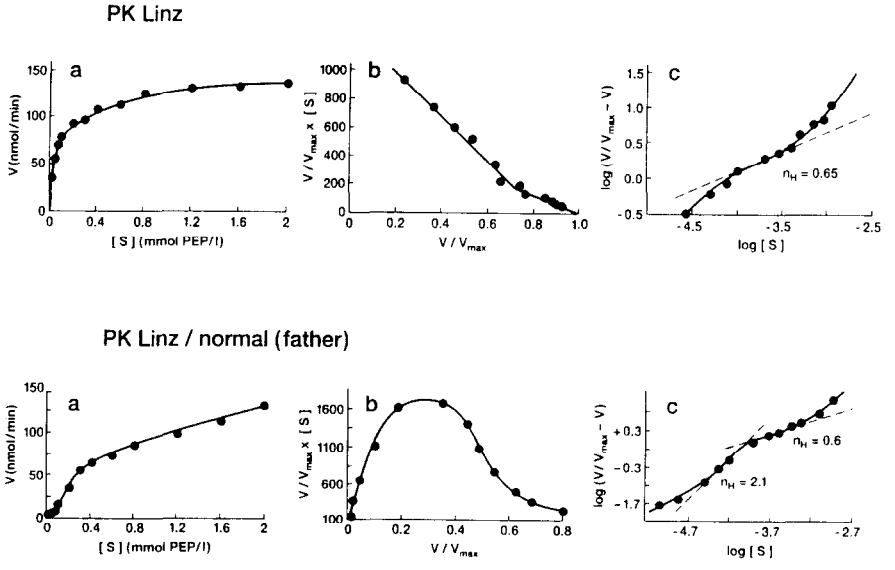

Figure 4: Upper part : Negative cooperative substrate binding of PK "Linz".
Lower Part: Mixed cooperative substrate binding of PK "Linz/normal" (paternal enzyme).
From left to the right the Michaelis Menten-, the Scatchard- and the Hill plot are given.

Non cooperative PEP binding

The patient with *PK "Bukarest* $_{1,2}$*"* [11] is a compound heterozygote for PK deficiency with different mutations in each allele so that one should expect the presence of two defective enzymes, reflected in the enzyme kinetics by a mixed cooperative substrate binding. The paternal enzyme (PK "Bukarest$_2$/normal") already exhibits mixed cooperative substrate binding (positive/non cooperative) due to the presence of the wild-type enzyme and a deficient enzyme. The genetic defect in the second allele is caused by a point mutation c1594CGG→TGG leading to the substitution Arg 532 to Trp (PK "Bukarest$_2$"). According to the crystallographic studies by Muirhead et al. [26] and site-directed mutagenesis studies by Walker et al. on *Bacillus stearothermophilus* PK [39] this defect is located in the alpha 5 helix of the C domain of the PK subunit and close to the binding site for the allosteric activators. Therefore, it is not surprising that a mutation, located in a structure that is conserved in all organisms investigated so far changes the allosteric properties of this enzyme variant from positive to non-cooperative substrate binding. In the maternal enzyme PK "Bukarest$_1$ /normal", the healthy allele encodes for normal PK, but a point mutation in the second allele c721GAG→TAG (yielding a stop codon), results in chain termination. If this has any effect at all, it causes the production of a truncated PK where probably a large part of the active site is missing (PK "Bukarest$_1$"). Therefore, PK "Bukarest$_1$ /normal" should exhibit only half the PK activity and a positively cooperative PEP binding arising from the wild-type enzyme. Experimentally only 45 % activity was found, but the enzyme had normal $K_{0.5PEP}$ and positively cooperative substrate binding. Consequently, the daughters enzyme PK "Bukarest$_{1,2}$", which is a mixture of the truncated maternal and the defective paternal enzyme variant, exhibits both, a lowered activity and an abnormal non-cooperative substrate binding (cf. lower part of **Figure 3**). This patient also exhibits a severe clinical course of the disease.

Problems of predictions deduced from enzyme kinetic studies

When predictions are deduced from enzyme kinetic studies alone, one must keep in mind that there is a remarkable influence of some physiologically important metabolites, such as G-6-P, 2.3-DPG, adenosine diphosphate (ADP) and ATP on the reaction kinetic pattern of the PK-catalyzed conversion of PEP to pyruvate. Therefore, regulatory alterations, as are observed in the case of some enzyme variants could, in principle, be caused not only by structural changes of the protein due to genetic mutations, but also by the presence of abnormally high levels of certain metabolic phosphates. To discriminate between these alternatives, additional enzyme kinetic studies must be performed with the parental enzymes. If the cooperative properties of these enzymes also deviate from the normal behavior, even though the metabolite concentrations are in the normal range, the altered enzyme regulation is then ascribed to a gene defect. In general, *G-6-P* acts as a negative allosteric effector, shifting the Hill coefficient towards smaller values: positively cooperative substrate binding to the enzyme of normal controls and to the enzymes of some of the patients becomes non cooperative. Non- or mixed cooperative enzyme variants show a negative cooperativity after addition of G-6-P, whereas already existing negative cooperativity becomes even more pronounced. *2.3-DPG* and *ADP* act as antagonists (in the sense of positive allosteric effectors) for G-6-P by shifting the Hill coefficient positively with increasing concentrations, i.e. negative becomes non- and non-cooperativity becomes positive cooperativity. Upon the addition of *ATP*, all enzymes, normal as well as defective, are converted to enzymes with positively cooperative PEP binding. Moreover, if the

Table 2: Statistical classification of several discriminating parameters correlated with the clinical course of PK deficiency

		Quantitative analysis Discrimination values and number of correctly classified cases		Discrimination analysis
Parameter	Normal range	Group 1 (mild course)	Group 2 (severe course)	Correct classification
Enzyme activity (% of normal)	85 - 115	≥ 33 (14/22 patients)	< 33 (22/22 patients)	80 %
Glucose-6-Phosphate (µmol/ml RBC)	0.026 - 0.042	< 0.11 (22/22 patients)	≥ 0.11 (19/22 patients)	93 %
2.3-Diphosphoglycerate µmol/ml RBC)	4.2 - 5.3	< 7.9 (17/22 patients)	≥7.9 (16/22 patients)	77 %
Enzyme cooperativity				
PEP-binding	positive cooperative	positive cooperative (28/28 patients)	negative cooperative/ non cooperative (16/26 patients)	82 %
ADP-binding	non cooperative	non cooperative (24/28 patients)	negative cooperative (23/26 patients)	87 %

the physiological range, a remarkable decrease in enzyme activity occurs at ATP concentrations greater than 2 mmol/l; by performing inhibition studies we could prove that ATP acts as a competitive inhibitor [34].

Biochemical parameters as prognostic tools for PK deficiency

Finally the question arises, which of the parameters investigated during the characterization of the variant enzymes gives a good correlation with the degree of hemolytic anemia; these parameters can serve as a prognostic tool apart from the hematological findings.

Table 2 summarizes all the evidence obtained from a statistical analysis of the biochemical data. The following procedure seems feasible in order to ensure a correct diagnosis and a correct prognosis of the course of PK deficiency:

1) The residual enzyme activity, that has been corrected according to the reticulocyte counts, is lower than 33 % of the normal value in all cases with a severe course. 2) The glucose-6-phosphate concentrations, which indicate the extent of the perturbation of glycolysis, are lower than 0.11 micromole/l in all cases with a mild clinical course; however, these concentrations are above this threshold in cases with a severe course. 3) Positively cooperative PEP binding was present in all cases with a mild clinical course, whereas non-cooperative, negatively cooperative or mixed cooperative substrate binding with a predominant negative part is found in most cases of severe hemolytic anemia. 4) Cooperativity of the co-substrate ADP binding (non-cooperative in most cases of mild hemolytic anemia and negatively cooperative in most cases with severe clinical course) describes correctly 87 % of all cases. Taking into account the combination of all the parameters given in Table 2, we can predict correctly the clinical manifestations of all 54 patients except one and should now be able to ensure an accurate prognosis of the disease already at an early age without the necessity of performing a detailed molecular genetic analysis [24].

From the evolutionary point of view it was interesting to note that some of the mutations observed so far can apparently be attributed to ethnical groups. In the eastern hemisphere the mutations c1151 C →T and c1261 C→A are predominant, whereas in the western hemisphere the mutations c1529 G →A and c721 G→T occur more frequently. When more data are available, one can probably build a pedigree of the gene defects, that cause pyruvate kinase deficiency.

Glucose phosphate isomerase deficiency

Glucose phosphate isomerase (glucose-6-phosphate ketol isomerase, EC 5.3.1.9, GPI) is an essential enzyme in all tissues, catalyzing the interconversion of fructose-6-phosphate (F-6-P) and glucose-6-phosphate. Both substrates play an important role in glycolysis, gluconeogenesis and in the pentose phosphate cycle. Normal GPI is a dimeric enzyme with a molecular weight of 134.000 dalton and binds F-6-P non-cooperatively. So far no isoenzymic forms of GPI have been observed. The crystal structure of the pig GPI was determined by Shaw and Muirhead at a 0.35 nm resolution [40] and demonstrates that GPI is composed of two identical subunits, each consisting of a larger and a smaller domain. As frequently found for glycolytic enzymes, each domain comprises a parallel beta-sheet core, surrounded by alpha helices linking the beta strands. The active site is located in a cleft between the large and the small domain close to the intersubunit binding site, and it is composed of chains from both subunits. Therefore, enzyme deficiencies could be due to alterations in the tertiary structure of the monomer itself, or they could be caused by defects in the intersubunit binding site.

The ***gene encoding human GPI*** has been assigned to the long arm of chromosome 19 [41] with a length of at least 50 kB [42]. The human GPI-gene consists of 18 exons, ranging from 44 bp to 153 bp in size, whereas the introns vary between more than 20 kb (intron 9) down to 87 bp (intron 16). According to the 1.9-kb GPI cDNA one would expect a protein consisting of 558 amino acids. Surprisingly, it was found that this sequence is identical with a sequence that also codes for a monomeric protein, neuroleukin (NLK) [5, 6, 7], with a MW of approximately 63.000 dalton. ***Neuroleukin*** is a neurotrophic factor that promotes the survival of embryonic spinal neurons, skeletal motor neurons and sensory neurons in cell culture. It also acts as a lymphokine product of lectin-stimulated T cells and induces immunoglobulin secretion [43]. Moreover, it was reported that GPI/NLK shares a sequence homology with the differentiation and maturation mediator for human myeloid leukemia cells [8] as well as with parts of the gp120 protein of the human immuno-deficiency virus type 1, HIV-1 [9]. NLK displays its neurotrophic activity as a monomer. In contrast to the dimeric GPI, which exhibits no NLK activity, the monomerized GPI does show NLK activity under reducing conditions, but lacks the GPI activity, although the GPI monomer still binds F-6-P [9]. Baumann et al. [8] suggested that a specific cleavage of the GPI dimer occurs in pathological cases and that this cleavage perturbs the catalytic center. These findings suggest that GPI / NLK may also play a role outside the carbohydrate metabolism.

GPI deficiency was first described in 1967 [45], and it is supposed to be the third most frequent cause of a hereditary non-spherocytic hemolytic anemia, presumably induced by a defective erythrocyte enzyme. So far approximately 60 patients with GPI deficiency have been characterized biochemically. In most cases the clinical picture of this autosomal recessive disease shows a variable degree of hemolytic anemia. Interestingly enough - keeping in mind the sequence analogy of GPI and NLK, and that apparently monomeric GPI is NLK - additional neurological impairment was found in some cases [2, 4]. Hydrops fetalis has also been reported [46].

Since the first description of ***DNA sequence abnormalities*** in 1993 [47] the analysis of the GPI gene performed with 17 patients - six homozygotes and 11 compound heterozygotes suffering from GPI deficiency - resulted in the discovery of 18 missense mutations, 2 nonsense mutations, one splice site mutation and a four - nucleotide deletion mutant [48, 49, 50]. In our laboratory a GPI - deficient patient with neurological disorders (GPI "Homburg") was available for biochemical and molecular genetic studies, together with his clinically unaffected parents. The biochemical and genetic properties of this GPI variant were compared with those that cause hemolytic anemia alone. Another goal of our studies, besides investigating the structure-function relationship of GPI, was to search for epitopes in the GPI subunit, where amino acid exchanges probably lead to either hemolytic impairment, or neurological disorders, respectively. Furthermore, the hypothesis should be examined, whether a specific cleavage of dimeric GPI occurs in pathological cases; such a cleavage should perturb the catalytic center and might lead to the conversion from an enzyme to a trophic factor.

Severe course of the hemolytic anemia caused by chain termination and splice site mutation

To start with, we studied the ***biochemical and molecular genetic properties of GPI variants*** from two patients who were compound heterozygous for GPI deficiency; both patients suffered from severe hemolytic anemia. The combination of a single amino acid substitution close to the catalytic site, together with a point mutation leading to a truncated or absent enzyme in both patients, probably explains the severity of the clinical course. Additionally, the investigations could be extented in the case of GPI "Zwickau" to the parental enzymes thus proving for the first time the compound heterozygosity of the parents at the molecular genetic level. The enzymes of both patients GPI "Zwickau" and GPI "Nordhorn" [51] revealed reduced GPI activities and remarkable thermolabilities. The G-6-P concentration was elevated 2.3 times for GPI "Zwickau" and 3.8 times for GPI "Nordhorn"; this demonstrates most likely a pertubation of glycolysis. GPI activity is also reduced in the heterozygote parents. Enzyme kinetic studies revealed that the defective enzymes of both patients, as well as the enzymes of the parents of GPI "Zwickau" exhibit the normal non-cooperative substrate binding.

Sequencing the patients' GPI genes showed four different point mutations, two of them involving highly conserved amino acids. The c1039 C→T substitution found in the gene of GPI "Zwickau" as well as in the father, has been described recently [49] and causes an Arg 347 to Cys substitution close to the putative catalytic site. The second

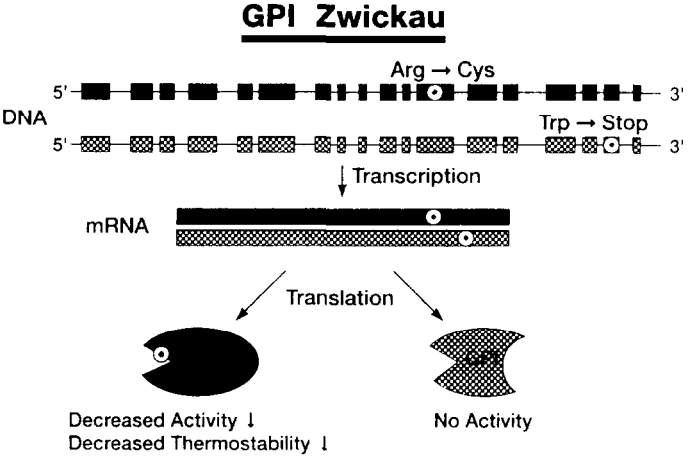

Figure 5 Point mutations in the gene of GPI "Zwickau"and their consequences for the enzyme properties. The paternal and the maternal allele are given in black and grey, respectively.

mutation in this patient, also present in the mother is a novel c1538 G→A substitution causing a change from Trp to stop codon at position 513, most likely resulting in premature RNA degeneration; this yields a truncated protein which does not show GPI activity, and probably no enzyme is made at all. This is schematically presented in Figure 5. Because the maternal enzyme is inactive, the product of the mother's allele has no influence on the thermostability of the patient's enzyme. Therefore only the exchange Arg 347 to Cys, inherited from the father, is responsible for the observed reduced thermostability and for the decreased enzyme activity. In the gene of GPI "Nordhorn" a c1028 A→G mutation was discovered, causing a Gln 343 to Arg substitution; this has also been previously described [50, 52]. As reported by Fujii et al. [53] this mutation produces a protein with a higher electrophoretic mobility; this has also been observed in our laboratory in the case of the mother's and the patient's enzyme. This alteration manifest itself in reduced GPI activity, reduced thermostability and an increase of the G-6-P concentrations; at the same time it indicates the heterozygosity of the mother for GPI deficiency. The second mutation was a novel splice site mutation at the border between intron 15 and exon 16: IVS15-(-2) A→C which leads to an aberrant splicing of exon 16; as schematically depicted in Figure 6 this results in a truncated enzyme that is most likely inactive, or possibly no enzyme at all. The biochemical characterization at the time of diagnosis, which was reported already 20 years ago [51] showed that the defective allele of the father encodes for a protein with either no GPI activity or no enzyme at all. This is supported by the normal thermostability of the paternal enzyme and the lack of a common band of the father's and the patient's enzyme in a starch gel. One may conclude that the intersubunit binding domain is not affected by the mutations described here, because in all cases presented in this study normal values for $K_{0.5\ F\text{-}6\text{-}P}$ and normal non-cooperative F-6-P binding were found [Huppke et al. Eur.J.Ped. in press].

GPI/NLK deficiency and neurological impairment

In an earlier study performed in our laboratory the enzyme variant GPI "Homburg" has been characterized clinically and biochemically. The patient suffered from severe hemolytic anemia and in addition displayed ***neurological symptoms*** [2, 54]. The variant showed reduced GPI activity

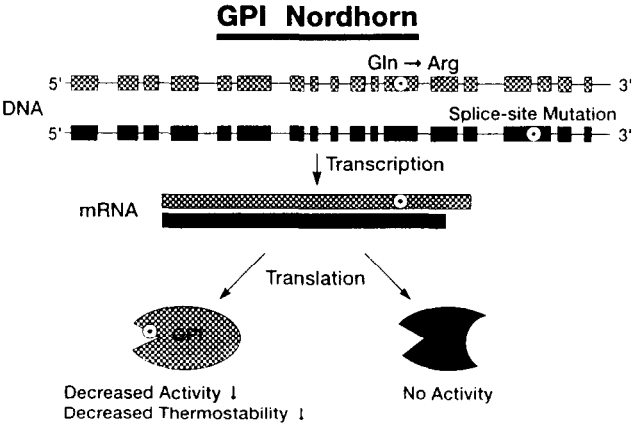

Figure 6 Point mutations in the gene of GPI "Nordhorn" and their consequences for the enzyme properties. The paternal and the maternal allele are given in black and grey, respectively.

(8 %) and a fourfold elevated G-6-P concentration indicating a rather serious perturbation of glycolysis. Molecular genetic studies have been performed, where the compound heterozygosity of the patient and the heterozygosity of the parents could be proven. A detailed comparison of the structural and functional alterations, based on the mutations and the location of the changes in the 3-D structure of the proteins together with their relation to the clinically picture is submitted for publication [Kugler et al. 1997]. One mutation rather distant from the active site leads to an incorrect folding of the subunit and therefore presumably the GPI and the NLK activities were either inhibited or even abolished. The second mutation found with GPI "Homburg" is located in an area close to those described in case of GPI "Zwickau" and GPI "Nordhorn". In these other cases, alterations near the active site occur which obstruct the conformational changes during the substrate binding process. This decreases the GPI activity, but still allows proper folding of the amino acid chain. Hence, the neurotrophic properties of the protein remain unchanged; therefore, the patients carrying these GPI variants exhibit severe hemolytic anemia, but they do not exhibit neurological impairment.

4. Conclusions

We succeeded in partially exploring the mechanisms by which pyruvate kinase deficiency leads to hemolytic anemia. This was achieved by biochemically characterizing the enzyme variants and detecting the genetic mutations that create alterations of the enzyme structure; the abnormalities result in an insufficient flux of glycolysis. The perturbations of glycolysis lead to a decreased energy supply of the red blood cells, and this gives rise to earlier lysis of the cells which consequently causes hemolytic anemia. Furthermore, we presented a complete correlation between the clinical picture, the function and the structure of some representative deficient enzymes.

In the case of GPI / NLK deficiency we could satisfactorily explain the origin of hemolytic anemias - similar to the way in which PK deficiency was explained with the aid of biochemical and molecular genetic methods - and correlate them with the severity of the clinical course of the disease. This knowledge can now be applied to the clinics; for instance, a prenatal diagnosis can be established. We are just beginning to understand the complex role of GPI/NLK with respect to neurological impairment. The characterization of one patient with neurological signs has been completed and seem to indicate, that a mutation leading to an incorrect folding of the protein abolishes GPI/NLK activity and probably is responsible for the neurological symptoms. Mutations, however, located in an area close to the active center, as described in the previous section manifest themselves in reduced GPI-activity and hence cause hemolytic anemia. The relationship with other diseases, where NLK shows sequence analogies either to the differentiation and maturation mediator for human leukemia cells or to the gp120 protein of HIV-1 (where patients suffering from these diseases also exhibit neurological impairment) awaits further investigations. Current efforts are in this direction.

Acknowledgements: The authors thank Prof. Dr. Manfred Eigen and Prof. Dr.med. Werner Schröter for many helpful discussions, and for their interest in and steady support of this project. These studies were supported by the Deutsche Forschungsgemeinschaft, Bonn Bad Godesberg (La 527,1-3).

References

[1] K.R. Tanaka, C.R. Zeres, Semin. Hematol. 27 (1990) 165

[2] W. Schröter, S.W. Eber, A. Bardosi, M. Gahr, M. Gabriel, F.C. Sitzmann, Eur. J. Pediatr. 144 (1985) 301

[3] A. Zanella, C. Izzo, P. Rebulla, L. Perroni, M. Mariani, G. Canestri, G. Sansone, G. Sirchia, Am. J. Hematol. 9 (1980) 1

[4] O. Shalev, R.S. Shalev, L. Forman, E. Beutler, Ann. Hematol. 67 (1993) 197

[5] M.E. Gurney, S.P. Heinrich, M.R. Lee, H.S. Yin, Science 234 (1986) 566

[6] M. Chaput, V. Claes, D. Portetelle, I. Cludts, A. Cravador, A. Burny, H. Gras, A. Tartar, Nature 332 (1988) 454

[7] P. Faik, J.I.H. Walker, A.A.M. Redmill, M.J. Morgan, Nature 332 (1988) 455

[8] M. Baumann and K. Brand, Cancer Res. 48 (1988) 7018

[9] Y. Mizrachi, J. Neurosci. Res. 23 (1989) 217

[10] E. Beutler, K.G. Blume, J.C. Kaplan, G.W. Löhr, B. Ramot, W.N. Valentine, Br. J. Haematol. 35 (1977) 331

[11] M. Lakomek, P. Huppke, B. Neubauer, A. Pekrun, H. Winkler, W. Schröter, Ann. Hematol. 68 (1994) 253

[12] J.V. Dacie and S.M. Lewis, 5th edn. Curchill Livingstone, Edinburg (1975)

[13] E. Beutler, 2nd edn. Grune and Stratton, New York (1975) 38

[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265

[15] M. Lakomek, H. Winkler, G. De Maeyer, W. Schröter, Br. J. Haematol 72 (1989) 445

[16] H. Lineweaver and D. Burke, J. Am. Chem. Soc. 56 (1934) 658

[17] B.H. Hofstee, M. Dixon, E.C. Webb, Nature 184 (1959) 1296

[18] G. Scatchard, Ann. NY Acad. Sci. 51 (1949) 660

[19] A.V. Hill, J. Biochem. 7 (1913) 471

[20] M. Lakomek, H. Winkler, S. Linne, W. Schröter, Am. J. Hematol. 31 (1989) 225

[21] H. Schmid-Schönbein, L. Weiss, H. Ludwig, Blut 26 (1973) 369

[22] M. Lakomek, E. Friederichs, H. Winkler, W. Tillmann, W. Schröter, Clinical Hemorheology 13 (1993) 55

[23] M.A. Innis, K.B. Myambo, D.H. Gelfand, M.A.D. Brown, Proc. Natl. Acad. Sci. USA 85 (1988) 9436

[24] M. Lakomek, B. Neubauer, A. v.d. Lühe, G. Hoch, H. Winkler, W. Schröter, Eur. J. Haematol 49 (1992) 82

[25] D.W. Allen, J.D. Groat, B. Finkel, B.H. Rank, P.A. Wood, J.W. Eaton, Am. J. Hematol. 14 (1983) 11

[26] H. Muirhead, D.A. Clayden, D. Barford, C.G. Lorimer, L.A. Fothergill-Gilmore, E. Schiltz, W. Schmitt, EMBO J. 5 (1986) 475

[27] T. Noguchi, K. Yamada, H. Inoue, T. Matsuda, T. Tanaka, J. Biol. Chem. 262 (1987) 14366

[28] H. Fujii, S. Miwa, Am. J. Hematol. 34 (1990) 301

[29] W.N. Valentine, K.R. Tanaka, S. Miwa, Trans. Assoc. Am. Physicians 74 (1961) 100

[30] S. Miwa, H. Kanno, H. Fujii, Am. J. Hematol. 42 (1993) 31

[31] B. Neubauer, M. Lakomek, H. Winkler, M. Parke, S. Hofferbert, W. Schröter, Blood 77 (1991) 1871

[32] H. Kanno, H. Fujii, A. Hirono, S. Miwa, Proc. Natl. Acad. Sci. USA 88 (1991) 8218

[33] S. Miwa, H. Fujii, S. Takegawa, T. Nakatsuji, K. Yamato, Y. Ishida, N. Ninomiya, Br. J. Haematol. 45 (1980) 575

[34] M. Lakomek, H. Winkler, A. Pekrun, N. Krüger, M. Sander, P. Huppke, W. Schröter, Enzyme Protein 48 (1995) 149

[35] L. Baronciani and E. Beutler, Proc. Natl. Acad. Sci. USA 90 (1993) 4324

[36] H. Kanno, H. Fujii, A. Hirono, M. Omine, S. Miwa, Blood 79 (1992) 1347

[37] C. Lenzner, P. Nürnberg, B.J. Thiele, A. Reis, V. Brabec, A. Sakalova, G. Jacobasch, Blood 83 (1994) 2817

[38] E. Beutler and L. Baronchiani, Human Mutation 7 (1996) 1

[39] D. Walker, W.N. Chia, H. Muirhead, J. Mol. Biol. 228 (1992) 265

[40] P.J. Shaw and H. Muirhead, J. Mol. Biol. 109 (1977) 475

[41] F.A. McMorris, T.R. Chen, F. Ricciuti, J. Tischfield, R. Creagan, F.H. Ruddle, Sience 179 (1973) 1129

[42] J.I.H. Walker, M.J. Morgan, P. Faik, Genomics 29 (1995) 261

[43] M.E. Gurney, S.P. Heinrich, M.R. Lee, H.S. Yin, Science 234 (1986) 574

[44] W. Xu, K. Seiter, E. Felman, T. Ahmed, J.W. Chiao, Blood 87 (1996) 4502

[45] M.A. Baughan, W.N. Valentine, D.E. Paglia, P.O. Ways, E.R. Simson, Q.B. DeMarsh, Blood 32 (1968) 236

[46] Y. Ravindranath, D.E. Paglia, I. Warrier, W. Valentine, M. Nakatani, R.A. Brockway, N. Engl. J. Med. 316 (1987) 258

[47] J.I.H. Walker, D.M. Layton, A.J. Bellingham, M.J. Morgan, P. Faik, Hum. Mol. Gen. 2 (1993) 327

[48] J.I.H. Walker, D.M. Layton, A.J. Bellingham, M.J. Morgan, P. Faik, Hum. Mol. Genet. 2 (1993) 327

[49] W. Xu, P. Lee, E. Beutler, Genomics 29 (1995) 732

[50] H. Kanno, H. Fujii, A. Hirono, Y. Ishida, S. Ohga, Y. Fukumoto, K. Matsuzawa, S. Ogawa, S. Miwa, Blood 88 (1996) 2321

[51] W. Schröter, H.H. Koch, B. Wonneberger, W. Kalinowsky, A. Arnold, K.G. Blume, W. Hüther, Pediat. Res. 8 (1974) 18

[52] L. Baronciani, A. Zanella, P. Bianchi, M. Zappa, F. Alfinito, A. Iolascon, N. Tannoia, E. Beutler, G. Sirchia, Blood 88 (1996) 2306

[53] H. Fujii, H. Kanno, S. Miwa, Blood 84 (1994) 544a

[54] B.A. Neubauer, S.W. Eber, M. Lakomek, M. Gahr, W. Schröter, Acta Haematol. 83 (1990) 206