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The Role of Erythrocyte Membrane Proteins in Haemolytic Anaemias in South African Populations

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CHAPTER 4: DISCUSSION

4.1 Pathogenesis of Hereditary Spherocytosis

HS occurs when an interaction between the erythrocyte lipid bilayer and the erythrocyte membrane skeleton is compromised, either through a protein deficiency or through the loss of a binding site. Without this support structure, portions of the erythrocyte membrane are lost and the cells become spherical (see figure 4.1) as the surface area to volume ratio decreases. Spherocytes cannot pass smoothly through the narrow sinuses of the spleen and are retained for extended periods of time. The spherocytes are ultimately phagocytosed and haemolysed by the phagocytes of the spleen resulting in anaemia due to increased destruction of the erythrocytes (Walensky *et al*, 2003). The HS kindred examined in this study had a decreased amount of band 3 in the erythrocyte membrane which diminished the binding of the lipid bilayer to the membrane skeleton.

4.2 HS Erythrocyte Morphology

While it is normal for up to two percent of erythrocytes to be spherical, particularly when aged, the salient feature of any HS blood smear is a higher than normal number of spherical cells. A number of morphologically abnormal cells may also be present. Acanthocytes (membrane-budding erythrocytes) are particularly prominent in splenectomised patients as the unsupported membrane portions are not removed through splenic conditioning. Echinocytes (erythrocytes with spiny protrusions) and poikilocytes (irregular erythrocytes) may also be seen. Of particular importance in band 3 deficient HS is the presence of mushroom-shaped (pincered) cells which are found in up to 80 percent of patients (Walensky *et al*, 2003). Pincered cells have been shown to disappear after splenectomy (Palek & Jarolim, 1995). In this study 18 of the 21 (86 percent) examined patients had pincered cells. Notably, the splenectomised mother of proband B, in concordance with the studies of Palek and Jarolim (1995), did not have pincered cells and the acanthocytes common in splenectomised patients were visible. It is

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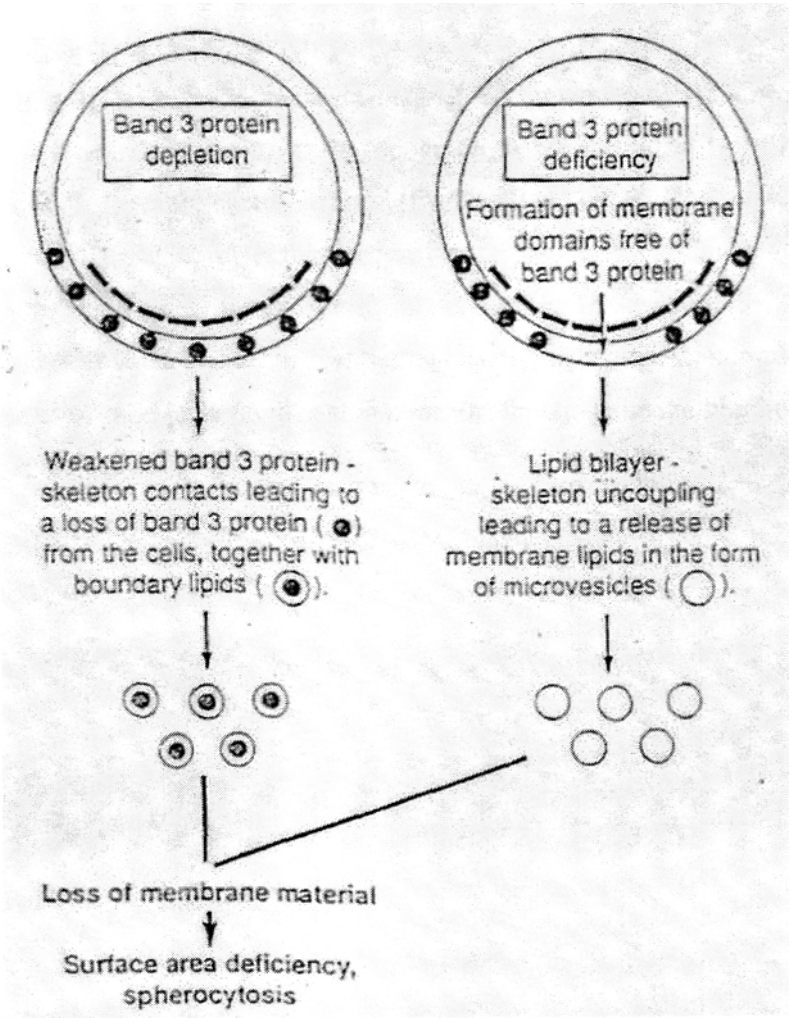


Figure 4.1 Schematic Model of Hypothetical Mechanisms of Spherocyte Formation in Band 3-Deficient HS (Palek & Jarolim, 1995)

possible that the irregularity of the erythrocyte membrane after splenectomy masks the presence of pincered cells seen before splenectomy.

4.3 Position of Band 3 Mutations Causing HS

Band 3 mutations resulting in HS were found in three unrelated kindred (six subjects). All of the mutations occur in exon 13 which encodes phenylalanine 480 to lysine 542 of the transmembrane domain of band 3 (figure 4.2). This region spans transmembrane domains three, four and five as well as extracellular loop two (Fujinaga *et al*, 1999). Two of these mutations lie in the previously defined codon 490 hotspot (Dhermy *et al*, 1997; Lima *et al*, 1999; Bracher *et al*, 2000) which encodes an arginine lying at the extracellular border of the fourth α-helix. The third is in codon 508 and is a novel nucleotide change which encodes a lysine lying near to the cytoplasmic border of the same α-helix.

4.4 HS due to Band 3 Codon 490 Hotspot Mutations

Probands A (Black) and B (Caucasian) both have mutations in codon 490 (encoding arginine) of the band 3 gene which account for their HS. Jarolim *et al* (1995) described a number of mutations in arginine codons (e.g. codon 760) which lie at putative membrane boundaries and are highly conserved, possibly acting as "stop transfer signals", amino acids found at the membrane boundaries of transmembrane proteins and that are important for the normal insertion of band 3 into the erythrocyte membrane. Arginine 490 lies at such a membrane boundary (located at the amino-terminal of the fourth transmembrane segment) and is located within the transmembrane region which is highly conserved among different anion exchange proteins (Lux *et al*, 1989). Both of the known codon 490 mutations, band 3 Pinhal (R490H; CGC→CAC) (Lima *et al*, 1999) and band 3 Bicetre (R490C; CGC→TGC) (Dhermy *et al*, 1997), were detected and are described in detail in section 4.4.3.

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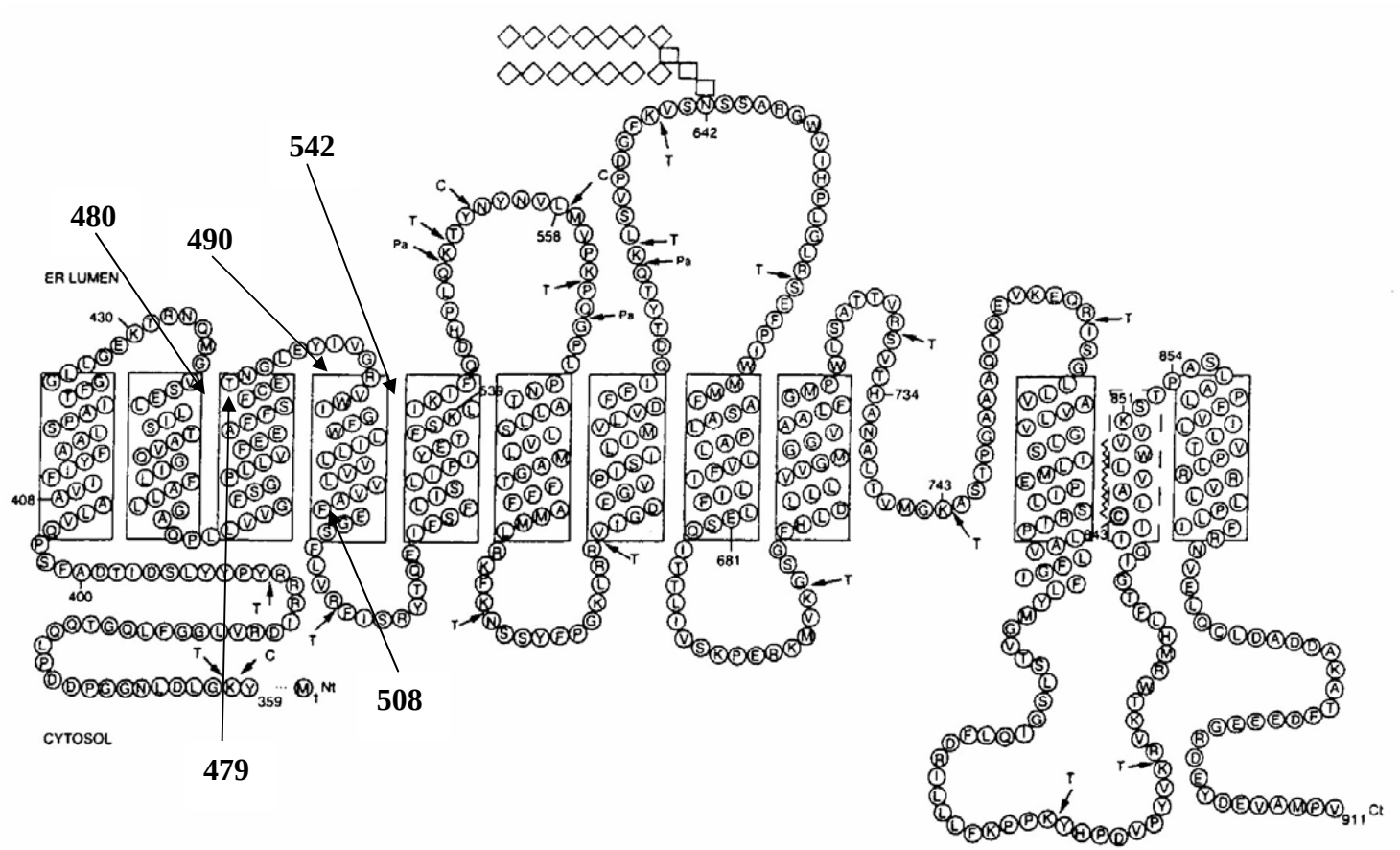


Figure 4.2: Band 3 Transmembrane Region

Arginine 490 (R) and glutamic acid 508 (E), phenylalanine 480 (F) and lysine 542 (K) (the first and last amino acids encoded by exon 13 respectively) and cysteine 479 are indicated by large arrows. (Fujinaga *et al*, 1999)

4.4.1 Clinical Severity

Proband A (R490H) has mild anaemia and a normal reticulocyte count. This contrasts with the patients examined by Lima *et al* (1999) where reticulocyte counts were increased and two patients had been splenectomised, indicating severe HS. Bracher *et al* (1999) described the mutation as band 3 Bloemfontein and noted variable clinical expression in the kindred. The severity ranged from mild (as observed for proband A in this study) to severe, the individual being splenectomised, which is similar to the kindred studied by Lima *et al* (1999). These findings imply that modulating factors alleviate or worsen the clinical severity of HS, as discussed in detail in section 4.5.4. Like proband A, the patients studied by Lima *et al* (1999) display pincered cells.

Proband B (R490C) has severe anaemia, being transfusion dependent. Spherocytes and pincered cells were visible on his blood smear. His mother has mild anaemia but is splenectomised, indicating that she formerly suffered from severe HS. Her blood smear showed the presence of a number of acanthocytes in concordance with splenectomy. No reticulocyte counts were available for this kindred but, based on the protein 4.1a/b ratio, both the proband and his mother had increased reticulocyte counts. The proband had a lower protein 4.1 a/b ratio (0.94, 68% of control) than his mother (1.11; 80% of control), indicating more reticulocytes, and it is assumed that the mother tended towards normal because she was splenectomised. The proband is transfusion dependent which indicates that even with increased numbers of reticulocytes being released, he has inadequate compensation of haemolysis. These profiles are similar to those of Dhermy *et al* (1997), splenectomised individuals having near normal reticulocyte counts and no pincered cells (similar to the mother) and unsplenectomised patients displaying higher numbers of reticulocytes in their blood and pincered cells (similar to proband B).

4.4.2 Band 3 Deficiency

When comparing the two kindred, proband A appears to have a greater band 3 deficiency, contrasting with the clinical symptoms where proband B and his

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mother have more severe HS. This incongruity is due to medical intervention, proband B being transfusion dependent and his mother being splenectomised. Proband A has adequate compensation of haemolysis and is neither transfusion dependent nor splenectomised. This means that although a number of band 3 molecules are lost through splenic conditioning, the erythrocyte membrane proteins analysed are all her own and her band 3 deficiency represents the actual decrease of protein. This is not true for proband B and his mother. The proteins analysed for the proband do not indicate the actual level of his band 3 deficiency as he receives frequent transfusions and the analysis includes the band 3 of the donor (normal) erythrocytes. While the mother does not have transfused erythrocytes (and hence donor membrane proteins), she is splenectomised. Band 3 is not removed through splenic conditioning, resulting in less membrane (and hence band 3) loss. Furthermore, although her erythrocytes have an abnormal shape, they survive for a closer to normal period of time which ameliorates her anaemia.

Contrasting the kindred studied to the previously described HS patients with codon 490 mutations, the band 3 deficiency (27 percent decrease) of proband A (band 3 Pinhal) is similar to those observed by Lima *et al* (1999) (20 to 33 percent decrease) and Bracher *et al* (1999) (19 to 39 percent decrease). The band 3 Bicetre kindred studied by Dhermy *et al* (1997) displayed a more severe band 3 decrease (28 to 33 percent) than the decrease observed for proband B (band 3 Bicetre) (17 percent decrease) and his mother (21 percent decrease). Since this kindred appears to be clinically similar to the patients examined by Dhermy *et al* (1997), some of those patients also being splenectomised, minor differences in the analysis of the proteins, or possibly the degree of compensated haemolysis, may account for the apparently different levels of deficiency. Assuming that only normal band 3 is inserted into and retained in the erythrocyte membrane, the band 3 decrease for the heterozygotes would be expected to be 50 percent and homozygotes would have no band 3 in the erythrocyte membrane. As this does not occur, it is believed that mutant band 3 can be inserted into the erythrocyte membrane but in lower quantities than normal band 3.

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4.4.3 Band 3 Pinhal and Band 3 Bicetre

Arginine residues, including arginine 490, are highly conserved in band 3 and often lie at the predicted membrane boundaries where they are thought to act as "stop transfer signals" for band 3 insertion into the membrane (Jarolim *et al*, 1995). Substitutions of these residues could produce a band 3 deficiency either by excluding band 3 insertion into the erythrocyte membrane during erythropoiesis or through removal of band 3 from the membrane during terminal differentiation. In this study the arginine is replaced by either a histidine (band 3 Pinhal) or a cysteine (band 3 Bicetre) and the different levels of band 3 deficiency may be explained by the different structures (see Figure 4.3) and charges of the side chains of these amino acids.

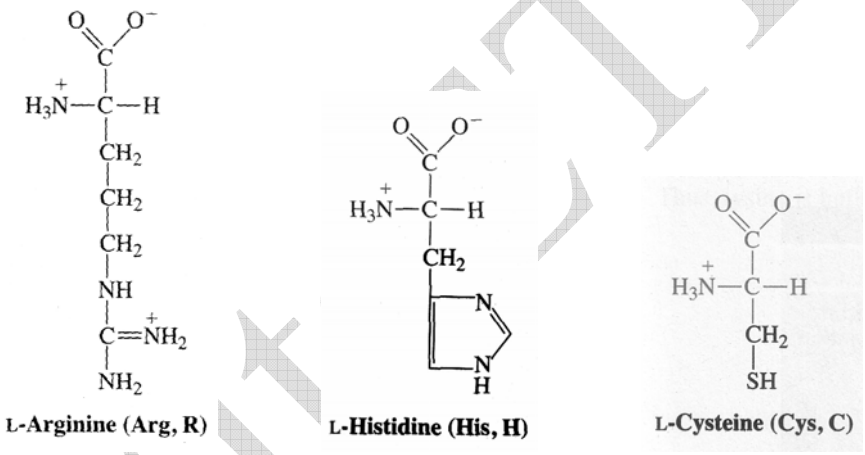


Figure 4.3: Linear Structures of Arginine, Histidine and Cysteine

The side chain of arginine is a guanidino group (three nitrogen atoms bound to a carbon atom) at the end of a linear hydrocarbon chain. Histidine contains a bulky imidazole ring and cysteine carries a sulphydryl group (Stryer, 1988). The decreased quantity of band 3 protein in the erythrocyte membrane observed in band 3 Pinhal probably arises as the bulky imidazole ring of histidine alters the spatial arrangement of the protein, arginine having a more linear structure. This would affect the α-helices of band 3, decreasing its insertion into the membrane. Both arginine and histidine have a basic charge (1+) and hydrophilic side chains which are suited to the hydrophilic environment outside of the lipid bilayer.

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Arginine is however a stronger base than histidine (Alberts *et al*, 1994) which may impact on the insertion of band 3 into the erythrocyte membrane.

On the contrary, cysteine is neutral and its sulphydryl-containing side-chain is slightly hydrophobic (Stryer, 1988). Band 3 may fold inwards to protect the cysteine from the external hydrophilic environment and this could hinder its insertion into the erythrocyte membrane. Alternatively, the altered band 3 conformation may be due to the high reactivity of the sulphydryl group which forms disulphide bonds with other sulphydryl groups when oxidised (Stryer, 1988). If cysteine 490 binds to another cysteine residue (such as cysteine 479 in the third α-helix of band 3, figure 4.2), the folding of band 3 would be greatly disrupted. More significant changes to the conformation of band 3 may be the underlying reason that band 3 Bicetre (cysteine) is generally linked to more severe HS than band 3 Pinhal (histidine).

4.4.4 Possible Mechanisms of Band 3 Deficiency

There are a number of possible reasons for the band 3 deficiency observed in each kindred. The most probable is that there is decreased insertion of the protein into the lipid bilayer of the erythrocyte as amino acid substitutions alter the conformation of band 3. As indicated in section 4.3, amino acid 490 lies at the extracellular border of the fourth transmembrane α-helix, a domain that must be inserted before helices one to three can be inserted (Ota *et al*, 1998). This may explain the decreased presence of membrane-bound band 3 in these kindred. The decreased insertion of the protein could alternatively be linked to decreased stability of band 3, the protein being degraded before insertion into the erythrocyte membrane. Pre-insertion degradation can occur either in the cytoplasm or in the endoplasmic reticulum if band 3 is not bound to ankyrin which, as discussed in section 1.4.5, may protect the protein from proteolytic enzymes (Hanspal *et al*, 1998).

The band 3 deficiency observed may be due to increased splenic sequestration because of the presentation of a senescence antigen, normally only found in aged

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erythrocytes. Kay (1993) suggested that protein degradation causes reorientation of band 3, creating a senescence antigen. Amino acid substitutions could imitate this reorientation in mutant band 3 proteins which are inserted into the membrane (Poole 2000). Beppu *et al* (1990) showed that sulphydryl oxidation of band 3 heightens the formation of the erythrocyte senescence antigen which could have an effect in kindred B (band 3 Bicetre) where arginine is replaced by cysteine.

At the level of protein production, the band 3 deficiency could be caused either by a promoter defect or by unstable mRNA. The promoter region of band 3 in exon 1 was examined using SSCP and appeared to be normal in all patients. RNA studies have been reported for both band 3 Bicetre (Dhermy *et al*, 1997) and band 3 Pinhal (Bracher *et al*, 1999) and the mutant mRNA transcripts were stable. Furthermore, Jarolim *et* al (1995) reported normal mRNA levels in five patients with HS linked to arginine substitutions.

4.5 HS due to a Novel Band 3 Mutation: E508K

Proband C (Black) was homozygous for a novel mutation in codon 508 (GAG→AAG, E508K). The parents of the proband were heterozygous for the nucleotide change.

4.5.1 Clinical Severity

Proband C has severe HS and was transfusion dependent until the age of 12 when she was splenectomised. The parents have the same mutation yet display different clinical profiles of HS: the mother has minimal symptoms while the father is moderately affected. Modulating factors (section 4.5.4) are believed to cause the different clinical profiles.

4.5.2 Band 3 Deficiency

Homozygous proband C suffers from extremely severe HS and cannot produce any normal band 3. Her apparent minor band 3 deficiency (ten percent) is inaccurate because of her transfusion dependency, as discussed for proband B

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(section 4.4.2). Ribeiro *et al* (2000) in their study of an infant homozygous for band 3 Coimbra and Perrotta *et al* (1998) in a study of an infant homozygous for band 3 Neapolis demonstrated that patients with homozygous mutant band 3 can survive with extreme medical intervention but, like proband C, have clinically severe HS. The E508K mutation is similar to the band 3 Coimbra mutation (Ribeiro *et al*, 2000) in that it also occurs within the highly conserved transmembrane region, very probably interfering with the insertion of band 3 into the erythrocyte membrane. The E508K mutation is the fourth homozygous case of band 3-deficient HS described and the second where the mutation impacts directly on the transmembrane region.

The parents of the proband, in concordance with their different clinical severities, had different protein profiles. The mother had 85 percent of normal band 3 while the father had only 65 percent of normal band 3. The protein profiles correlated with the clinical profiles (the lower the band 3, the worse the HS) but the reason for the different levels of band 3 expression remains elusive. SSCP and restriction enzyme analysis of the kindred did not reveal any other mutations and, while it is possible that the father, like the band 3 Coimbra-Mondego compound heterozygotes (Allosio *et* al, 1997), has a second mutation or modulating factor (section 4.5.4), none were located. Furthermore, the nucleotide change was not found in a cohort of control subjects (78 Black alleles and 40 Caucasian alleles), indicating that E508K is not a polymorphism, and the mutation is thus believed to cause the band 3 deficiency in this kindred. The homozygous proband is more severely affected than her heterozygous parents which also implies that the band 3 deficiency and resultant anaemia are caused by the E508K mutation.

4.5.3 Mutant Band 3 E508K

Amino acid 508 lies near the cytoplasmic border of the fourth α-helix of band 3 (figure 4.2) and once again, diminished insertion of this helix could abolish the insertion of the first three helices and hence the entire band 3 protein (Ota *et al*, 1998). As with the amino acid substitutions of codon 490, the different structures of glutamic acid and lysine (figure 4.4) would affect the folding of band 3.

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Glutamic acid has a carboxyl (COOH) side chain while lysine has an amino (NH_2) side chain. These groups are ionised (COO^- and NH_3^+ respectively) at physiological pH7.5 (Stryer, 1988) and these divergent charges are believed to be the reason for the altered conformation of band 3, causing its impaired insertion into the erythrocyte membrane.

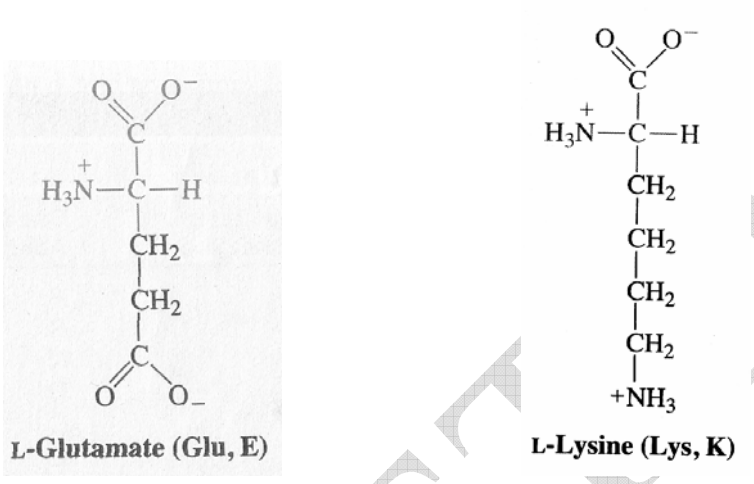


Figure 4.4: Linear Structures of Glutamic Acid (Glutamate) and Lysine

Furthermore, lysine residues have been observed to form non-sulphur cross-links in a number of proteins (e.g. collagen) (Stryer, 1988). The presence of a lysine residue instead of a glutamic acid residue at position 508 may further alter the conformation of the α-helices of band 3 through such cross-linking. It is possible that both the charge of the amino acid and cross-linking affect the insertion of band 3 into the erythrocyte membrane. Like the band 3 Pinhal and band 3 Bicetre mutant proteins, it is possible that either the RNA or the translated protein is unstable and is degraded, causing the band 3 deficiency (section 4.4.4), but RNA studies could not be performed on this kindred as no fresh blood samples could be obtained.

4.5.4 Possible Modulating Factors for the Codon 508 Mutation

The differences in the degree of band 3 deficiency (and hence the clinical severity) in the parents indicated that a second mutation or polymorphism could modulate the severity of the disorder. The most probable scenario is that the father (and possibly the proband) carries a modulating factor which worsens his

condition. Alternatively, the mother may have a factor which ameliorates her band 3 deficiency, possibly by up-regulating the production of band 3 (both normal and mutant) so that there is more protein available for insertion into the erythrocyte membrane. As none of the proband DNA remained, only the DNA of the parents were examined for modulating factors. According to restriction enzyme digests, none of the polymorphisms known to increase band 3 deficiency (band 3 Genas, band 3 Mondego and band 3 Montefiore) were present in this kindred. Compound heterozygotes with, for example, the band 3 Mondego polymorphism in *trans* to band 3 Coimbra (Allosio *et al*, 1997) or the band 3 Genas polymorphism in *cis* to band 3 Lyon (Allosio *et al*, 1996) show more severe clinical symptoms that their counterparts without the polymorphism.

Another type of modulating factor was recently described by Pagani *et al* (2003): the composite exonic regulatory element of splicing. These are polymorphisms or silent substitutions within the gene which can act to silence or enhance gene expression. In a cohort of cystic fibrosis patients, supposedly silent nucleotide changes in CFTR exon 12 were convincingly associated with variable phenotypes. The nucleotide changes did not affect the expected amino acid sequence but were strongly linked to variable degrees of exon skipping during pre-mRNA splicing (Pagani *et al*, 2003). SSCP analysis indicated that the exons and also the promoter region of band 3 of the parents of proband C were normal, although nucleotide changes can be overlooked if they make only small changes to the conformation of the exon.

Mutations in a different protein may also modulate the severity of band 3 protein deficiency. The most likely modulators are glycophorin A and ankyrin, both of which are linked to the transport of band 3 to the cell membrane (discussed in detail in section 1.4.5). If band 3 transport from the endoplasmic reticulum is delayed, the protein would have extended glycan chains as a result of excess post-translational modification due to the extended time spent in the presence of endoplasmic reticulum glycosylating enzymes. Similarly, if transport from the endoplasmic reticulum is increased, the glycan chains would be decreased in

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length (Bruce *et al*, 1994). Erythrocytes with lengthened glycan chains can be seen as displaying a senescence antigen, increasing the chance of sequestration in the spleen and possibly affecting band 3 insertion into the erythrocyte membrane.

4.6 The CpG Mutations

It is of importance that codon 490 (CGC) includes a cytosine-guanine (CpG) dinucleotide, which is linked to a number of frequently occurring point mutations (hotspots). Coetzer *et al* (1991), for example, described codon 28 of α-spectrin as a CpG hotspot associated with four mutations linked to hereditary elliptocytosis. The CpG dinucleotide is also implicated in a number of band 3 gene hotspots, including codon 150 (Allosio *et al*, 1996), codon 490 (Dhermy *et al*, 1997; Lima *et al*, 1999), codon 760 (Jarolim *et al*, 1995; Kanzaki, 1997) and codon 808 (Jarolim *et al*, 1995; Kanzaki, 1997), all of which encode arginine (CGN, N indicating any nucleotide), and codon 837 (ACG) (Jarolim *et al*, 1996; Iwase, 1998; Yawata *et al*, 2000) which encodes threonine.

The manner in which the CG dinucleotide is mutated essentially involves the deamination of a methylated cytosine (5-methylcytosine) to a thymine (see Figure 4.5) on either the sense or the antisense DNA strand (Duncan & Miller, 1980; Holliday & Grigg, 1993). Methylation of cytosine is common in both pro- and eukaryotes and is important in the deactivation of certain genes. The cytosine residue located before the guanine residue is particularly susceptible to methylation, 5-methylcytosine being found predominantly in these dinucleotides. (Holliday & Grigg, 1993) Furthermore, a CG dinucleotide is more likely to become methylated if the dinucleotide on the complementary strand is already methylated. This occurs through the activity of the methylation maintenance enzyme, which ensures that methylation is maintained throughout cell division, indicating that methylation has an important function in cell activity (Alberts *et al*, 1994).

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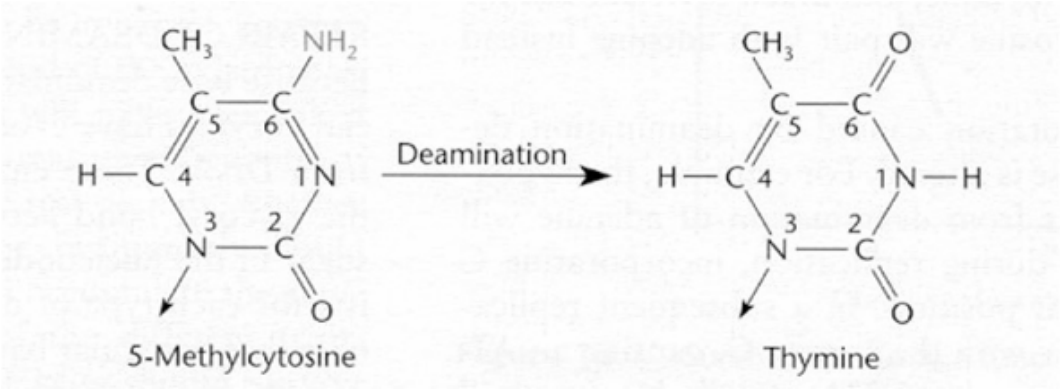


Figure 4.5: Spontaneous Deamination of 5-Methylcytosine to Thymine

The pairing of guanine and thymine resulting from the deamination of 5-methylcytosine can be repaired by thymine DNA glycosylase in mammalian cells but the efficiency of this enzyme is low, possibly because of the difficulties encountered in differentiating between normally and abnormally paired thymine. It is hypothesised that a DNA binding protein recognises the GT mispairing and allows thymine DNA glycosylase to excise the incorrectly inserted thymine (Alberts *et al*, 1994). Because of the susceptibility of 5-methylcytosine to deamination, CpG dinucleotides are found at a lower than expected frequency in most of the genome and are often associated with mutations. Only one percent of the genome carries a higher than expected number of CpG dinucleotides: the CpG islands which are regions of up to a few thousand bases found in the 5' region of many genes. They tend to lie within gene promoter regions and are highly conserved (Holliday & Grigg, 1993).

Mutations of the CpG dinucleotide are only observed on both DNA strands of the arginine codon. The nucleotide sequence of this codon (CGN) explains its propensity for mutation, the CpG dinucleotide comprising the first two nucleotides of the triplet. Hence, no matter which strand is mutated, either the first or the second nucleotide is changed, altering the amino acid encoded. Mutations of the CpG dinucleotide were observed on both the sense and the antisense DNA strands in this study. Band 3 Pinhal (C<u>G</u>C→C<u>A</u>C) is an example of deamination of cytosine on the antisense strand (i.e. G<u>C</u>G→G<u>T</u>G) whereas the band 3 Bicetre mutation (<u>C</u>GC→<u>T</u>GC) occurs on the sense DNA strand.

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It must also be noted that while codon 508 (GAG) does not contain a CpG dinucleotide, codon 507 reads TTC causing a CG dinucleotide at the codon boundary (TTCGAG). The mechanism of the mutation observed in codon 508 is identical to that described above and occurs on the antisense DNA strand (i.e. AAGCTC→AAGTTC). The formation of a CpG dinucleotide over two codons is also seen in the band 3 Coimbra mutation at codon 488 (GTG→ATG) where codon 487 is ATC. This evidence, in conjunction with the data collected in this study and the known CpG hotspots, emphasises the significance of CpG dinucleotides as mutation hotspots.

4.7 Anion Exchange Function

Changes in the conformation of mutant band 3 which is inserted into the erythrocyte membrane may alter the anion transport function of the protein. Notably, Poole *et al* (2000) state that the second extracellular loop of band 3 is highly conserved and is necessary for anion exchange activity. Assuming the insertion of mutant band 3 into the erythrocyte membrane, anion transport activity in the kindred discussed above could be lost as both codon 490 and codon 508 are associated with the second loop. Codon 490 lies at the extracellular border of the lipid bilayer where the second loop reinserts into the membrane whereas codon 508 lies in the α-helix following this loop. Sulphate flux studies, as a measure of anion exchange, would have been of great interest but no fresh blood samples could be obtained from any members of the three kindred.

4.8 Protein 4.2 Deficiency

A deceased quantity of protein 4.2 was observed in all patients and is a secondary phenomenon of the band 3 deficiency. Protein 4.2 is often depleted in patients with band 3-deficient HS as attachment sites for protein 4.2 are lost with the removal of band 3 from the membrane. The protein 4.2 decrease is seldom of the same degree as that of band 3 as protein 4.2 can also bind to other membrane proteins (Walensky *et al*, 2003). The mutations detected in this study affect the

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membrane domain of band 3 rather than the cytoplasmic domain where the protein 4.2 binding sites are located. The deficiency of protein 4.2 is thus related to a decreased number of binding sites as less band 3 is inserted into the membrane. This is corroborated by evidence that patients with a greater band 3 deficiency had less membrane-linked protein 4.2.

4.9 Analysis of 12 Band 3-Deficient Hereditary Spherocytosis Patients

4.9.1 Exclusion of Known and Novel Mutations

The remaining 12 patients examined had mild to moderate HS and none had a severe band 3 deficiency, the decreases similarly ranging from mild to moderate. The mutations underlying these deficiencies were not determined but a number of known mutations were excluded.

Restriction enzyme analysis on amplified DNA from the probands was used to exclude Band 3 Cape Town (E90K), Band 3 Osnabruck/Lyon (R150X), Band 3 Tuscaloosa (P327R), Band 3 Pribram (intron 12, -1g→a) and Band 3 Most (L707P) as well as mutations in the codon 490 and codon 760 hotspots. SSCP analysis was used to screen for possible nucleotide substitutions in the remainder of the gene. Exons two to 20 of the band 3 gene cover the entire coding region of the gene and exon one contains the band 3 gene promoter. All of these regions were evaluated and only two patients displayed abnormal SSCP patterns, proband D in exon four and proband E in exon nine (discussed in section 4.9.2).

The normal SSCP patterns indicated that no known or novel exonic mutations were present in these patients. The amplified PCR product usually included part of the intron, the majority of primers being intronic, and the splice sites are thus also likely to be normal. It is however possible that nucleotide changes were overlooked as SSCP analysis is not infallible, primarily because it relies on DNA conformation which can be affected by the nucleotide substitution and the DNA sequence surrounding the mutated nucleotide (Saad *et al*, 1993).

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4.9.2 Nucleotide Changes in Two Patients

Exon 4 of proband D and exon 9 of proband E were sequenced, revealing heterozygous nucleotide changes in both cases, neither of which were believed to cause their band 3 deficiency. The conformational change in exon four of proband D was the band 3 Memphis I polymorphism (K56E) which is located within the cytoplasmic (ankyrin-binding) domain and is a common polymorphism with no known phenotypic effects. It is linked to slow moving band 3 in polyacrylamide gels, an artefact caused by the decreased ability of the band 3 Memphis protein to bind to SDS. This results in decreased denaturation of the protein and hence a different mobility through the polyacrylamide gel (Jarolim *et al*, 1992). Proband E has a silent change (GCA→GCC) in exon 9 (codon 250) that does not alter the amino acid encoded which is alanine (GCN). It is possible that the codon 250 nucleotide change affects pre-mRNA splicing and causes decreased production of band 3, as described by Pagani *et al* (2003).

4.10 Analysis of Protein 4.1-Deficient Hereditary Elliptocytosis Patients

Immunoblotting indicated protein 4.1 of a normal size in nine HE kindred. This indicated that neither the shortened unnamed variant (McGuire *et al*, 1988) nor lengthened protein 4.1 Hurdle-Mills (McGuire *et al*, 1988) were present. The known point mutations, protein 4.1 Madrid (Dalla Venezia *et al*, 1992) and protein 4.1 Lille (Garbarz *et al*, 1995), and protein 4.1 Aravis (Lorenzo *et al*, 1994), where an entire codon is lost, were tested using restriction enzyme digests but no causative mutations were located. The last of the known protein 4.1 mutations, protein 4.1 Algeria (Conboy *et al*, 1986) and protein 4.1 Annecy (Dalla Venezia *et al*, 1998), are both linked to the loss of the initiation codon. The immunoblot for these mutations appears normal as a quantity of normal protein 4.1 would be present. The only means of examining these proteins would be mRNA analysis and no fresh blood samples could be obtained for this procedure.

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4.11 Conclusions

The study of hereditary haemolytic anaemias in the South African population is important as the causes underlying the disorders differ from other countries. For example, band 3 defects in the American and European populations account for approximately 20 percent of HS cases, ankyrin and spectrin abnormalities being the most common (Tanner, 1997; Delaunay, 1999), whereas in South Africa up to 50 percent of HS patients are band 3 deficient (Coetzer, 1999). Furthermore, HS is very common in the Caucasian population but rare in other race groups in America and Europe (Walensky *et al*, 2003), yet has been described in a number of South African Blacks (Coetzer, 1999). In this study one third (5/15) of the patients identified was Black. This may be explained by the origins of the Black populations: those studied in Europe and Northern America originate from North and West Africa and are genetically different from the South African Black population. Similarly, while HE affects all populations, protein 4.1-deficient HE has only been described in Caucasian and Algerian populations (Walensky *et al*, 2003), but has been detected in number of Black kindred in South Africa (Coetzer, 1999). HE is also generally linked to spectrin dimer defects, protein 4.1 deficiencies only accounting for approximately 20 percent of HE cases (Delaunay, 1999). In South Africa, in contrast, up to 50 percent of HE patients have a protein 4.1 deficiency (Coetzer, 1999), including many Black patients (3/9 in this study).

In this study three mutations causing a band 3 deficiency were identified, one of these (E508K, GAG→AAG) being a novel mutation. All of these mutations are located in exon 13, illustrating the importance of the encoded amino acids for the insertion of band 3 into the erythrocyte membrane. Codon 490 was once more highlighted as a mutation hotspot as two such mutations, band 3 Pinhal (R490H, CGC→CAC) and band 3 Bicetre (R490C, CGC→TGC), were identified in this study. Band 3 Bicetre has only been described in the Caucasian population (Dhermy *et al*, 1997 and own observations), whereas band 3 Pinhal has been described in Caucasian (Lima *et al*, 1999) and Cape Coloured (Bracher *et al*, 2000) kindred. In this study band 3 Pinhal was identified in a Black African

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proband, indicating that the codon 490 hotspot affects both Caucasian and Black population groups. All the mutations occurred in a CpG dinucleotide, emphasising the susceptibility of this dinucleotide to mutagenic 5-methylcytosine deamination.

The number of mutations (more than 50) described within the band 3 gene is far greater than those described in the protein 4.1 gene (a mere seven). A possible reason for this is that erythroid band 3 is only expressed in the erythrocyte and the kidney whereas erythroid protein 4.1 is widely dispersed, a variety of isoforms arising from extensive alternative splicing. Mutations in the protein 4.1 gene could thus have further-reaching effects resulting in non-viable foetuses. Alternatively, the size of the coding regions of the band 3 and protein 4.1 genes could play a role. Band 3 is encoded by a smaller gene (20kb) than protein 4.1 (more than 200kb) but the band 3 protein contains 911 amino acids whereas erythroid protein 4.1 is only 589 amino acids in length. The likelihood of an exonic region of the band 3 gene (with its smaller introns) being affected by a nucleotide change is thus higher than the likelihood of a protein 4.1 exon being affected.

HS and HE are heterogeneous at both the level of the protein defect and the genetic cause of such defects. While clinical studies can indicate the type of haemolytic anaemia, no morphological picture is conclusively linked to a specific protein defect. Membrane protein analysis, a labour-intensive and costly technique, is required to identify the protein abnormality and is important to both the accurate diagnosis and the prognosis of the disorders. PCR screening is difficult because of the large size of the genes and the family-specific mutations associated with each protein defect, and is presently only feasible in a research laboratory. Identification of the mutation would ultimately be useful for treatment using gene therapy, particularly as only invasive surgery or transfusions currently alleviate severe inherited haemolytic anaemias. The genes of band 3 and protein 4.1 are very large and replacing the entire gene would be difficult; ideally the affected exon could be identified and restored through genetic recombination.

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