Chapter 1
General Introduction
Erythrocyte and erythrocyte membrane

Erythrocytes are the major component of blood, ideal for many studies of membrane structure due to the absence of internal membranes. During the penultimate stage of development of erythrocytes from the multipotent stem cells in the bone marrow it undergoes disassembling of internal membraneous organelles and the nucleus is physically expelled owing to its unique property unlike other cells. The mature human erythrocyte is a circular, biconcave, non-nucleated disc and spends its circulatory lifetime of approximately 120 days performing its major function of oxygen delivery throughout the whole body system before being deglycosylated and removed in the spleen and the liver (1). Figure 1 shows the average size and shape of a normal erythrocyte. Erythrocytes must possess a remarkable ability to undergo cellular deformation which is evident from the fact that the diameter of human red cell (8μm) far exceeds that of capillaries (2-3μm) of microvasculature through which it must pass in the process of delivering oxygen to the tissues.

Figure 1: Average size and shape of normal erythrocyte (taken from www.easynotecards.com).
Erythrocyte is composed of a single external membrane, the plasma membrane, surrounding a cytoplasm containing a highly concentrated solution of Hb, some glycolytic enzymes and minor amounts of other proteins and enzymes. Thus, all the structural elements of the red cell are in one way or other linked to the cell membrane. In fact, the red cell membrane serves as the best-defined model for understanding the organization and interactions that maintain cell shape and integrity of the structures inside and outside the cell. The erythrocyte membrane is highly specific in the four particular aspects for the functional role of transporting dissolved gases between the cells. First is its characteristic flattened biconcave shape, responsible for its ability to undergo the large passive deformation. Secondly it is highly elastic, permitting the cell to withstand without rupture the high sheer forces it experiences in circulation. Both of these properties is reflected into the geometry, the viscosity of the intracellular environment and the material properties of its membrane. Hence erythrocyte can be effectively considered as having a phospholipid bilayer membranous bag containing a highly concentrated Hb solution wrapped inside a two dimensional filamentous protein network called erythrocyte membrane skeleton or “cytoskeleton” lying on its cytoplasmic face (2, 3).The membrane cytoskeletal proteins and the transmembrane proteins inserted in lipid bilayer form a network lining the inner face of the membrane and the membrane associated proteins or linking proteins link together the other two type of proteins to form a 3D protein network to effect the intricate biorheological properties of erythrocytes. Thirdly the membrane is highly permeable to oxygen and also bicarbonate ions due to the presence of anion transporters, Band 3, a large dimeric glycosylated globular integral protein, which exchanges bicarbonate and chloride ions across the erythrocyte membrane. Finally the presence of glycoporin, major erythroid integral protein, accounts for the very high negative surface charge density of erythrocyte as the amino terminal extra cellular domain of this small protein is highly glycosylated with the oligosaccharides bearing the negatively charged terminal sialic acid sugar residues. Besides, erythrocytes performs a number of other vital functions for its survival including (a) effective glycolysis for energy production (b) cation pumping against electrochemical gradients (c) synthesis of glutathione and other metabolites (d) nucleotide catabolism reactions (e) maintenance of Hb iron in its functional, reduced, ferrous state; (f) protection of enzymatic and structural proteins from oxidative damage, denaturation and (g) preservation of membrane phospholipid asymmetry. About 52% of the membrane mass is protein, 40% is lipid, and 8% is carbohydrate, the carbohydrate is invariably in the form of glycolipids or glycoprotein and the density of the membrane is directly proportional to the
amount of protein in the membrane (4). The structural and functional aspects of the various lipid and protein components have been well understood (5-17).

The major lipid components are unesterified cholesterol and phospholipids, present in nearly equimolar quantities. Free fatty acid and glycolipids are present in small amounts (5). The phospholipid composition of membrane is phosphatidylcholine (PC), 30%; sphingomyelin (SM), 25%; phosphatidylethanolamine (PE), 28%, and phosphatidylycerine (PS), 14%. Other phospholipids such as phosphatidic acid, phosphatidylinisitol-4-phosphate, and phosphatidylinisitol-4,5-diphosphate make up about 2-3% of the total. Most surprisingly, though cholesterol is distributed almost equally between the two leaflets of membrane, these phospholipids are asymmetrically distributed in the membrane, with more than 75% of the choline containing uncharged phospholipids PC and SM found in the outer monolayer of the lipid bilayer, while 80% of PE and all PS, the charged phospholipids, reside in the inner monolayer (6). According to recent studies several different types of energy-dependent and energy-independent phospholipid transport proteins have been shown to generate and maintain phospholipid asymmetry (18, 19). A representative asymmetric distribution of phospholipids as determined experimentally is shown in Figure 2.

![Figure 2: Composition and asymmetry of lipids in erythroid membrane (taken from rbclab.com).](image-url)
The regulation and physiology of membrane phospholipid asymmetry is manifested in various phosphatidylserine-related pathophysiologies. The cooperative activities of three transporters regulate the membrane asymmetry: the ATP-dependent nonspecific lipid floppase, which slowly transports lipids from the cell’s inner-to-outer leaflet. The ATP-dependent aminophospholipid-specific translocase, which rapidly transports PS and PE from the cell’s outer-to-inner leaflet; and the Ca$^{2+}$-dependent nonspecific lipid scramblase, which allows lipids to move randomly between both leaflets. At physiological Ca$^{2+}$ concentrations, PS asymmetry is enhanced because of an active translocase and floppase but inactive scramblase, while at increased concentration of intracellular Ca$^{2+}$ induces PS randomization across the cell’s plasma membrane. The exposure of PS at the cell’s outer leaflet induces coagulation and thrombosis and marks the cell as a pathologic target for elimination by phagocytes. The over exposure of PS in outer leaflet symbolizes the phagocytosis of red cells by macrophages. Hence loss of lipid asymmetry leading to exposure of PS on the outer monolayer has been suggested to play a role in premature destruction of thalassemic and sickle red cells (20-22). The exclusive localization of PS to the inner monolayer also inhibits the adhesion of normal red cells to vascular endothelial cells, thereby ensuring unimpeded transit through the microvasculature (23, 24).

In contrast to phospholipids, cholesterol diffuses across the membrane in a physiologic time-scale (a half-time of seconds or less) (17). The membrane red cell is unable to synthesize lipids de novo, but a number of lipid renewal pathways produce considerable turnover of various phospholipids with no net change in lipid composition. Furthermore, the cholesterol content of the membrane is dependent on the exchange that takes place between plasma cholesterol and membrane cholesterol (25).

**Erythrocyte membrane proteins:**

Erythrocyte membrane proteins are the earliest membrane proteins studied and documented in the last 35 years both from the normal and pathological point of view (26). Simple SDS-PAGE gels shows about a dozen of characteristic bands in the red blood cell membrane which were earlier named according to the position protein band in the gel. Latest studies using proteomics techniques have revealed presence of more than 850 proteins in the erythrocyte membrane (27). Depending on localization and their functional roles the erythrocyte membrane proteins are broadly classified into two categories a) Integral membrane proteins and b) Membrane skeleton proteins. Figure 3 shows the major membrane cytoskeleton proteins.
**Figure 3:** A schematic representation of an erythrocyte cytoskeleton enclosed within the phospholipid bilayer (taken from www.studyblue.com)

**Integral membrane proteins**

Integral membrane proteins are tightly bound to membranes by hydrophobic forces through their hydrophobic domains. Integral proteins exhibit diverse functional heterogeneity, serving as transport proteins, as adhesion proteins involved in interactions of red cells with other blood cells and endothelial cells, as signaling receptors, and other still undefined activities. The major integral membrane proteins that are transporter protein include include band 3 (anion transporter), aquaporin 1 (water transporter), Glut1 (glucose and L-dehydroascorbic acid transporter), Kidd antigen protein (urea transporter), RhAG (gas transporter, probably of carbon dioxide), Na⁺ K⁺-ATPase, Ca⁺²-ATPase, Na⁺-K⁺-2Cl⁻ cotransporter, Na⁺ Cl⁻ cotransporter, Na⁺ K⁺-cotransporter, K⁺Cl⁻ cotransporter, and Gardos Channel (28-30). Integral proteins can either stick into the membrane from one side or not exit the other or they may be transmembraneous and project through both sides. Individual transmembrane proteins are known to contribute to the structural integrity of the membrane and/or the transport of small molecules across the lipid bilayer. The major integral proteins providing the structural integrity includes glycophorins (GP-A, GP-B and GP-C), duffy antigen (blood group antigen,
also known as chemokine receptor) and decay accelerating factor (DAF) (protects cell from
damage by complement (31, 32).

Band 3, the anion exchanger, is the major integral protein, constituting about 25% of total
membrane proteins and this protein is composed of three dissimilar and functionally distinct
domains i.e. the hydrophilic cytoplasmic domain, the acidic C terminal domain and the
hydrophobic transmembrane domain. The two clearly established functions of band 3 in the
membrane are (1) anion transport, resulting in one-for-one exchange of HCO₃⁻ or Cl⁻ across
the membrane; and (2) physical linkage of the lipid bilayer to the underlying membrane
skeleton and may have some role in macrophage-mediated removal of IgG bound erythrocytes
(33, 34). Four sialic acid-rich glycoporphins (glycophorin A, B, C and D) compose a class of
integral membrane proteins termed glycophorins, constituting approximately 2% of total
erythrocyte membrane proteins (35). Glycophorins keep blood fluid, carry carbohydrates on
extramembrane surface and help in blood group determination (36). A number of other
integral membrane proteins are present in the erythrocyte membrane. Aquaporin-1, present in
the erythrocyte membrane, belongs to a family of membrane channel proteins that serve as
selective pores for water transport.

**Erythrocyte membrane skeletal protein**

Underlying the plasma membranes of cells there is a self-assembled network of proteins named
the membrane skeleton. The cytoskeleton which gives the membrane its characteristic
mechanical properties is involved in cell function, including cell-cell interactions, motility,
receptor organization, endocytosis, exocytosis and cell division, etc. Unlike other cells
erythrocyte does not contain transcellular cytoskeleton and have no microtubules or intermediate
filaments.In the absence of internal organelles or other transcellular structures the human
erythrocyte relies on its plasma membrane and a network of membrane associated proteins
which is called membrane skeleton. Spectrin, ankyrin, actin, tropomyosin and few other proteins
like dematin, tropomyosin, tropomodulin etc. are the principal components of the erythrocyte
membrane skeleton (37, 38). Lateral interactions among these proteins constitute the spectrin-
based composite structure that is anchored to the bilayer through vertical interactions.The
membrane cytoskeleton is roughly hexagonal lattice majorly formed by spectrin tetramers, five
to six were attached to a short, 37-nm-long actin filament composed of 12–14 actin molecules
(39, 40). Now this spectrin-actin network is coupled to the membrane bilayer primarily by
association of spectrin with ankyrin, which in turn is bound to the cytoplasmic domain of the
anion exchanger. The anion exchanger is associated into dimers (41), which associate with separate sites on the membrane binding domain of ankyrin to form pseudo-tetramers (42-45). Anion exchanger dimers also are associated on their cytoplasmic surface with band 4.2 (46). Additional membrane connections are provided at the spectrin-actin junction by a complex between protein 4.1, p55, a member of the MAGUK family, and glycophorin C (47-49). According to electron microscopy several proteins responsible for capping actin and defining the length of actin filaments as well as stabilizing spectrin-actin complexes have been localized to spectrin actin junctions (50, 51). Each spectrin-actin junction is stabilized by the formation of a ternary complex with band 4.1(52, 53) and this stabilization occurs through the direct interaction of band 4.1 with both spectrin subunits and actin. Adducin associates with the fast-growing end of actin filaments in a complex that caps the filament and promotes assembly of spectrin (54-57) and it is a target for the calcium-dependent regulatory protein, calmodulin; as such, its ability to promote spectrin-actin interactions is regulated by intracellular calcium concentrations. However, in contrast to band 4.1, adducin is much less abundant in erythrocyte. A nonmuscle isoform of tropomyosin is associated with the sides of actin filaments (58). Tropomyosin is of the same length as actin filaments as seen in electron micrographs and is a candidate to function as a morphometric ruler defining the length of actin filaments in erythrocyte membranes. Tropomodulin caps the slow-growing end of actin filaments in a ternary complex involving tropomyosin (59-62). A representative electron microscopic presentation of the membrane skeleton is shown in Figure 4A. Spectrin tetramers are cross-linked at nodes (junction points) by short filaments of F-actin and band 4.1/adducin.

Approximately six spectrins interact with each node (Figure 4). At the central region of the spectrin tetramers are bound ankyrin/anion exchanger complexes (63). Figure 4B describes the membrane-cytoskeleton connections in erythrocytes. Ankyrin is linked through its ank-repeat region to dimers of the anion exchanger (gray). Each ankyrin is capable of cross-linking two anion exchanger dimers. The 15th helical repeat of β-spectrin (yellow) interacts with ankyrin. At the NH2-terminal region of β-spectrin is a binding site for protein 4.1 (red). Protein 4.1 forms a ternary complex with the transmembrane protein glycophorin C and the membrane-associated guanylate kinase p55 (green). The spectrin-actin junction junction is shown in Figure 4C. Short filaments of F-actin containing 14–16 monomers tether spectrin at the nodes shown in A. The negative (-) end of the filaments is blocked by tropomodulin, and nonmuscle tropomyosin lies along the filament. β-spectrin binds actin via its NH2-terminal CH domains (CH1 and CH2) and the first two triple helical repeats (Rpt1 and Rpt2). Approximately six
spectrins would fit a short actin filament if the spectrins were arranged along the side of the filament as indicated in this figure. This is consistent with the six spectrins per node indicated in Figure 4. Spectrin-actin interaction is promoted by protein 4.1 and adducin. Adducin has a high affinity for the positive (+) end of the filaments and possibly recruits other proteins to the positive end (64).

Figure 4: Organization of the erythrocyte membrane skeleton, (A) Electron microscopic depiction of membrane skeleton, (B) Membrane skeleton connections in erythrocytes, (C) The spectrin-actin junction (Adapted from the reference, 64). The physical characteristics and the functional roles of the well known membrane skeletal proteins of human Red Blood Cell (RBC) are shown in Table1.
Table 1: Membrane skeletal proteins of human RBC

<table>
<thead>
<tr>
<th>Name of proteins</th>
<th>Subunit mol wt.(KDa)</th>
<th>Oligomeric status</th>
<th>Functional role</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-spectrin</td>
<td>260</td>
<td>α₂β₂ tetramer</td>
<td>Major protein that forms the meshwork of membrane skeleton</td>
<td>65</td>
</tr>
<tr>
<td>β-spectrin</td>
<td>225</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>43</td>
<td>Oligomers of 12 to 17 units</td>
<td>Cross liked by spectrin into a quasi hexagonal membrane skeletal network</td>
<td>53</td>
</tr>
<tr>
<td>Adducin</td>
<td>105</td>
<td>Heterodimer</td>
<td>Barbed end capping protein of actin and promotes spectrin-actin associations</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>29</td>
<td>Heterodimer</td>
<td>On binding actin stabilizes it and regulates spectrin – actin interactions</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropomodulin</td>
<td>43</td>
<td>Monomer</td>
<td>Binds tropomyosin and inhibits TM-actin interactions.</td>
<td>59</td>
</tr>
<tr>
<td>ankyrin</td>
<td>215</td>
<td>Monomer</td>
<td>Interacts with spectrin and Band 3.</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>186</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>160</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein 4.1</td>
<td>78</td>
<td>Monomer</td>
<td>Helps in spectrin-actin complex formation</td>
<td>49</td>
</tr>
<tr>
<td>Pallidin</td>
<td>72</td>
<td></td>
<td>Deficient erythrocytes have shortened lifespan</td>
<td>68</td>
</tr>
<tr>
<td>Dematin</td>
<td>48</td>
<td>Trimer</td>
<td>Actin bundling activity</td>
<td>69</td>
</tr>
</tbody>
</table>

The spectrin based skeleton

About $10^5$ spectrin heterotetramers and its higher oligomers cross link and inter-triangulate approximately 30,000 actin protofilaments. Linkage of the meshwork overlying the lipid bilayer is usually mediated both along spectrin, through Band 3-ankyrin and at the spectrin-actin nodes via protein 4.1 associations with Glycophorin C and p55. Besides the actin binding proteins the mobile proteins on the cell surface are aquaporin and the GPI-linked protein CD59 and sterically...
excluded from those regions of the membrane with high density of cytoskeleton connected proteins such as Band 3. Spectrin-actin interfaces are normally linked to the bilayer by a series of protein associations involving protein 4.1, glycophorin C and probably p55. Macromolecular complexes, ankyrin based and 4.1R based are believed to be responsible to hold the structural integrity. Band 3 and RhAG link the bilayer to the membrane skeleton through the interaction of their cytoplasmic domains with ankyrin, and glycophorin C, XK, Rh, and Duffy through their interaction with protein 4.1R (70-72). Two other proteins, adducing and dematin also act as linking proteins as they interact with Band 3 and Glut 1 (72, 73). These membrane protein linkages with skeletal proteins may play a role in regulating cohesion between lipid bilayer and membrane skeleton.

**Spectrin:**

Spectrin isoforms have been identified in a wide variety of cells and tissues and distributed in cells ranging from plants, bacteria, and primitive ameoba to human. Erythroid spectrin was first isolated by Marchesi and Steers in 1968 as a membrane associated protein from a Hb free ghost. It is expressed at the level of 200,000 copies per cell (74). Spectrins are extended, flexible molecules; 200 in length and 3–6 nm across with actin-binding domains at each end (75-77). Spectrin consists of α and β subunits, which are both related to α actinin (78-82). The α and β subunits are associated laterally to form antiparallel heterodimers, and heterodimers are assembled head-head to form heterotetramers. In mammals, there are two α and five β subunits of human spectrin (αI, αII, βI, βII, βIII, βIV, and βV). The αI and αII-spectrin subunits are encoded by SPTA1 and SPTAN1, respectively (83, 84). SPTA1 is expressed in erythroid cells. The genes for α1∑1 and β1∑1 chains are localized in chromosomes 1 and 14 respectively. Erythroid spectrin is a combination of two α1∑1 and β1∑1 chains (α2 β2). The α1∑1 chain is erythroid specific. In contrast to SPTA1, the SPTAN1 encodes several αII-spectrin isoforms that are expressed in all nonerythroid cells. SPTB, SPTBN1, SPTBN2, and SPTBN4 encode βI–βIV spectrins, respectively, and SPTBN5 encodes βV spectrin that is expressed at a low level in many tissues. Multiple isoforms of spectrin are shown in Table 2.
Table 2: Human Spectrin genes, subunits and heterotetramers (Adapted from Ref no. 64):

<table>
<thead>
<tr>
<th>Spectrin subunits</th>
<th>Gene</th>
<th>Human Chromosome</th>
</tr>
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<tbody>
<tr>
<td>$\alpha_1$</td>
<td>SPTA1</td>
<td>1</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>SPTAN1</td>
<td>9</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>SPTB</td>
<td>14</td>
</tr>
<tr>
<td>$\beta_{-G/\beta_2}$</td>
<td>SPTBN1</td>
<td>2</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>SPTBN2</td>
<td>11</td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>beta IV</td>
<td>19</td>
</tr>
<tr>
<td>$\beta_{-H/\beta_5}$</td>
<td>BSPECV</td>
<td>15</td>
</tr>
</tbody>
</table>

Spectrin repeat

$\alpha_1\Sigma_1$ and $\beta_1\Sigma_1$ chains each have three domains of different structure and function. The subdomains are rod, the elongated backbone of each polypeptide, and self association which involves the portion of each polypeptide chain corresponding what has been defined as the head of the dimers and either the actin binding domain at the amino terminus of the $\beta_1\Sigma_1$ polypeptides or an EF hand structural region at the carboxy terminus of the $\alpha_1\Sigma_1$ polypeptides. Each spectrin subunit is comprised of an elongated backbone made up of a succession of repetitive units (roughly 106 amino acids long) defined as spectrin repeats, flanked by non homologous N- and C-terminal sequences (83, 85). The predominant structure found in the $\alpha$ chain is the elongated rod domain which begins at the amino terminus of the polypeptide. The repeats are to be believed to be structurally identical units despite their variable degrees of conservation and each is composed of three $\alpha$ helices. Usually alpha spectrin contains about 20 repeats while the beta spectrin has 16 repeats and the size of such segments varies in the range between 99 and 114 amino acid residues. All the repeats are made of three helices of which A and C are parallel and B is antiparallel (86). Helix B is longer in size and it contains a conserved proline residue in the middle which makes the structure a little kinked. The three helices are slightly curved and wrap around each other in a left-handed supercoil (87). Consecutively arranged repeats are connected through the junction of helix C and helix A of the following repeat in an uninterrupted helical structure (88, 89).Studies on the secondary structure of
spectrin by CD spectroscopy yielded the value of 60-70% α-helical content (90). Repeat 10 domain has a short and unique stretch of amino acids, homologous to the non kinase SH3 domain of the src family of proteins and have the β structure (83). Repeats 20-22 of α1Σ1 chain are the most variable when compared to the other repeats. Repeat 22 is made of unique structural domain consisting of two EF hand motifs. The β chain has a smaller mass and the chain which is comparatively shorter than α chain, is also assembled from three structural regions. The amino terminus sequence from 1-272 residues, composes actin binding domain, followed by the rod domain consisting of 17 homologous repeat motif and the carboxyl terminus of the β chain which consists of 52 amino acid residues constituting a single α helix.

Atomic resolution of the structure of triple-helical repeat domains obtained by X-ray crystallography (87, 91, 92) as well as NMR (80) provides a striking confirmation of the structure originally predicted by Speicher and Marchesi (93). Triple helical repeats are comprised of two parallel and one antiparallel α-helices, which are stabilized by interactions between hydrophobic residues spaced in a heptad repeat pattern found in other examples of paired helical structures (figure 5). Tandem triple helical repeats of spectrin are comprised of antiparallel α-helices connected by an extended α-helix (87). Comparison of hydrodynamic properties of single and multiple domains suggest that serial repeats are flexible and configured such that the average end-end length is reduced compared with values predicted for rigid rods (94). One possible source of flexibility is bending of the α-helix interconnecting domains. A novel mechanism for shortening end-end distances by rearrangement of helices has been proposed by Grum and coworkers (87) based on alternative structures observed by crystallography. An extended series of triple helical repeats may also exhibit a superhelical twist (87, 95). Spectrin repeats have recently been demonstrated by atomic force microscopy to reversibly unfold and refold when subjected to forces in the range of 35 pN (96). Spectrin with triple helical domains has the potential to function as molecular springs that can store energy and dampen deformations resulting from mechanical stress.
Structural Domains of spectrin subunits:

α-spectrin contains 22 domains among which domains 1–9 and 11–21 are comprised of triple helical repeats also found in β and β-H spectrins. Domain 10 is an SH3 (src homology domain 3) motif and the COOH-terminal domain 22 is related to calmodulin (97) (Figure 6). Domain 11 of vertebrate α2-subunits contains a 35-residue extension with the cleavage site for Ca$^{2+}$-activated protease and a calmodulin binding site (98, 99). β-spectrin contains 19 domains beginning with a highly conserved NH2-terminal actin-binding domain comprised of two adjacent calponin homology domains (100, 101), followed by 17 consecutive triple-helical repeat domains and ending with a COOH-terminal domain which includes a PH domain (Figure 7). Ankyrin-binding sites are at a site in the midregion of the tetramer (102, 52) and have been found to locate in the repeat number 15 of β1-spectrin (103). Repeat 17 is a partial helical repeat that pairs with the COOH terminus of α-spectrins to form a noncovalent triple-helical structure.

Figure 5: Structure of spectrin repeats. (88)

Figure 6: Structural domains of spectrin dimer (159).
α spectrin contains an EF-hand motif located at the NH₂ terminus of that are juxtaposed to the actin-binding domain on the adjacent β-subunit. The EF-hand domain of spectrin is structurally similar with calmodulin and contains four EF hands. From NMR studies it is evident that, in the absence of calcium, EF-1 α helices are tightly packed, whereas the EF-2 helices are less compact and are involved in side-to-side interactions with the EF-1 helices. The amino-terminal EF-hands are functional and also exhibit a Ca²⁺-dependent conformational change (97, 104) as this binding causes a redistribution of hydrophobic interactions within EF-1 which results in an opening of the helix-turn-helix structure that is, in turn, propagated to EF-2. These conformational changes may modify the interface between segments α22 and β1, and may in particular modify the loop structure between EF-1 and EF-2 which plays an important role in interchain binding at the tail end of the spectrin subunits (105, 100). This may explain the regulatory role of Ca²⁺ in the interaction between filamentous actin and spectrin (104).

The F-actin binding region is located in the NH₂-terminal actin-binding domains of the β spectrins and this region can be divided into two regions which are termed as calponin homology (CH) domains (107), actin binding proteins that regulate smooth muscle contraction (figure 7). These are similar in sequence to a region of the smooth muscle actin binding protein calponin; such tandem pairs occur in other proteins that have lateral associations with actin filaments, including dystrophin, utrophin, α-actinin, and fimbrin. As a single copy the CH domain is also present in the signalling protein such as Vav. Like PH and SH3domains the CH domain are classified as a protein module which is present both in cytoskeletal and signal transduction proteins.

X-ray crystallography has resolved the atomic structures of the NH₂-terminal (108) and COOH-terminal (100) CH domains of β-spectrin at 2A resolution (101). It is dominated by four α helices, each consists of 11-18 residues, connected by long loops. The β-spectrin CH domains are also likely to interact with actin. The actin binding activity of β spectrin is restricted to the β chains (90, 107). A minimal actin-binding fragment of erythrocyte spectrin has been produced by limited trypsin digestion and derives from residues 47–186 (109). These residues represent the NH₂-terminal CH domain (known as CH1). Several considerations suggest that the site of contact with F-actin involves the junction between CH domains, with the first domain providing most of the interactions (100, 110). The second CH domain may contribute by enhancing the affinity for F-actin and/or have a regulatory role (100).
Figure 7: Crystal structure of a CH domain from human beta-spectrin at 2 Å resolution (101).

The Pleckstrin homology domain (figure 8) is particularly abundant in cell signalling proteins. PH domains can be involved in reversible anchorage of proteins containing PH domains to cell membrane as this domain recognize phosphorylated head groups of phosphatidyl inositol. β spectrin contains a pleckstrin homology (PH) domain, described first by Haslam et al. and by others, located in the COOH-terminal segment, which is deleted in certain alternatively spliced isoforms (111-113). These domains extend out from spectrin rods in the mid-region of spectrin tetramers and are placed within 10 nm of each other. PH domains are 100-120 residue folding units first resolved in pleckstrin, which is a major protein kinase C substrate in platelets, and subsequently been found in many proteins (114). An interesting feature of proteins with PH domains is a role in signaling and proximity to plasma membranes. The first three dimensional structures determined of PH domains were the N terminal PH domain of human pleckstrin and the domain of murine β spectrin, both by NMR. PH domains include a seven-stranded β-sheet arranged as a β-barrel which is capped by a COOH-terminal α-helix. The conserved tryptophan in the C terminus of the domain is the part of the hydrophobic core. The N and C termini of PH domains are very close in space, facilitating the insertion of this domain into new proteins. Solution of PH domain structure from other proteins indicates that the different PH domains share the same fold, but variations in loop lengths and composition connecting the β strands provide substantial variability in potential interaction surfaces (115). Consistent with structural predictions, binding activities of PH domains of spectrin and other proteins are distinct both with respect to interactions with various phosphatidylinositol lipids and to proteins (114). The variable loops play an important role in
ligand binding of PH domains and responsible for the functional variability to this family. The loops can also carry additional secondary structural elements, like the short α-helices in the loop 3-4 of β-spectrin and loop 5-6 of PLCδ1 and Btk PH domains.

Figure 8: Crystal structure of a PH domain from human β-spectrin at 2 Å resolution (111).

The Src homology domains 2 and 3 are among the first signalling modules reported. They were identified as regions outside the catalytic kinase domain of cytoplasmic protein tyrosine kinases by sequence homology with other signalling proteins (112, 116). SH3 domains, initially observed in the Src protein tyrosine kinase, are present in many proteins involved in cell signaling and mediate interactions with proline-rich stretches in a variety of target proteins (117). SH3 domains are inserted within repeat 9 of the α-subunit between helix B and C, human α-spectrins (93). The SH3 domain contains 62 amino acid residues. The structure of the α-spectrin SH3 domain has been resolved at an atomic resolution of 1.1 Å by X-ray crystallography (118) and NMR (119, 120). The three-dimensional structure of spectrin SH3 domains is a compact β-barrel and exhibits the same overall fold as other SH3 domains. The structure of SH3 domain consists of five antiparallel β-strands and a short 310-helix, one sheet, three beta turn, two beta bulges and three beta hairpins (Figure-9). The β-strands form two β-sheets that are almost perpendicular to each other in a sandwich structure. The most unique structure of this domain is the accumulation of conserved aromatic residues on one of its surface which has proved to be the interaction site for the ligands of SH3 domain (121). It binds with the proline rich peptides and has been classified as metal binding protein and it is also present in cytoskeletal proteins providing a possible link between the signal transduction pathways and the morphological changes of the cell (118, 122). The SH3
domain of erythroid α-spectrin interacts with a tyrosine kinase-binding protein, hssh3bp1/e3B1/Abi1(123) and that of nonerythroid spectrin with a low-molecular weight phosphotyrosine phosphatase (124), c-Src (a tyrosine kinase), Na+/H+ exchangers and Na+ channels (ENaC) in epithelial cells.

**Figure 9:** Crystal structure of a SH3 domain α-spectrin at 1.12 Å resolution (118).

The lateral interactions between the first two repeats of β spectrin and most C-terminal repeats of α spectrin form the spectrin filament heterodimers (125, 126). The biologically relevant tetramer is formed by head-to-head dimer interactions. These α–β interactions results in the reconstitution of a complete triple helical repeat as present along the spectrin molecule (127-129). The crystal structure of the tetramerization site (Figure-10) showed that the interaction of the approx. 30 amino acid residue N-terminus of α spectrin form a single helix and approx while 70 residue C-terminal double-helical domain of β spectrin (β17) leads to the formation of a triple-helix bundle (130). Such structure got resemblance with a fully folded spectrin repeat, and is stabilized predominantly by hydrophobic contacts. The lateral association of α and β spectrin subunits is highly conserved and occurs between vertebrate spectrins (131). The high affinity between α and β subunits indicates that spectrin does not exist as independent monomeric chain but is an obligatory heterodimer or tetramer. Recently it has been reported that replacement of an invariant Trp or other residues also affects the interaction between the helices and inhibits the self association, resulting in the reduction of spectrin tetramer concentration as manifested in the erythrocytes of patients suffering from hereditary elliptocytosis (HE) (132) where defects in spectrin tetramer formation have been established (133, 134). Mutations in α and β spectrin defined in these patients would be predicted to disrupt helical pairing predicted from
biochemical studies (135, 136, 92). These mutations include substitution of prolines, which would be expected to disrupt an α-helix, as well as mutations in residues predicted to provide contacts between helices.

Figure 10: Crystal structure of Self associating domain of spectrin at 2.8 Å resolution (130).

The Ankyrin binding region in spectrin, is highly conserved, is located within the 14th and 15th repeats the β chain of spectrin, (137, 103). This domain was found to have the general features of tandem triple-helical repeats both in its crystal structure (Figure-11), and in solution (138-140, 128). The highly conserved motif within the loop has a key role in maintaining the appropriate tilt angle between the two repeats due to interactions with the linker. Again some structural data showed that the recognition of ankyrin by spectrin requires shape complementarity, unlike induced fit model as the distinctive inter-repeat kink is reported to be important for ankyrin spectrin recognition (141). The major docking site for ankyrin is the conservative patch of anionic amino acid residues on helix C of the 14th repeat together with a few residues within the linker and loop flanked by helices B and C of repeat 15. As mentioned above, ankyrin(s)-mediated interactions with the vast majority of spectrin ligands – various transmembrane proteins such as channels, transporters and receptors – play an important role in normal cell/organism function.
Figure 11: Crystal structure of the spectrin/ZU5-ankyrin R complex at 2.75 Å resolution (128).

Spectrin interacting proteins:

Spectrin is a multi-domain protein with a wide distribution in different types of cellular systems as discussed earlier. In fact various types of protein molecules interact with spectrin with varying affinity and perform some important cellular functions. The functional role of spectrin has been understood from the physical and chemical properties and summerized in table 3 suggesting participation of spectrin in various biological processes such as in signal transduction and propagation and regulation of many cellular processes.

Table 3: Biochemical and biophysical properties of erythroid spectrin

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Binding constants and other biophysical properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer-dimer</td>
<td>800 nM</td>
<td>127, 142</td>
</tr>
<tr>
<td>Binding dissociation Constant, $K_d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F actin</td>
<td>$1-2 \times 10^{-6}$ M</td>
<td>109</td>
</tr>
<tr>
<td>$K_d$, binding site stoichiometry</td>
<td>1:10-12 actin subunits in filaments</td>
<td></td>
</tr>
<tr>
<td>Ankyrin</td>
<td>$2.5 \times 10^{-6}$ M (dimer)</td>
<td>128</td>
</tr>
<tr>
<td>$K_d$</td>
<td>$2.7 \times 10^{-6}$ M (tetramer)</td>
<td></td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>Binding location</td>
<td>Kd, Stoichiometry, Binding Site</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>Binding location</td>
<td>Kd, Stoichiometry, Binding Site</td>
</tr>
<tr>
<td>Adducin</td>
<td>2 per spectrin</td>
<td>2×10^-6 M, 2 per spectrin, Actin binding site, β chain</td>
</tr>
<tr>
<td>Protein 4.1</td>
<td>2×10^-6 M</td>
<td>2×10^-6 M, 2 per spectrin, Actin binding site, β chain</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>2×10^-6 M</td>
<td>2×10^-6 M, 2 per tetramer, α chain, between repeats 11 and 12</td>
</tr>
<tr>
<td>Protein 4.2</td>
<td>0.30 µM, 2 per tetramer carboxyl terminal EF-hand domain in the α-spectrin</td>
<td></td>
</tr>
<tr>
<td>Dematin</td>
<td>400 nM, spectrin–actin complex</td>
<td></td>
</tr>
<tr>
<td>Glucose transporter protein</td>
<td>4.6×10^-4 - 4.6×10^-5 M-1 (dimer) 7.3×10^-4 M-1 (tetramer) 2 per tetramer α-chain, between repeats 11 and 12</td>
<td></td>
</tr>
<tr>
<td>Ligands</td>
<td>2×10^6 M, 650 molecules per spectrin Hydrophobic sites of spectrin</td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>6 µM, 1 per spectrin Self association domain of spectrin</td>
<td></td>
</tr>
<tr>
<td>PRODAN</td>
<td>2×10^-7 M, 1 per spectrin</td>
<td></td>
</tr>
</tbody>
</table>

**Stoichiometry**
- Phosphorylation of ankyrin reduces the affinity 1 per dimer B-chain, 15th repeat

**Binding location**
- Carboxyl end for spectrin-actin
- Actin binding site, β chain
- α chain, between repeats 11 and 12
- Self association domain of spectrin
<table>
<thead>
<tr>
<th>Binding site</th>
<th>Stoichiometry</th>
<th>K_d, Binding site</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1 mM</td>
<td>50-100</td>
<td>152</td>
</tr>
<tr>
<td>2,3 DPG</td>
<td>1 mM</td>
<td>50-100</td>
<td>152</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>0.35 µM (erythroid spectrin), 200</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>Tetracaine</td>
<td>0.47 µM</td>
<td>100</td>
<td>154</td>
</tr>
<tr>
<td>Malachite green</td>
<td>0.25 µM</td>
<td>1</td>
<td>155</td>
</tr>
<tr>
<td>Hemin, K_d</td>
<td>0.5 µM</td>
<td>1 per spectrin</td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>1.47 µM</td>
<td>1 per spectrin</td>
<td></td>
</tr>
<tr>
<td>PP-IX</td>
<td>1.95 µM</td>
<td>1 per spectrin</td>
<td></td>
</tr>
<tr>
<td>Lipids</td>
<td>K_d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>33±5 nM</td>
<td>156-162</td>
<td></td>
</tr>
<tr>
<td>PE/PC</td>
<td>577±132 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS/PC</td>
<td>146±15 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>99±13nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC/PS (7:3) (high salt)</td>
<td>140±12 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC (high salt)</td>
<td>170±1 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC/PS (7:3) (low salt)</td>
<td>1120±480 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol/SM/PC</td>
<td>54.3±14 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE/PC (10% Chol)</td>
<td>150±10 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE/PC (20% Chol)</td>
<td>152±36 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE/PC (50% Chol)</td>
<td>231±78 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ankyrin binding domain (PE/PC)</td>
<td>50±12 nM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Hemoglobin**

Hemoproteins are the group of metalloproteins containing heme prosthetic group that is bound to the protein either covalently or non-covalently. Hb, catalase, cytochrome b5 etc belong to that class of protein. Again heme is the prosthetic group for many different heme containing enzymes like cytochrome p450, catalase, peroxidases and several proteins involved in electron transfer. The heme contains a reduced iron atom, in the centre of a highly hydrophobic, planar, porphyrin ring. The planar protoporphyrin IX ligand serves as a tetradentate one. The 5-th coordination position of Fe (II) is occupied by the N atom of the HisF8, and lastly the octahedral coordination about Fe (II) is completed by water or carbon-dioxide or oxygen or other suitable ligands. In Hb, for example, the protein provides a single axial histidine ligand and the vacant sixth coordination site is available for oxygen binding. In case of electron carrier hemoproteins heme contains two strong axial ligands and generally does not bind to molecular oxygen, for example, cytochrome c are six-coordinate with histidine and methionine ligation.

All these proteins having closely similar iron porphyrin moieties encapsulated with different polypeptides and the differences in the way the proteins interact with the heme group are manifested in the diversity of their different functions. Because of having diverse range of their biological functions the structure and functions of these proteins are well characterized. Oxygen, nitric oxide, carbondioxide and hydrogen sulphide bind to the iron atom of hemoproteins. On binding to the heme group they can modulate the function of those hemoproteins. Three main biological function of hemoproteins are; 1) electron transport (cytochorome b5), 2) transport of oxygen (Hb, myoglobin), 3) catalysis of redox reactions (horseradish peroxidise, cytochrome P450). Since hemoproteins enable electron transfer they can also act as redox regulators, like catalase, peroxiredoxin 2 etc, which destroy the reactive oxygen species formed and to reduce met-Hb to its original Fe(II) form.

Vertebrate Hbs (Hb: C\textsubscript{34}H\textsubscript{32}N\textsubscript{4}O\textsubscript{4}Fe: mol.wt. ~64,450 kDa) are tetramers- consist of four polypeptide subunits: two α like globin subunits (i.e. α /ζ) and two β like globin subunits (i.e. β /γ /δ /ε) though under some conditions homotetramer also exists (Figure 12). Each polypeptide chain contains a heme group as the prosthetic group- in which a hexa- coordinated Fe(II) lies at the center of the heterocyclic Fe (II) protoporphyrin IX complex. The planar protoporphyrin IX ligand serves as a tetradentate one. The 5-th coordination position of Fe(II) is occupied by the N atom of the HisF8, and lastly the vacant sixth
coordination site is available for water or carbon-dioxide or oxygen or other suitable ligands. The polypeptide chains around the heme group protect the Fe(II) from being oxidized to Fe(III) and provide a pocket into which the oxygen can bind reversibly. Each polypeptide subunit is stabilized by non-covalent interactions (163) and the αβ contacts are stabilized by electrostatic interactions and hydrogen bonding (163).

![Figure 12: Structure of Hemoglobin tetramer](taken from www.slideshare.net)

In erythrocyte cytosol Hb (5mM) constitutes approximately more than 98% of total cytosolic proteins. During passing through capillaries in the alveoli of lungs, Hb reversibly binds oxygen with a conformational change where the heme moiety moves slightly out-of-plane to accommodate the 6th ligand. Now Fe (II) in contact with free O₂, forms a redox system with Fe (II) being oxidized to Fe (III). In tissues since the CO₂ concentration is high O₂ molecule is replaced by CO₂ which is triggered by alkaline pH of the CO₃²⁻ions. It is reported that the reaction may take a complex free radical pathway via Fenton’s reaction resulting into generation of reactive oxygen species (ROS) i.e. peroxides, superoxides and free radicals etc. (164). Hb auto-oxidation takes place in the erythrocytes and about 3% of total Hb undergoes oxidation to form met-Hb everyday where the Fe (II) of the heme moiety gets irreversibly oxidized to Fe (III). The continuous conformational change in Hb usually accounts for the ligation of a water molecule or a small anion with the heme cavity, which in turn mediates an electron transfer from Fe (II) to O₂ to form superoxides triggering the redox reactions that follow. In addition to oxidizing Hb, ROS species exert degradation of the
membrane lipids and also the cytoskeletal proteins resulting in premature destruction of erythrocytes. Although the genetic machinery is absent in matured erythrocytes, it has thus become extremely important for the cell to pre-load itself with defense machinery to overcome these problems throughout its lifetime which includes a combination of redox regulators present in sufficient amounts.

Table 4: Various types of human Hbs (165):  

<table>
<thead>
<tr>
<th>Types of Hbs</th>
<th>Composition</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early embryonic Hbs:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb Portland</td>
<td>ζ2γ2</td>
<td>--</td>
</tr>
<tr>
<td>Hb Gower-I</td>
<td>ζ2ε2</td>
<td>--</td>
</tr>
<tr>
<td>Hb Gower-II</td>
<td>α2ε2</td>
<td>--</td>
</tr>
<tr>
<td>Fetal Hbs:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbF</td>
<td>α2γ2</td>
<td>85%</td>
</tr>
<tr>
<td>HbA</td>
<td>α2β2</td>
<td>5-10%</td>
</tr>
<tr>
<td>Adult Hbs:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA</td>
<td>α2β2</td>
<td>97%</td>
</tr>
<tr>
<td>HbA2</td>
<td>α2δ2</td>
<td>2%</td>
</tr>
<tr>
<td>HbF</td>
<td>α2γ2</td>
<td>1%</td>
</tr>
</tbody>
</table>

Under normal condition, in human, at various stages of development, various types of Hb appear to satisfy the specific necessity of oxygen delivery system in the organism in a highly controlled order. All human Hbs possess similar tetrameric structure- consisting of two α like chains and two β like chains.

The ζ is the first α like globin chains to be expressed which is then converted to αα, while the β like pathway has two switches, firstly ε to γ and later γ to β- following the course of normal development (Table 4). HbF is a mixture of two molecules- the Gγ chain (residue
136 is Gly) and the Aγ chain (residue 136 is Ala), which are coded for by different genes. Under some special physiological condition, HbF imparts advantageous phenotype even in adults i.e. as in HPFH (hereditary persistence of fetal Hb) and in δβ-thalassemia (165).

**Structure of hemoglobin:**

Hb A has two identical α chains and two identical β chains. Each α chain has 141 amino acid residues (mol.wt. 15.126 kDa), while each of β chain having 146 amino acid residues (mol.wt. 15.867 kDa). Each α chain is made of the following helical segments: A, B, C, E, F, F', G, and H and each β chain consists of all above mentioned helical segments along with an additional D helix. The packing of chains in the Hb molecule is such that close interlocking contact of side chain exists between unlike subunits, but there is little contact between α and α or β and β. Again the αβ contacts are of two types: the α₁β₁ (or α₂β₂) and α₂β₁ (or α₁β₂). The α₁β₁ contacts involve B, G and H helices and the GH corner which are called ‘packing contacts’ - as they represent subunit packing that remains unaltered when the transition from its deoxy- to oxy- configuration of Hb molecule occurs. Whereas, the α₁β₂ contacts involving mainly helices C and G and FG corner are termed as ‘sliding contacts’, because they undergo the major changes when the ligation state of the heme is varied. Now the fixed ‘packing contacts’ are more extensive than the variable ‘sliding contacts’ which means that 126 atoms from 32 residues make up the packing contacts in deoxyhemoglobin while 107 atoms from 27 residues form the sliding contacts (166).

Approximately, one-fifth of the total surface area of the isolated subunits is buried in the process of making the Hb tetramer (166) while 60% is involved in packing contacts and 35% in sliding contacts. The rest 5% represents the small amount of contact between like subunits. These contacts are not only hydrophobic, hydrogen bonds and salt-bridges are also important in holding the subunits together approximately one-third of each of the contacts involves polar side-chains (166). Here some positions (166) having the same residues in several species studied are listed (Table 5).
Table 5: Invariant amino acid residues in Hb observed throughout almost all vertebrates (166):

<table>
<thead>
<tr>
<th>Position</th>
<th>Amino acid residue</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>His</td>
<td>proximal heme-linked histidine.</td>
</tr>
<tr>
<td>E7</td>
<td>His</td>
<td>distal histidine near the heme.</td>
</tr>
<tr>
<td>CD1</td>
<td>Phe</td>
<td>heme contact.</td>
</tr>
<tr>
<td>F4</td>
<td>Leu</td>
<td>heme contact.</td>
</tr>
<tr>
<td>B6</td>
<td>Gly</td>
<td>allows the close approach of B and C helices.</td>
</tr>
<tr>
<td>C2</td>
<td>Pro</td>
<td>helix termination.</td>
</tr>
<tr>
<td>HC2</td>
<td>Tyr</td>
<td>cross links the H and F helices</td>
</tr>
<tr>
<td>C4</td>
<td>Thr</td>
<td>uncertain</td>
</tr>
<tr>
<td>H10</td>
<td>Lys</td>
<td>uncertain</td>
</tr>
</tbody>
</table>

These invariant residues of the Hb molecule have functional significance. Several of them directly affect the oxygen-binding site, like Tyr HC2, which stabilizes the molecule by forming a hydrogen bond between the H and F helices. His F8 and His E7 are the residues that act as direct and indirect ligands to the heme iron respectively. Positions B6 and E8 can only be Gly because they are the contact points where helices B and E cross, leaving no room for side chains between them. Pro residue at C2 position makes the sharp bend between B and C helices. Another Pro group performs the same role at the beginning of the G helix, but can be found at either position G1 or G2 (166).

**Hemoglobin interacting protein(s):**

Erythrocyte contains a highly concentrated solution of Hb whose expression and normal function(s) are highly regulated by interaction with other protein(s). Hb interacts with several cytoskeletal proteins like protein 4.1, band 3, spectrin, actin, ankyrin. Band3 is the most abundant protein of the human erythrocyte membrane ($10^6$ copies/cell) and it helps to mediate the electroneutral exchange of chloride and bicarbonate across the lipid bilayer (167,
The cytoplasmic domain of band3 has the high affinity for Hb (169-171). According to a report the NH2-terminal peptide of band 3 is the binding site for deoxyhemoglobin. The binding site spans a distance of 18 Å, includes amino acid residues like Arg 1040 and Arg 1046 as well as the basic residues within the DPG binding site of Hb. Cross-linking the two β82 lysine residues at the entrance to the central cavity blocks the binding of the peptide which interferes with the binding of the 43kDa band 3 fragment indicating that the acidic amino-terminal residues in the intact cytoplasmic domain also bind at this site. Moreover oxygen binding studies has shown that the isolated peptide as well as the entire cytoplasmic domain of band3 binds with a much lower affinity to oxyhemoglobin as compared to deoxyhemoglobin. The dissociation constant for the deoxyhemoglobin-band3 complex was found to be 5×10⁻⁴ M (172).

The studies on binding and interactions of Hb to spectrin were first reported by Chaimanee and Yuthavong in 1977 (173). Hb has been found to affect the self-association of spectrin dimer to form the tetramer under in vitro conditions which may arise from steric or allosteric globin binding at or near the head region and hence a defective self-association has been found in both α thalassemia and β thalassemia(174-176). Several reports indicate the association of spectrin with Hb under different experimental and physiological conditions, e.g. in case of senescent erythrocytes and under oxidative stress (177-179). Again there are reports which also indicate an irreducible complexation of the globin chains of Hb with spectrin by an oxidative mechanism causing further damages to the erythrocyte by the hydroxyl radical generated from the hydrogen peroxide by the heme iron in the complex globin during aging (180-182). Progressive accumulation of spectrin-globin complex results in progressive echinocyte formation, increase in membrane rigidity as well as increasing adherence and phagocytosis of altered erythrocytes by monocytes (183-184). However, in a cellular context this would probably require that the location of the complex to be at or near the dimer-dimer junction because of the limited effective range of the free radical.

AHSP, acting as a chaperone protein, is a small protein of 102 amino acids and synthesized in red blood cell precursors. It binds specifically to α Hb subunits preventing the formation of α Hb-cytotoxic precipitates (185, 186). The AHSP and α- Hb are both monomers in solution and form a heterodimer complex with an association constant of 10⁷ M⁻¹ at 20°C but does not bind with β- Hb or HbA, compatible with a physiological role (187). AHSP in its native state is conformationally heterogeneous in solution, an elongated
antiparallel three \( \alpha \)-helix bundle complex. The \( \alpha \)-helix is particularly the C-terminal residue. The third \( \alpha \)-helix is arranged with a very rare right-handed twist. It also interacts with another chaperone protein HSP 70. Carbonic anhydrase 1 was shown to interact with Hb. Recent study suggests Hb also interacts with numerous oxidoreductases like catalase, SOD-1, Peroxiredoxin 2 (Prdx2), flavin reductase, NDPK (alternatively known as NM23), PNP, SELENBP1, ALDH-1 (188).

**Motivation and Objective of the Present Work:**

**Spectrin binding of heme derivatives**

Free heme exerts toxic effects like lipid peroxidation, DNA damage and protein aggregation. In case of severe hemolysis, occurring during pathological states like sickle cell disease, ischemia reperfusion and malaria, levels of free heme increase inside erythrocyte. In this study our aim was to investigate whether spectrin, being the major erythroid cytoskeleton protein has any role as acceptor of free heme. We have compared the interactions of three heme derivatives, hemin chloride, hematoporphyrin and protoporphyrin-IX, with dimeric and tetrameric spectrin, elaborated in Chapter 3. We also attempted to understand the molecular basis of this kind of spectrin-porphyrin interaction. We’ve also noticed heme-induced structural changes in the membrane skeletal protein. The present study takes us a step ahead towards the understanding the potential of erythroid spectrin to act as the heme acceptor with functional implications in cell signaling and other membrane mediated processes. Erythroid spectrin could thus act as a potential accepter of heme, particularly relevant under disease conditions.

**Membrane interaction of heme derivatives**

Free heme plays notorious role in lipid peroxidation, destabilization of cytoskeleton by impairing lipid bilayer which leads to hemolytic anemia. Although heme oxygenase is primarily responsible for heme detoxification, it gets overwhelmed in presence of high concentration of free heme. Under this condition free heme partitions into lipid bilayer and starts to exert its damaging effect. In this study the effect of lipid composition and acyl chain length on heme partitioning to small unilamellar vesicles were examined to explore the partitioning behavior of these ligands. Again the extent of membrane damage exerted by hemin in terms of leakage and fusion was not explored till date. Though the degrading effect of heme on lipid bilayer is known, the effect of aminophospholipids of different headgroup
(PS, PE) and cholesterol on partitioning of heme into membrane was not still properly understood. In this part we have estimated the partitioning of hemin along with its two analogues, hematoporphyrin and protoporphyrin into different membrane models, as a function of acyl chain length, concentration of phospholipids, different head groups and incorporation of cholesterol, elaborated in Chapter 4. Here we also tried to explore if any detoxification role of spectrin to prevent membrane leakage caused by these heme derivatives as our earlier research showed that spectrin could act as potential heme accepter. Again we compared the extent of membrane distortion exerted by heme and its two derivatives using different liposomes. Hemin and hematoporphyrin had much higher leakage efficiency than protoporphyrin IX. We also checked the involvement of heme derivatives in membrane fusion and lipid mixing assay ruled out their role as fusogen.

**Spectrin and Hb binding of the anti-leukemic drug, Imatinib mesylate**

Anti leukaemia drug, imatinib mesylate has been shown to bind to the membrane skeletal protein, spectrin and to the most abundant erythroid protein, HbA in its oxy-form, elaborated in Chapter 5. Such bindings are detected by monitoring the imatinib induced quenching of the tryptophan fluorescence of the proteins with increasing concentrations. The thermodynamic parameters associated with such binding, molecular docking studies, circular dichroism studies were performed. This is the first study showing favourable interactions of this anticancer agent with the two major erythroid proteins which could throw light in understanding the mechanism of action and toxicity of this drug those are to be optimized for cancer treatment.

**Regulation of peroxidise activity of hemoproteins**

Chaperones and redox regulatory proteins are found to be up-regulated in Hemoglobinopathies. It was also reported earlier that Hb interacts with spectrin and other heme proteins involved in redox regulation inside erythrocytes. In addition, erythroid spectrin was earlier shown to exhibit chaperone like property. Taken together, we’ve studied the functional significances of such spectrin interactions of heme proteins in the erythrocyte cytosol by measuring the peroxidise activity of few selected heme proteins e.g. HbA, α- and β-globin chains, catalase and cytochrome C with horseradish peroxidise, taken as the positive control, in presence and absence of two important phosphate metabolites like ATP and 2,3 diphosphoglycerate, elaborated in Chapter 6.
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