Chapter 11
Embryonic and Fetal Human Hemoglobins: Structures, Oxygen Binding, and Physiological Roles

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Abstract During the past two decades, significant advances have been made in our understanding of the human fetal and embryonic hemoglobins made possible by the availability of pure, highly characterized materials and novel methods, e.g., nano gel filtration, to study their properties and to correct some misconceptions. For example, whereas the structures of the human adult, fetal, and embryonic hemoglobins are very similar, it has generally been assumed that functional differences between them are due to primary sequence effects. However, more recent studies indicate that the *strengths* of the interactions between their subunits are very different leading to changes in their oxygen binding properties compared to adult hemoglobin. Fetal hemoglobin in the oxy conformation is a much stronger tetramer than adult hemoglobin and dissociates to dimers 70-times less than adult hemoglobin. This property may form the basis for its protective effect against malaria. A major source of the increased strength of fetal hemoglobin resides within the A-helix of its gamma subunit as demonstrated in studies with the hybrid hemoglobin Felix and related hybrids. Re-activating fetal hemoglobin synthesis in vivo is currently a major focus of clinical efforts designed to treat sickle cell anemia since it inhibits the aggregation of sickle hemoglobin. The mechanisms for both the increased oxygen affinity of fetal hemoglobin and its decreased response to DPG have been clarified. Acetylated fetal hemoglobin, which makes up 10–20% of total fetal hemoglobin, has a significantly weakened tetramer structure suggesting a similar role for other kinds of protein

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acetylation. Embryonic hemoglobins have the weakest tetramer and dimer structures. In general, the progressively increasing strength of the subunit interfaces of the hemoglobin family during development from the embryonic to the fetal and ultimately to the adult types correlates with their temporal appearance and disappearance in vivo, i.e., ontogeny.

Keywords Hemoglobin · Sickle cell · Malaria · Acetylation · Oxygen affinity · Nano gel filtration · Ontogeny

Introduction

Adult hemoglobin has provided us with an appreciation of how a protein accomplishes its physiological goals of oxygen transport from lungs to tissues with such high efficiency (Perutz 1989). Additional insights into protein versatility can be realized from comparisons of the adult type with the fetal and embryonic hemoglobins to ascertain how changes in amino acid sequence at strategic locations can enable a particular hemoglobin type to maximize binding of oxygen when its supply is limiting. The family of human hemoglobin tetramers is an excellent system in which to study this variable extent of oxygen binding; they all possess a very similar structural architecture as a platform on which to confer type-specific properties (Fig. 11.1a). The human hemoglobins number eight in all; many, but not all, are present at various stages of development. That they bind different amounts of oxygen (Fig. 11.2) has been known for many years but the mechanism whereby this is achieved is conjectural. This chapter describes subtle differences at the subunit interfaces of normal adult, fetal, and embryonic human hemoglobins that impart important physiological properties optimized for one particular type of hemoglobin. Independent of its increased oxygen binding properties, fetal hemoglobin provides a therapeutic effect in sickle cell anemia and possibly in malaria, as also described in this chapter. The early literature describing the embryonic hemoglobins is relatively sparse with some conclusions questionable.

In order to obtain reliable data on the fetal and embryonic hemoglobins, sufficient quantitites of native and well characterized materials as well as sensitive, accurate methods to measure their properties must be available. Fetal hemoglobin purified from umbilical cord blood (Bookchin and Nagel 1971; Bookchin et al. 1975) and characterized by mass spectrometry has made it possible to study this hemoglobin in great detail. Embryonic hemoglobins are the least understood type due to difficulty in procuring them, a problem that has now been resolved since they are available in their native states from transgenic mice (He and Russell 2001) making it possible to study and compare them with the well known adult hemoglobin.

The methodology used in these studies is based on gel filtration chromatography but using very low hemoglobin concentrations to study differences in the basic foundations of the subunit assemblies of the three hemoglobin types that are responsible

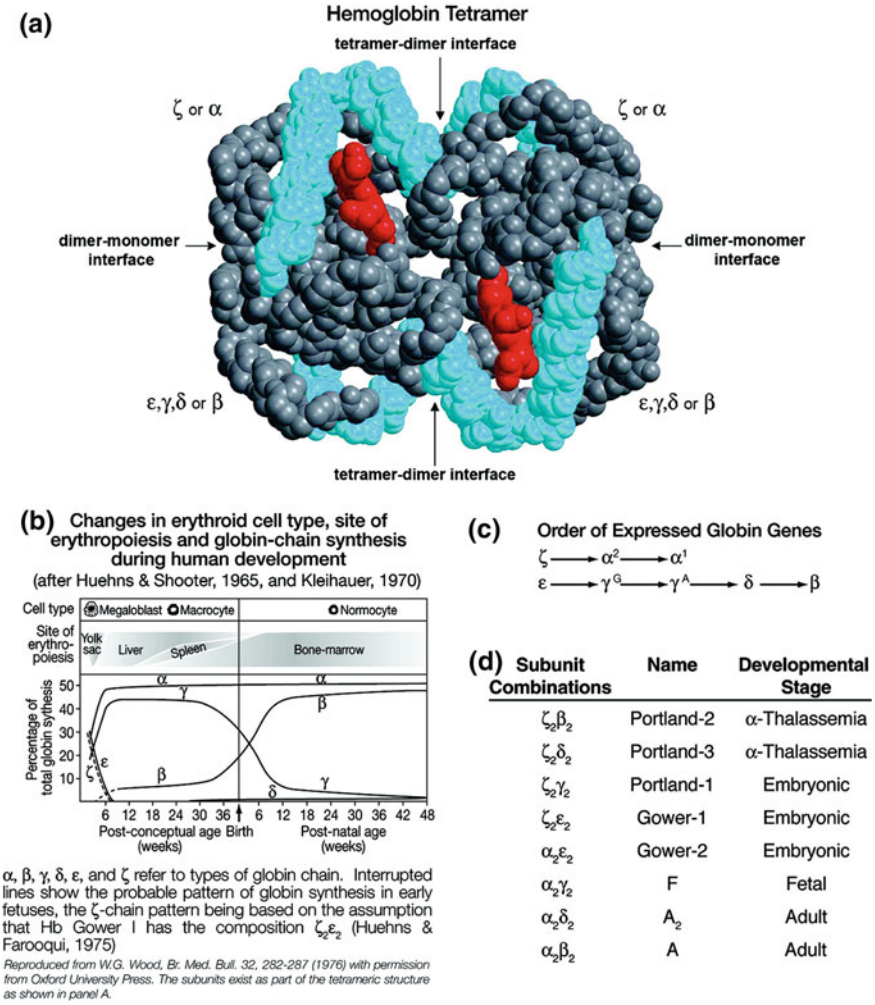

Fig. 11.1 Part A-Hemoglobin tetramer with the different types of globin subunits indicated by Greek letters and the locations of the two types of subunit interfaces designated with arrows. Part B-Changes in the expression of subunits as a function of time. Part C-Order of globin subunit expression. Part D-Names of the eight different types of human hemoglobins formed by combinations of various subunits as a function of time. Reproduced from Manning et al. (2009) with permission

for their specific oxygen bonding characteristics. Avoiding the high hemoglobin concentrations usually employed, which greatly exceed their equilibrium dissociation constants, permits one to observe all stable assembly intermediates since it shifts the equilibrium away from the predominant tetramer species and towards its constituent dimer and monomer components, as described below. We refer to this experimental approach as nano gel filtration, whose basis is reminiscent of the principle used a

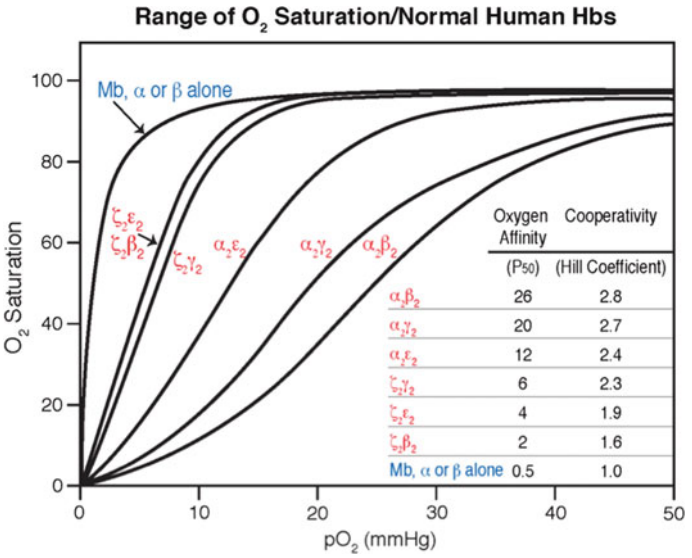

Fig. 11.2 Oxygen binding curves of human embryonic, fetal, and adult hemoglobin tetramers (red labels). The oxygen binding curves of the individual alpha and beta subunits have blue labels. Reproduced from Manning et al. (2017) with permission

century ago to observe the fundamental property of the bending of light whose magnitude was accurately measured during a solar eclipse when the intensity of the sun's intense glare, which would ordinarily obscure such observations, was minimized (Dyson et al. 1920); this experiment was an important proof of Einstein's theory of general relativity.

Hemoglobin Ontogeny

The individual globin subunits are expressed at precise times (Fig. 11.1b) and in a particular order (Fig. 11.1c) in a process known as ontogeny. The depiction in Fig. 11.1c represents the basic elements of the system that expresses the human globin subunits but it is an oversimplification since the human alpha cluster and beta cluster globin genes, which are present on chromosomes 16 and 11, respectively, are embedded in nucleosome arrays that are subject to opening by promotors or closing by repressors; these bind to specific gene regions distant from the expressed loci (sometimes referred to as the Locus Control Region, LCR) (Stamatoyannopoulos and Grosveld 2001). There are three examples of a reciprocal pattern of gene expression in Fig. 11.1b (epsilon – > gamma, gamma –> beta, and zeta –> alpha) where expression of one gene is decreasing and, concurrently, expression of another gene is increasing. Such behavior is referred to as gene switching (Ikuta et al. 1996, Baron 1996) and is

a very active area of research especially as it relates to fetal hemoglobin expression in sickle cell anemia; this will be discussed later in this chapter.

Other aspects in the transition from Fig. 11.1b, c involve globin gene clusters, consisting of expressed (exons) and unexpressed (introns) regions that are subject to a process known as splicing to produce the functional intact genes shown in Fig. 11.1c. Neither this topic nor post-translational modifications (referred to as epigenetics), nor RNA polymerase with associated transcription factors for messenger RNA synthesis will be covered in this chapter. Instead, we will evaluate the final expressed products of the system shown in Fig, 1D and, specifically, how they differ from one another regarding oxygen binding and subunit interfaces.

Subunit Assembly

The expressed globin subunits bind heme and then pair together to form dimers held together by a dimer-monomer interface (Fig. 11.1a) which, in the case of adult Hb A, has an equilibrium strongly in favor of dimer formation (Perutz 1989). Pairs of the same dimers combine to form tetramers having a flexible tetramer-dimer interface that can rearrange into two conformations in equilibrium, oxy (R) and deoxy (T), which either bind or release oxygen, respectively (Perutz 1989). This chapter will cover the assembly process for the entire human hemoglobin family and describe the physiological consequences of the differences between them.

Even though there are eight possible combinations of hemoglobin subunits, only six have been found during normal development (Fig. 11.1d). The remaining two, Hb Portland-2 and Hb Portland-3, are only present in patients with alpha-thalassemia where the alpha gene has suffered deletions so the zeta gene continues to be expressed. Why these two hemoglobins are not normally expressed is unclear; a suggestion is offered here.

Some individual hemoglobin subunits are unstable and prone to aggregation, unfolding, or oxidation at the heme moiety leading to experimental difficulties in studying their assembly to tetramers directly. For example, individual beta and gamma subunits self-assemble (Adachi et al. 2000). Through the careful studies of many investigators (Antonini and Brunori 1971; Edelstein et al. 1970), such problems for individual subunits for adult Hb A have largely been solved but subunits comprising the other hemoglobins could still pose problems. Once formed, however, tetramers are generally quite stable. Hence, our experiments begin with intact tetramers and we study the dissociation process by dilutions in order to shift the tetramer <--> dimer <--> monomer equilibrium to the right. Such an experimental approach, however, requires a highly sensitive detection system in which the quality of the data and the ensuing conclusions depend on the resolution and reproducibility of the methods employed.

In this chapter a simple high resolution gel filtration method coupled with software for elution curve analysis (Manning et al. 1996) has been invaluable in yielding new insights into the fetal and embryonic types of human hemoglobin and how they differ

from adult Hb A. In spite of earlier claims based on simulations that such gel filtration methods could not yield accurate results (Zimmerman and Ackers 1971), our data (Manning et al. 1996) for adult Hb A agreed well with previous results obtained by other methods. It has been generally assumed that all human hemoglobins have not only the same overall structure shown in Fig. 11.1a but also similar strengths at both types of interfaces, but in this chapter we demonstrate otherwise using this methodology.

Oxygen Binding Comparisons

The most important physiological properties of the three types of human hemoglobin- embryonic, fetal, and adult- are their oxygen binding capacities (Fig. 11.2), which differ considerably and have evolved so that each can optimally bind the oxygen to which it is exposed at a given time during development (Wood 1976; Bunn and Forget 1986). For example, adult Hb A (alpha2beta2,) (curve farthest to the right) has a midpoint value (50% oxygen saturation defined as P_{50}) of 26 mm Hg (Fig. 11.2 inset), which is close to the average oxygen tension of venous blood. Fetal and embryonic hemoglobins bind oxygen more avidly than adult Hb. In order to achieve maximum binding of available oxygen, their oxygen binding curves are shifted to the left (Fig. 11.2).

This is advantageous since the amount of oxygen in the environments to which they are exposed are lower than that used by air-breathing adults. In order for a hemoglobin to be functional, the middle part of the oxygen binding curve where the oxygen binding (y-axis) is most sensitive to changes in oxygen tension (x-axis), i.e. having the highest degree of cooperativity, should be near the available oxygen tension. Thus, if an embryonic hemoglobin was present in an adult, it would not function as a useful oxygen carrier because at the high ambient oxygen tension that adults use, an embryonic hemoglobin would be fully saturated with oxygen and unable to release it. Also, preterm neonates with greater than normal amounts of fetal hemoglobin (Fig. 11.1b) and lower 2,3-DPG may also have difficulty releasing oxygen.

The mechanisms by which these variable oxygen saturations are achieved are unclear and inspection of the structure in Fig. 11.1a offers no insight. Speculation has focused on primary sequence differences of fetal and embryonic hemoglobins compared to adult hemoglobin and how those changes could influence the heme environment where oxygen is bound. However, definitive experimental evidence is lacking for this perspective. In this chapter we describe the likely sources of their increased oxygen affinities as arising from how their subunits interact.

Fetal Hemoglobin–Oxygen Binding Without DPG

The moderately increased oxygen affinity of Hb F (Fig. 11.2) (Poyart et al. 1978; Chen et al. 2000) enables the transfer of oxygen from maternal hemoglobin to the fetus. How this important left shift in the oxygen binding curve of Hb F is imparted has long been the subject of speculation, but its mechanism was only clarified when recombinant DNA technology became available. To understand this mechanism, we start with the equilibrium between the R (oxy) and T (deoxy) tetrameric states, whose ratio is defined as the allosteric constant, L. When Hb either binds or releases oxygen, the tetramer-dimer interface (Fig. 11.1a) rearranges its subunit contacts and the position of the R/T equilibrium changes (Perutz 1989). The single amino acid difference between fetal and adult hemoglobins at this interface is the substitution of an Asp in the gamma subunit of Hb F for a Glu in the beta subunit of Hb A at position 43. A recombinant Hb A with this single substitution tightens this interface in the R-state (Chen et al. 2000) thereby shifting the R/T equilibrium so that there is less of the T-state and more of the R-state and the affinity for oxygen increases (Poyart et al. 1978). The other four sequence differences at the subunit interfaces are at the dimer-monomer interface. A recombinant hemoglobin with these four additional substitutions in addition to the Glu–> Asp substitution did not have a further increased oxygen affinity (Chen et al. 2000) indicating that the single Asp for Glu substitution at the tetramer-dimer interface was sufficient to account for the increased oxygen binding of Hb F.

Different Responses of Fetal and Adult Hemoglobins to DPG

Since it is the oxygen binding properties of adult and fetal hemoglobins within the red cell that is physiologically important, the effect of the allosteric regulator 2,3-diphosphoglyceric acid (2,3-DPG), which is normally present in red cells at equimolar concentrations with hemoglobin (Benesch and Benesch 1967), requires consideration. It binds with perfect geometry in a 1:1 ratio with the deoxy form of Hb A in a cleft between the two beta chains (Arnone 1972) and moves the oxygen binding curve further to the right. DPG has a diminished effect on fetal Hb F compared with that of adult Hb A, which further increases the difference between the two regarding oxygen binding. Since the DPG binding pocket in fetal Hb contains a Ser instead of a His at position 143, it was conjectured (Bunn and Forget 1986) that this substitution was responsible for the reduced DPG effect on Hb F. When this hypothesis was tested directly by two independent labs using recombinant mutants (Adachi et al. 1997; Fang et al. 1999), the role of the Ser—> His substitution at position at 143 was excluded as contributing to the reduced effect of DPG on Hb F. Instead, the E43D substitution at the tetramer-dimer interface, mentioned above as the main contributor to the increased oxygen binding of Hb F (Chen et al. 2000), is responsible for the lowered DPG response.

Effect of Fetal Hemoglobin in Sickle Cell Anemia

The structure of sickle hemoglobin (Hb S) has been solved (Padlan and Love 1985; Harrington et al. 1997); tetramers in the deoxy state initially adhere to one another through hydrophobic bonding involving the V6 beta mutation sites as donors and F85-L88 beta acceptor sites on adjacent tetramers. The aggregate is subsequently stabilized by a number of other inter-tetrameric interactions both laterally and longitudinally. In the oxy state, this aggregate does not form due to unfavorable contacts. As a result, oxygen binding to hemoglobin S aggregates in sickle cells compared to normal cells is decreased, i.e., the oxygen binding curve shifts to the right compared to that for normal adult Hb A (Fig. 11.2) The presence of such aggregates in red cells causes them to assume a crescent sickle shape in the venous circulation thereby occluding capillaries resulting in oxygen deprivation. One approach, which has been reviewed recently (Eaton and Bunn 2017), to treating this disease is to prevent this aggregation (polymerization) directly. Another approach is based on the observation that fetal hemoglobin impedes this polymerization process because tetramers of Hb F with gamma instead of beta subunit have unfavorable contacts for aggregation (Bookchin and Nagel 1971; Poillon et al. 1993). Hence, increasing fetal Hb F production in vivo is currently the focus of several major efforts to treat sickle cell anemia. The model on which this approach is based is an example of an "experiment of nature" described next.

Hereditary Persistence of Fetal Hemoglobin (HPFH)

As depicted in Fig. 11.1b, fetal Hb expression persists until several months after birth when gamma subunit production nearly ceases except for a small amount (~1%) which continues to form even in normal individuals (Bunn and Forget 1986; Akinsheye et al. 2011). It is not known how this residual expression continues rather than being completely suppressed. A small percentage of patients with sickle cell anemia have elevated levels of Hb F (up to 30%) and these individuals have clinically fewer episodes of sickle cell crises. Even though the mechanism is not understood, this observation forms the basis of research efforts into treating sickle cell anemia by re-starting gamma subunit synthesis, as described next.

Manipulating Fetal Hemoglobin Gene in Sickle Cell Anemia

The first generation of inducers of fetal Hb synthesis built upon the clinical observations that those sickle cell patients who also had increased amounts of Hb F (HPFH) experienced decreased severity of the disease, as described above. Since it had been reported that gamma subunit production was associated with decreased levels of

DNA methylation (van der Ploeg and Flavell 1980; Groudine et al. 1981), the strategy of using DNA hypomethylating agents to induce Hb F synthesis was devised; studies with 5-azacytidine (DeSimone et al. 1982; Lavelle et al. 2008) ensued and this goal was realized but toxicity issues led to termination of its usage. Subsequent studies focused on this approach by testing analogs of azacytidine and, subsequently, similar agents such as hydroxyurea; this pharmaceutical agent, Hydrea, is currently in use as a treatment for sickle cell anemia and can lead to a significant elevation in Hb F but is associated with a higher frequency of stroke. Based on another aspect of gene expression, i.e., the acetylation of histones within active genes, butyrates and related compounds, which inhibit histone deacetylation, were also tested. These studies have been reviewed recently (Musallam et al. 2013).

Another effort to express higher levels of Hb F and thereby lower severity in sickle cell patients involves the gamma gene repressor named BCL11A (Sankaran et al. 2008, 2010) whose interaction with a specific DNA region has been identified. This strategy is to prevent binding of BCL11A thus increasing gamma subunit expression. Patients with HPFH have mutations in one of the two regions that bind this repressor thus leading to incomplete termination of HbF expression (Brendel et al. 2016; Liu et al. 2018). This is a new and currently active avenue of study.

Other Genetic Approaches in Sickle Cell Anemia Treatment

Rapid progress in genetic techniques recently has opened new approaches to correct the effects of the sickle gene mutation. Among these are the use of corrective genes whose expressed globins do not participate in Hb S aggregation (normal beta, the T87Q gene (described below) or the gamma gene), gene corrections e.g. by CRISPR-Cas9 or direct gamma gene induction. Recent reviews on these approaches are in (Demirci et al. 2018; Lettre and Bauer 2016; Telen et al. 2019).

One of the main contributors to the inhibition of Hb S aggregation by Hb F is the T87Q gamma substitution of Hb F (Nagel et al. 1979; Adachi et al. 1996). This is part of the Val-6 binding site on adjacent tetramers, which initiates Hb S aggregation. A recent genetic approach directed at treating sickle cell anemia has been the use of lentiglobin (Leonard and Tisdale 2018), where this substitution is fused with the remainder of the normal Hb A beta subunit, so both donor and acceptor sites that have been altered. This approach has shown encouraging initial results (Ribeil et al. 2017).

Another natural hemoglobin that inhibits Hb S aggregation just as effectively as Hb F is Hb A2, a minor hemoglobin occurring normally (Nagel et al. 1979; Adachi et al. 1996). Hb A2 also contains the Glu-6 site as well as the T87Q substitution so both donor and acceptor sites present in Hb S are replaced. Thus, genetic manipulations to increase the expression of Hb A2 as a treatment for sickle cell anemia are also worthy of consideration.

Fetal Hemoglobin, Malaria, and Thalassemia

A general understanding of thalassemia is helpful prior to a discussion of fetal hemoglobin and its relationship to malaria. Deletions at the alpha loci result in the disease alpha thalassemia (Higgs et al. 2005) and deletions at the beta loci lead to beta thalassemia. Of particular interest is the experiment of nature, e.g., in beta thalassemia, the *preceding* gene continues to be expressed (Fig. 11.1c), so fetal hemoglobin is produced in moderately high amounts. In alpha thalassemia, the zeta gene is expressed so the embryonic hemoglobin, Portland-2 (zeta2beta2) (as well as beta4 tetramers (HbH) and gamma4 tetramers (Hb Barts), are produced (Randhawa et al. 1984). Why hemoglobin Portland-2 is not found in normal embryos has not been explained but below we offer a suggestion.

The two alpha genes normally expressed in humans are identical but the two gamma genes encode for two different gamma subunits with either Gly or Ala at position 136. The Gly/Ala ratio changes from 75/25 before birth to 40/60 in adult red cells (Schroeder et al. 1968; Steinberg et al. 2014), another example of gene switching. It is not known whether these two types of Hb F have any different properties since neither has been studied separately.

In 1976 a link was proposed between decreased malaria occurrences and increased fetal hemoglobin concentrations in red cells both in newborns and in those afflicted with beta-thalassemia compared (Pasvol et al. 1976; Mosca et al. 2009). As shown in Fig. 11.1b, normal newborns still retain a significant amount of Hb F in their red cells. Hence, the decreased incidence of malaria in populations with high levels of beta-thalassemia is thought to be due to prevention of the normal switch from gamma to beta subunit production due to beta gene deletions. Thus, gamma subunit synthesis continues so Hb F concentration in such red cells is comparatively high and growth of the malaria parasite is impeded thereby conferring resistance. A rationale for this observation was provided recently (Shear et al. 1998) which stated that the protease of the malaria parasite digests Hb F very poorly because, as we had reported earlier (Dumoulin et al. 1997), Hb F is a tetramer with very strong subunit contacts and therefore not readily prone to dissociation (see below). Hence, the presence of protease-susceptible subunits formed by dissociation of Hb A in normal cells permits growth of the malaria parasite in adult cells. When cells contain high amounts of Hb F, subunits are present in much lower concentrations because Hb F dissociates 70-fold less than Hb A (Dumoulin et al. 1997) and a nutrient source for the parasite is depleted. This source of the increased strength of the fetal Hb tetramer is described below.

It has been known for many years that individuals who are heterozygous for sickle cell anemia, i.e., one sickle gene from one parent and one normal gene from the other parent are less likely to develop malaria (Allison 1954). This observation has been studied by many investigators; the mechanism has been reviewed recently (Luzzatto 2012).

Fetal Hemoglobin, a Strong Tetramer

All human hemoglobin tetramers have a very similar architecture (Fig. 11.1a) even though their oxygen-carrying capacities are significantly different (Fig. 11.2). A study of the assembly properties of each tetramer using a common laboratory instruments and a high resolution gel support with very high sensitivity and resolving capability coupled with software for elution curve analysis has provided evidence how this increased strength is achieved (Manning et al. 1996). The first proof of concept that this procedure was capable of detecting very small changes in subunit contact strengths was with the liganded form of fetal hemoglobin (Dumoulin et al. 1997). In spite of an earlier erroneous report (Bunn 1969) that Hb F and Hb A had the same dissociation properties, Fig. 11.3 shows that their tetramer-dimer dissociation properties are vastly different (~70-fold). Perutz had reported earlier that the deoxy

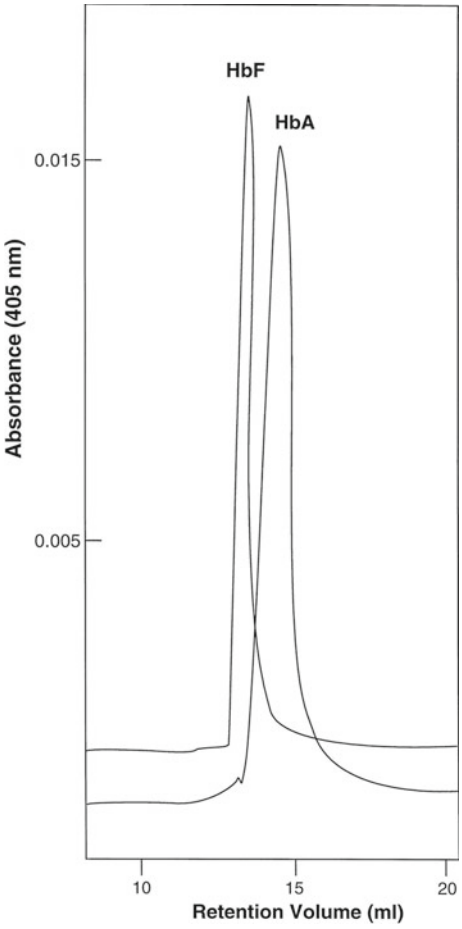

Fig. 11.3 Nano Gel Filtration of Human Fetal (Hb F) and Adult (Hb A) Hemoglobins. At the very dilute concentration used (500 nM), Hb A is predominantly dimeric and Hb F is tetrameric, which forms the basis for their separation. Reproduced from Dumoulin et al. (1997) with permission

form of fetal hemoglobin had a more compact overall structure (Frier and Perutz 1977) but the corresponding structure of the oxy conformation of Hb F was never solved. The molecular basis for this increased strength is described next.

Sources of Fetal Hemoglobin Enhanced Strength

In order to ascertain which segments of the gamma subunit sequence of Hb F endowed it with the 70-fold enhanced strength of its tetramer-dimer subunit contacts (Fig. 11.3), we substituted the 5 amino acids that were different in both types of interfaces between Hb F and Hb A; hence, this recombinant Hb A/F had the single difference at the tetramer-dimer interface and the four differences at the dimer-monomer interfaces (Dumoulin et al. 1997). Its oxygen-binding properties in response to DPG was the same as that for Hb F and not Hb A, despite the fact that it possessed all of the DPG binding residues of Hb A. Its tetramer-dimer dissociation was increased only 5-fold towards that of Hb F instead of 70-fold if all of the increased tetramer strength were due to the five interface differences. This observation clearly indicated that segments other than the subunit interfaces themselves contributed to the increased stability of the Hb F tetramer. This source of the other site(s) responsible for increased tetramer strength was investigated with a recombinant Hb called Hb Felix (Dumoulin et al. 1998), as described next.

Hemoglobin Felix

The recombinant Hb Felix has the A-helix (amino acids 1–18) of the gamma subunit of Hb F (comprising eight amino acid differences compared with the beta subunit of Hb A) joined to helices B-H (the remaining sequence of Hb A) (Dumoulin et al. 1998). Studies on this hybrid hemoglobin have provided many new insights into hemoglobin function. This recombinant Hb is completely stable and has the native conformation of Hb. A completely unexpected finding was that its tetramer strength is close to that of Hb F despite the fact that the A-helix is not part of the tetramer-dimer interface. This suggests that the A-helix influences the tetramer-dimer interface due to a long-distance effect perhaps by protruding deeper into the central cavity than the A-helix of Hb A (Dumoulin et al. 1998; Manning et al. 1998, 1999). In dissecting which amino acids of the A-helix were responsible for this enhanced effect, individual and combination substitutions were performed (Yagami et al. 2002). The major contributors were the V1G, P5E, and E7D at sites 1, 5, and 7 respectively. The DPG response of Hb Felix is unaffected by the A-helix substitution.

Acetylated Fetal Hemoglobin

A fraction (~15%) of Hb F normally present in red cells and referred to as Hb F1 is acetylated at the N-terminal Gly of its gamma subunit; adult Hb A has Val at this position; Val is not a favorable substrate for the enzyme that adds an acetyl group to N-terminal sites. It is not clear whether acetylated Hb F1 has a function although it does have some very interesting properties. Hence, Hb F1 has a reduced binding of DPG compared to Hb F (Bunn and Briehl 1970); thus, its oxygen affinity is higher than that of HbF in the presence of DPG. Another important feature of Hb F1 is its significantly reduced tetramer strength, about 30-fold increased dissociation constant compared to that of unacetylated Hb F rendering it similar to adult Hb A in this respect (Manning and Manning 2001). An interpretation of this post-translational modification is that it represents an unfavorable energetic event for the Hb F type of hemoglobin since it weakens major subunit interactions. In terms of influencing gene expression (Fig. 11.1c), such a property could disfavor gamma subunit expression thereby lowering Hb F formation in favor of beta subunit expression and Hb A formation, i.e. "switching" (Manning et al. 2009), as described more fully below.

Embryonic Hemoglobins Are Weak Tetramers with Increased Oxygen Affinity

Most of the early information on embryonic hemoglobins is derived from studies conducted about 50 years ago (Huehns et al. 1964; Huehns and Shooter 1965; Huehns and Faroqui 1975) when purification and characterization methods were incompletely developed; hence not all of the embryonic hemoglobins were identified correctly. In addition, difficulties in procuring them due to their rarity and ethical considerations hampered investigations on them. Later studies by Brittain and colleagues using improved methodology made a significant advance (Brittain 2002). Our studies on the embryonic hemoglobins (Manning et al. 2007) were made possible through the work of He and Russell (2001) who transfected the human globin genes into mice permitting the isolation of adequate amounts of the embryonic hemoglobin in the native state.

There are three embryonic human hemoglobins—Portland-1 (zeta2gamma2), Gower-1 (zeta2epsilon2), and Gower-2 (alpha2epsilon2) normally present during the first few months of life but two others—Portland-2 (zeta2beta2) and Portland-3 (zeta2delta2) have not been found during normal development, but reported only in cases of severe alpha-thalassemia when alpha subunits are not synthesized (Fig. 11.1d). Gene switching facilitated by promotors and repressors to change subunit types is usually invoked to explain the normal appearance and disappearance of the first three hemoglobins (Fig. 11.1b) but there has been no adequate explanation for the absence of the other two hemoglobins during normal development. In collaboration with Dr. J. E. Russell, we isolated, characterized, and studied all

the embryonic hemoglobins except for Hb Portland-3 and compared them with fetal and adult hemoglobins. Each embryonic hemoglobin purified from transgenic mouse blood showed a single band upon electrophoresis, a single elution peak during chromatography, and had the correct mass and circular dichroism pattern (Manning et al. 2007). An unexpected observation during purification was that some of them, in contrast to adult hemoglobin, did not remain tetrameric due to their ease of dissociation, as described below.

The embryonic hemoglobins bind oxygen extremely avidly (Hofmann et al. 1995; Hofmann and Brittain 1996; Brittain 2002) (Fig. 11.2), thus providing them with the ability to capture the very small amounts of oxygen to which they are exposed in embryonic tissue. However, it has never been established on a molecular basis how this property is attained. It would be potentially useful to have this information as a basis for further investigations in situations where the oxygen supply is low.

We compared purified embryonic hemoglobins with fetal and adult hemoglobin using a number of proven methods and made the following conclusions:

(1) From a structural standpoint, Hb Gower-2, alpha2epsilon2 (Sutherland-Smith et al. 1998), which occurs during normal development, is the most thoroughly studied embryonic Hb. Its structure is remarkably similar to that of adult and fetal hemoglobins with minor differences but there is nothing obvious from the structure how its very high oxygen affinity is bestowed. Models attempting to interpret its primary sequence differences from adult hemoglobin have been suggested to explain its increased oxygen binding capacity but these are not conclusive.

(2) Attempts to solve the structure of Hb Portland-1, alpha2gamma2, were hampered by its dissociation to free gamma subunits which then re-associated to gamma4 (Hb Bart's) (Kidd et al. 2001). Gamma subunits undergo self-assembly to form stable dimers and tetramers. This finding is consistent with the weakness of the subunit interfaces of the embryonic hemoglobins that we reported.

(3) Generally, both tetramer-dimer and dimer-monomer subunit interfaces are weak in the embryonic hemoglobins compared to corresponding interfaces in fetal and adult hemoglobin. This was evident when we reported a systematic study of these properties for all the embryonic hemoglobins (Fig. 11.4) (Manning et al. 2012) by high resolution gel filtration.

(4) When embryonic hemoglobins are mixed, they slowly dissociate to individual subunits which compete with each other to recombine and form the energetically most favorable tetramer. For example, when three embryonic hemoglobins containing alpha, gamma, or beta subunits are mixed, Hb A is formed but Hb F is not, suggesting that Hb A is the more stable tetramer (Manning et al. 2009). If normal adult and fetal hemoglobins are mixed in the same manner, they do not exhibit this behavior.

(5) The tetramer-dimer subunit interfaces dissociation constants of adult, fetal, and embryonic hemoglobins do not show a regular pattern but the values for the embryonic hemoglobins are about 10-30 times weaker than those for fetal and

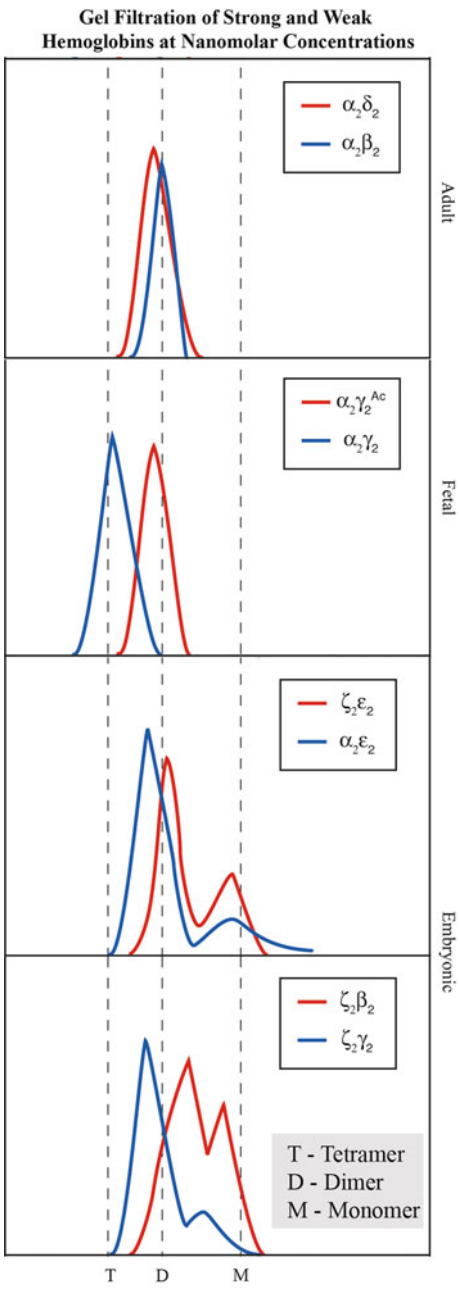

Fig. 11.4 Nano Gel Filtration of Strong and Weak Hemoglobins. At the concentrations used (10–100 nM), the human adult and fetal hemoglobins in the upper two panels have strong tetrameric and dimeric structures. The embryonic hemoglobins in the lower two panels generally have weak structures shown by a significant monomer population. Reproduced from Manning et al. (2017) with permission

adult tetramers (Manning et al. 2007) thus correcting some erroneous reports (Brittain 2002).

(6) Dissociation of the dimer-monomer interfaces have an ordered degree of strength corresponding to embryonic < fetal < adult (Fig. 11.4) (Manning et al. 2007). The alpha-beta and alpha-gamma dimers are very stable but the corresponding embryonic zeta-containing dimers are weak. In fact, the dimer-monomer dissociation equilibrium constant of Hb Portland (zeta-beta) was accurately determined to be 1.4 nM. That such a measurement can be made indicates that this interface is so weak that the equilibrium between dimers and monomers is freely reversible; in contrast, the adult Hb dimer-monomer dissociation constant (alpha-beta) is beyond the detection limits of current instruments. It is possible that the acetylation of the N-terminus of the zeta subunit contributes significantly to the weakness of the embryonic hemoglobin structures by analogy to the acetylation of fetal hemoglobin (Hb F1) described above.

(7) There is a correlation between dimer dissociation rates and oxygen binding. Thus, in addition to showing decreased oxygen affinity from left to right, Fig. 11.2 also depicts the increasing strengths of the interface between monomers in the same direction. The individual subunits and myoglobin represent the completely dissociated state whereas adult Hb has the strongest dimer interface; the embryonic and fetal hemoglobins have intermediate strengths. The alpha-epsilon dimer behaves like a transition Hb meaning that it shows an *intermediate* dimer strength and also has an *intermediate* degree of oxygen affinity (Fig. 11.2) clearly indicating a correlation between dimer strength and oxygen binding (Manning et al. 2010, 2017).

(8) A comparison of tetramer and dimer interface strengths of all the human hemoglobins by gel filtration at nanomolar concentrations dramatically demonstrate the order of their stability (Fig. 11.4) to be embryonic < fetal < adult (Manning et al. 2012).

(9) The embryonic hemoglobins have moderately reduced Hill coefficients (n values of 1.6–2.4 compared to n value of 2.8 for adult Hb, (see inset to Fig. 11.2) (Brittain 2002; Manning et al. 2017). This feature further enhances the ability of the embryonic hemoglobins to bind oxygen efficiently.

Linkage Between Oxygen Binding Affinity and Dimer Interface Strength

The degree of dimer interface strength has a significant influence on oxygen binding potential of the *normal* human hemoglobins. The wide range of oxygen binding potential shown in Fig. 11.2 starts from the left with the very high affinity of the individual subunits (and myoglobin) and proceeds to the far right with the low oxygen affinity of the adult hemoglobin tetramer; the embryonic and fetal hemoglobin tetramers are intermediate between these two extremes that parallels the strength

between different monomer types in the heterodimer of each hemoglobin (Manning et al. 2017). Hence, whereas the embryonic dimers with high oxygen affinity are quite weak and dissociate fairly readily, the fetal and adult dimers with lower affinities dissociate very little and have a low affinity. Hence, the variations in dimer strengths have a corresponding relationship to oxygen binding potential (Manning et al. 2007). Therefore, an unexplored area for future research is whether oxygen itself (Genbacev et al. 1997; Simon and Keith 2008) might have some role in promoting expression of a hemoglobin type.

The very tight binding of oxygen to the embryonic hemoglobins cannot readily be explained as arising from local effects of individual amino acid substitutions, e.g. on the heme moiety, but rather due to more global effects on the bonds that hold the subunits together. The well known oxygen binding curves of the human hemoglobin family (Fig. 11.2) are better envisaged as a spectrum of tetramers with different binding potential that depends on the degree to which the individual subunits bond together. Moderate weakening of the adult Hb A tetramer with its relatively low oxygen affinity will increase the population of dimers and monomers in the overall equilibrium not readily discernable by inspection of the structure but demonstrable by gel filtration at extremely dilute concentrations (Fig. 11.4). This equilibrium shift leads to an increase in oxygen binding. A major effect of amino acid differences between different subunit types is to determine how tightly they bind to one another, which in turn affects how readily they bind oxygen, an allosteric effect. It is likely that different properties of other hemoglobin types remain to be discovered when they are studied from a different perspectives.

Cooperativity and the Bohr Effect

Cooperativity can be envisaged as arising like a compressed spring representing the tightness of the interactions between subunits to confer low oxygen binding; release of this tension facilitates the binding of oxygen (Perutz 1989). The assembly process from individual subunits, which do not display cooperativity, to tetramers with positive cooperativity is an important feature of the oxygen binding curve and achieves its maximum in the midrange of the sigmoidal curves (Fig. 11.2); it is expressed as the Hill Coefficient (n). The curves from right to left (adult–> fetal–> embryonic) have decreasing cooperativity (Fig. 11.2 inset), which is consistent with a decrease in their overall tetramer strength since their subunit contacts are weaker in that same direction. The equilibrium between the deoxy and the oxy states, T/R, is defined as the allosteric constant, L. For adult Hb A this value is 5×10^5 whereas for the embryonic hemoglobins, it is 1–2 orders of magnitude lower (Brittain 2002). This decrease is consistent with a loss in tetramer strength for the embryonic hemoglobins. The Bohr Effect of Hb F is about 20% greater than that of Hb A at physiological chloride concentrations (Poyart et al. 1978) and likely enhances oxygen supply to the fetal circulation.

Relationship Between Dimer Interface Strength and Ontogeny

The stability of the dimer-monomer interface between the various hemoglobin subunits is clearly demonstrated by gel filtration at very low concentrations (Fig. 11.4). This pattern also resembles their chronological appearances (Fig. 11.1b), i.e., there is a correlation between the stability of this type of interface in terms of how long the dimer interface remains intact and the physiological duration of that particular hemoglobin in vivo. Stronger dimer pairs supplant weaker ones in the order embryonic – > fetal –> adult. Thus, the strength and ease of formation of dimers appears to be related to ontogeny, which would represent a thermodynamic contribution to gene expression. We refer to this as an "intrinsic" contribution to gene expression in contrast to "extrinsic" contributions, such as with promotors and repressors (Manning et al. 2012). Even though the term "switching" strictly refers to individual gene expression, it is exemplified as the production of a hemoglobin type when two subunits combine, i.e., embryonic to fetal type or fetal to adult type. The enhanced ability of fetal Hb to form tetramers as demonstrated by its very low tetramer-dimer dissociation constant may contribute to the switch from embryonic to fetal type of hemoglobin during development. The slow acetylation of fetal Hb weakens its tetrameric structure considerably (similar to that of Hb A, discussed above) and could subsequently contribute to the switch to the adult type of Hb, which is not acetylated. In addition, whether the change from Gly136 to Ala-136 in the two types of gamma genes has a role in switching remains to be studied. However, it is clear that considering both acetylation and the presence of two types of gamma subunits, fetal hemoglobin represents a heterogenous system whereas adult hemoglobin is not; i.e. an energetically more favorable state likely to favor its formation.

Conclusions

Hemoglobin has proven to be a continuing source of new and important information when viewed from different perspectives with novel approaches. Although the oxygen transport mechanism is well known for the adult type of hemoglobin, increased oxygen binding by the embryonic hemoglobins involves an additional parameter; a looser tetrameric structure that leads to higher oxygen affinity. Hence, the concept put forth here is that the hemoglobin structure is a platform capable of variable oxygen carrying abilities as a result of subtle alterations holding it together, i.e., the bonding between the subunits that comprise the tetramer. Thus, not all natural hemoglobin tetramers are alike in terms of their subunit strengths although their overall structural architectures may be practically identical and this flexibility is the source of distinct properties for a given hemoglobin type, which can provide it with advantages in a particular physiological circumstance, such as sickle cell anemia, malaria, and ontogeny.

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