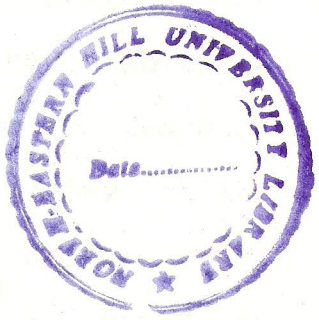
FUNCTIONAL AND CONFORMATIONAL STUDIES OF HEMOGLOBINS OF COW AND WATER BUFFALO



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A Thesis Submitted in Fulfilment of the Degree of

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Certified that the thesis titled " FUNCTIONAL AND CONFORMATIONAL STUDIES OF HEMOGLOBINS OF COW AND WATER BUFFALO " submitted by Mr. ABANI K. BHUYAN for the award of the degree of DOCTOR OF PHILOSOPHY in Biochemistry of the North-Eastern Hill University, embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the degree of Ph. D.. This work has not been submitted for any degree of any University.

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ACKNOWLEDGEMENT

I very sincerely thank Prof. R. Lalthantluanga for accepting me to work in his laboratory. His generosity, support, suggestions and guidance made it possible to complete this thesis. I must mention that he introduced me to 'Hemoglobins' while I was still an M.Sc. student. That 'Hemoglobin Introduction' became not only the genesis of this thesis, but also paved the way to my current research on macromolecular spectroscopy.

I gratefully acknowledge Mr. P. K. Prabhakaran's massive contribution to my present knowledge and experimental skill. He has been a teacher, a friend and a well wisher. Mr. Rishi Das also helped me a lot. Thanks to him.

Thanks are extended to Dr. M.Y.Khan for the pain he took to train me while I was a neophyte in M.Sc. I thank him also for his encouragement, suggestions and manifold help. Thanks are also due to Drs. K.Chatterjee, G.D.Sharma, B.K.Dutta and R.N.Sharan for giving me class room courses as part of Pre-Ph.D work.

Prof. J.Subramanian (presently at the Department of Chemistry, Pondicherry University) kindly allowed me to work in his laboratory during collection of EPR data. I thankfully remember his effective lectures on magnetic resonance spectroscopy. I also acknowledge the help extended to me by Dr. A.Lemtur (presently at RSIC) and by Dr. D.T.Khating of Department of Physics, NEHU.

I further extend my thanks to Prof. A.N.Rai and to late Prof. M.K.Khare for providing required facilities and help.

Finally, I thank my friends for their generosity.

This work was supported by University Grants Commission, New Delhi, through a National Educational Test Research Fellowship (Roll No. 490051 of 1984) awarded to me.

Abani K. Bhuyan

PREFACE

This thesis documents a few structural and functional properties of normal adult human hemoglobin and of cow and buffalo hemoglobins. Both cow and buffalo hemoglobins have been chosen with an object of uniquely categorizing the bovine group with regard to conformational and functional allostery of hemoglobins. Human hemoglobin, an extensively studied system and which represents the group of intrinsically high oxygen affinity hemoglobin, has been chosen as a standard to compare and contrast the experimental results on bovine hemoglobins. Primary structural data on buffalo hemoglobin remained unavailable since the writing of this thesis began. Nevertheless, amino acid sequence of cow or ox hemoglobin has been used to discuss the results on the basis of phylogenetic relatedness of cow and buffalo.

The thesis consists of four chapters and two appendices. Chapter 1 concisely describes the mammalian hemoglobin tetramer and warrants the objective of this work. Chapters 2 and 3 deal with structural and conformational analyses of the three hemoglobins. In Chapter 4 detailed functional studies are presented.

Each chapter has been prepared to include an introductory section followed by materials and methods, results and discussion. The introductory section presents a brief review of past work on the subject of that chapter. Materials and methods have been described specifically. Data and figures are briefed in the section 'Results'. Findings, both consistent and contradictory, are presented in detail

under the discussion section. Each chapter carries a list of references cited therein.

A summary of critical roles played by the distal histidine (His E7) in structural and functional properties of hemoglobin is presented in Appendix I. This appendix also highlights the current application of ultrafast spectroscopy and expression of globin genes in E.coli. Finally, Appendix II details the electrophoretic methods.

CONTENTS

A NOTE ON NOMENCLATURE

Three-lettered abbreviations for amino acids have been used throughout, except in Figures 1.4 and 1.5 where amino acid sequences are shown using standard one-lettered form.

The position of an amino acid in the sequence is identified with the segment to which it belongs. For example, HisF8 stands for the histidine at the 8th position of F-helix. Similarly, PheCD1 identifies phenylalanine as the first residue of CD corner.

α or β preceds or follows an amino acid to indicate the subunit which the residue belongs to. For instance, αHisF8 indicates that the histidine belongs to α subunit. Likewise, MetNA2β would identify the methionine residue at the second position of N-terminal nonhelical segment of β subunit. This mode of designation of a residue, however, has not been strictly maintained. In many places words have been used. Thus, MetNA2β could also be written as MetNA2 of β subunit.

Abnormal hemoglobins have been mentioned either through words or by arrows to indicate the amino acid replacements, for example, Hb Hammersmith (βPheC7 $\longrightarrow$ Ser). This means, in Hemoglobin Hammersmith, the PheC7 of β subunits has been replaced by a serine.

Besides, the following short forms have been used in quite a few places:

Short form	stands for
HbO_2	oxyhemoglobin
deoxyHb	deoxyhemoglobin
NOHb	nitrosyl complex of hemoglobin
COHb	carbonmonoxyhemoglobin
CNHb	cyanomet derivative
AqHb	aqueomet form of hemoglobin
HbA	normal adult human hemoglobin
HbC	cow hemoglobin
Hb-A and Hb-B	the two phenotypes of cow hemoglobin
HbB	buffalo hemoglobin

Otherwise indicated, usual convention and notations have been followed throughout.

CHAPTER 1

INTRODUCTION AND OBJECTIVE

1.1. HEMOGLOBIN OF MAMMALS

The mammalian hemoglobin (MW=64,500) is a tetrameric respiratory protein of red blood cells composed of four subunits, namely α_1, α_2, β_1 and β_2. Each subunit consists of a polypeptide chain and a heme group. The heme complex in hemoglobin is iron protoporphyrin IX, illustrated in Figure 1.1. The α and β polypeptide chains normally contain 141 and 146 amino acids, respectively. The sequences are different in the two chains. There are eight helical sections labeled A through H in the β chain. The D-helix is absent in the α chains (see Figure 1.4). Some of the helical stretches are separated by nonhelical regions, while there are no intervening nonhelical links between other helices. Each chain also carries short nonhelical stretches at the N and C termini.

The heme establishes a major contact with the polypeptide chain by means of a covalent bond between the coordination site 5 of the heme iron and the $N_{\epsilon 2}$ nitrogen on the imidazole group of a histidine residue called proximal or heme-linked histidine, which is the eighth residue of the F-helix (HisF8). The heme is also in van der Waals contact with another histidine (HisE7) on the distal side (Figure 1.2). Furthermore, eighteen other amino acid side chains,

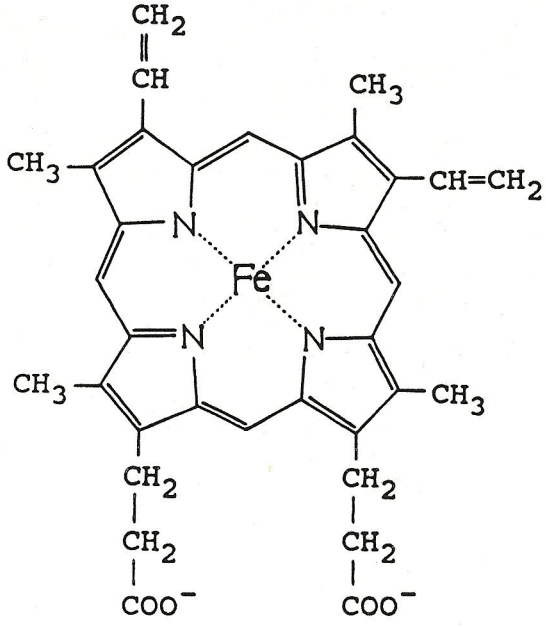


Figure 1.1. Ferrous protoporphyrin IX is the heme chromophore in hemoglobin. This isomer of protoporphyrin has four methyls, two vinyls and two propionates as side chain constituents.

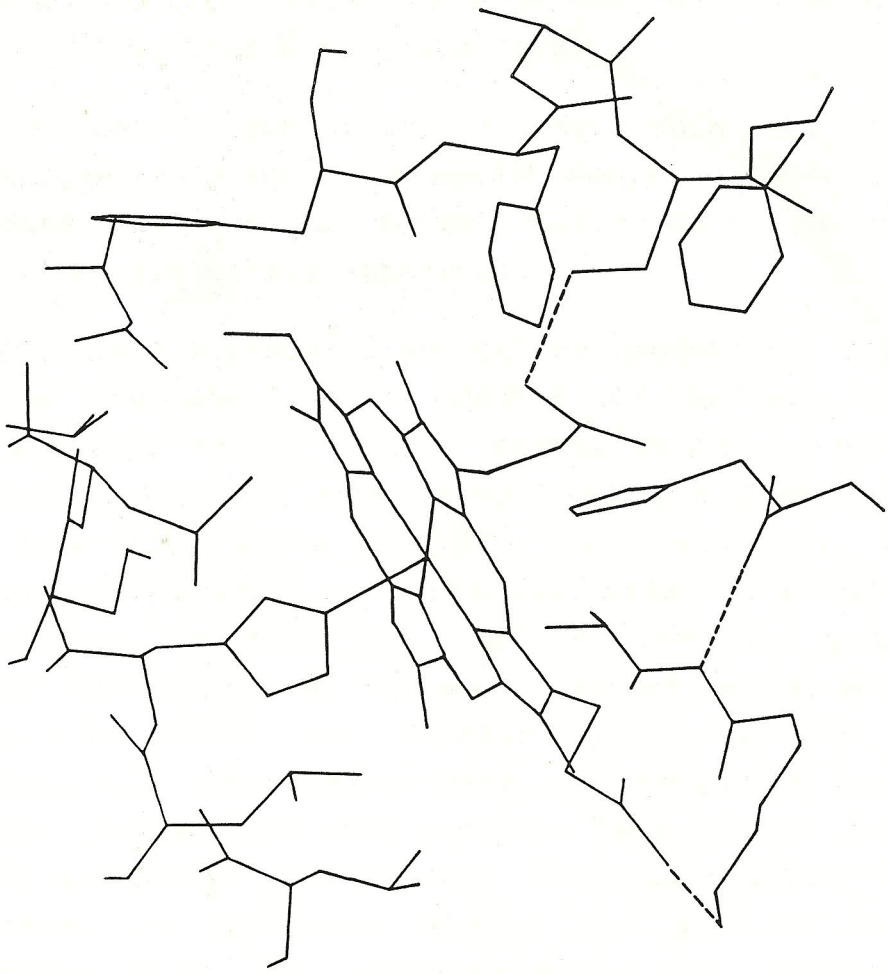


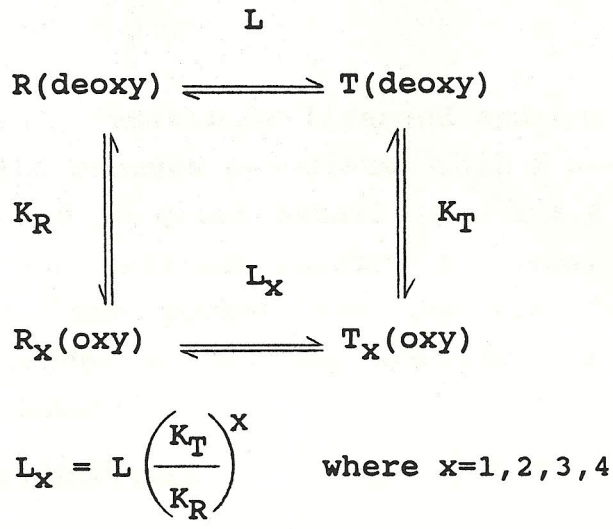
Figure 1.2. The heme group with surrounding residues of β chains of horse aqueomethemoglobin. The residues belong to the CD corner and E- and F helices. This stick diagram, which depicts the compactness of the heme pocket and some of the contacts between the heme and globin, has been reproduced after modification from Dr. Max Perutz's Croonian Lecture of 1968 (see ref. 15). For specific positions of Phe CD1, ValE11, HisE7 and HisF8, see Figure 1 of Appendix I.

most of which are nonpolar, make contacts with the heme. There is a hydrogen bond bridging the propionate side chain of the heme porphyrin with the side chains of HisCD3 of α subunits and LysE10 of β subunits (1-3).

The four subunits of the hemoglobin molecule are packed tetrahedrally by intersubunit nonpolar interactions, salt bridges and hydrogen bonds. The final shape of the tetramer is approximately spherical.

The heme ligands, also called homotropic ligands, bind at the sixth coordination position of the iron which is either in ferric or in ferrous state. Depending upon the ligand field splitting, each valency state may assume high spin or low spin state. Oxygen, carbonmonoxide, nitric oxide, alkylisocyanides, and nitrosoaromatic compounds form ferrous derivatives while H_2O, OH^-, F^-, and N_3^- complexes are ferric derivatives of the heme. Except deoxyhemoglobin (deoxyHb), in which the ferrous heme iron is five-coordinated, all other derivatives of hemoglobin are six-coordinated. Oxygen, the physiological heme ligand, combines reversibly with heme iron to form dioxygen complex. During this process, the high spin ferrous iron goes to low spin configuration with the same valency. The reaction of hemoglobin with oxygen is cooperative with 3.6 kcal/mol per heme as the free energy of cooperativity. The cooperative behavior is manifested in a sigmoid equilibrium curve for the reaction of hemoglobin with oxygen.

At the root of cooperative effect lies a structural concept, namely, an equilibrium between two different structures of the hemoglobin molecule, the R (relaxed) and T (tense) structures. The oxygen affinity of R form is larger than that of T. Their respective association constants with oxygen are K_R and K_T. For $R \rightleftharpoons T$, the equilibrium constant $L=T/R$ (scheme 1.1).



$$L_x = L\left(\frac{K_T}{K_R}\right)^x \qquad \text{where } x=1,2,3,4$$

Scheme 1.1

Structural arrangements of the four subunits are different in R and T forms. In traditional nomenclature, the relaxed and tense forms of the individual subunits (i.e., tertiary structure) are labelled by r and t, respectively. The R $\longrightarrow$ T transition is associated with a rotation of the $\alpha_1\beta_1$ dimer relative to the $\alpha_2\beta_2$ dimer by 12-15^0 and a translation of one dimer relative to the other by 0.8 Å (1). During this transition, the contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$ remain rigid; however, the dimers move relative to each other at $\alpha_1\beta_2$, $\alpha_2\beta_1$, $\alpha_1\alpha_2$ and $\beta_1\beta_2$ contacts.

The allosteric or heterotropic ligands, namely, H^+, CO_2, Cl^-, and organic phosphates lower the oxygen affinity of hemoglobin significantly. More precisely, K_T varies as a function of all of these effectors, while execpt [H^+] the other three ligands do not have much effect on K_R (4,5). These ligands bind to the globin part of the molecule to exert their effects. In deoxyHb, one molecule of organic phosphate salt bridges with an array of positively charged groups in a cavity between the two β chains (6-8). Any replacement or substitution of these amino acids in the β chains results in lesser interaction of organic phosphates

with hemoglobin.

Structural details of liganded and unliganded forms of hemoglobin and changes associated with R $\rightleftharpoons$ T transition have been published in great detail (see 3,4,9-23). Here we discuss briefly the stereochemistry of heme, the role of residues in the heme pocket and the functional role of heterotropic ligands, since they are more relevant to the subject of this thesis.

1.1.A. Stereochemistry of heme:

In deoxyhemoglobin the heme porphyrin is domed (24), so that the iron moves out of the mean porphyrin plane towards His F8. The porphyrin plane flattens up as oxygen binds to iron. This causes the iron to move toward the plane. The inward and outward movements of the iron are controlled by variable Fe-N_{porph} bond lengths and by constraints of the globin chains. As a consequence of toward plane movement of the iron, the distance between His F8 and porphyrin plane shortens by 0.5-0.6 Å in oxy- than in deoxyHb. The bond lengths and distances between planes which are used to describe heme stereochemistry can be found in reference 3. It is gratifying that many of the stereochemical changes in crystal structures of hemoglobin are consistent with those found in solutions probed by spectroscopic methods (see, for example, 25-28). A review of the literature also points out the fact that heme stereochemistry may be significantly different in different porphyrins and hemoglobin types. For example, nickel-porphyrin is planer and the Ni-$N_{\epsilon 2}$ (of HisF8) distance is 3.2 Å, which pushes the R $\rightleftharpoons$ T equilibrium towards right (3,27). In cobalt-porphyrin, the Co-N_{porph} bond is shorter by 0.1 Å than the Fe-N_{porph} bond. As a result, the displacement of Co from porphyrin plane is less than that in iron-porphyrin (29). In Hb Boston, an abnormal human hemoglobin where HisE7 of α subunits is replaced by Tyr

(30), the iron atom is linked to the tyrosine instead of HisF8 (31), and the allosteric equilibrium is strongly favored towards the T structure (32,33).

1.1.B. Stereochemical mechanism and the role of the heme:

The stereochemical mechanism first proposed by Max Perutz in 1970 (9) held that the oxygenation of hemoglobin is accompanied by structural changes in the subunits triggered by shifts of the iron atom relative to the porphyrin and, in the β subunits, also by the steric effects of oxygen itself. The salt bridges which constrain the oxygen-free form are broken by the energy of heme-heme interaction with the release of H^+. There have been much controversy regarding the role of iron displacement in governing R $\rightleftharpoons$ T equilibrium. However, experimental evidences still favor the distances of the iron and the proximal histidine from the porphyrin as the only determinant of allosteric equilibrium in the α subunits (3).

1.1.C. Role of residues on distal side of the heme pocket:

The heme pocket on the distal side is basically formed of PheCD1, HisE7, and ValE11 (Figure 1.2, see also Figure 1 of Appendix I). The role of PheCD1 in regulating the structure and function of hemoglobin is not definitively known. However, this near-heme-aromatic residue changes its orientation substantially with respect to the porphyrin ring in both α and β chains during R $\longrightarrow$ T transition (35). In R structure of α and β chains, the PheCD1 is in van der Waals contact with the carbons of porphyrin. In T form it swings away from the heme. Replacement or substitution of this residue, as in Hb Hammersmith (βPheC7 $\longrightarrow$ Ser) and Hb Bucuresti (βPheC7 $\longrightarrow$ Leu), leads to decrease in oxygen affinity and loss of cooperativity (36-38). It has been suggested that PheCD1 is directly involved in a charge transfer interaction with the heme so as to increase the electron density of the antibonding π^* orbitals of the

porphyrin rings in the R structure (39). Thus, this interaction has been assumed to contribute to the free energy of cooperativity. However, Perutz et al. (3) have argued that the distance between PheCD1 side chain and the porphyrin is 3.8 Å - 4 Å, a distance too large for charge transfer interaction to occur.

Displacement of the distal histidine and the valine is necessary for oxygen binding to the ferrous hemes of β chains (40). Valine imposes steric hindrance at the heme site of β subunits in T structure (9). The heme site of R structure is flexible enough to adjust the relative positions of the heme and ValE11. As a consequence, oxygen can bind favorably. HisE7, on the other hand, forms hydrogen bond with the bound oxygen (41,42). It also produces steric effect and acts as a gate for entry of ligands (43-46). The distal histidine, by swinging out of the way, makes an opening for oxygen to pass through, and this movement of the histidine requires a tertiary structural change (47). The displacements of HisE7 and ValE11 relative to the heme in T structure are initiated through a movement of the E-helix, which is brought about by tertiary structural change.

How HisE7 and ValE11 affect the oxygen equilibrium may be seen from oxygen affinity measurements with engineered hemoglobins (48-51). For example, replacement of the distal histidine by glycine lowers K_R leaving K_T nearly unchanged. On the other hand replacement of HisE7 by Val raises K_T and lowers K_R. When ValE11 is replaced by Ala, K_T rises with no change in K_R. Thus, these residues have individual contribution toward maintenance of the $R \rightleftharpoons T$ equilibrium. It is very important to note that HisE7 is seriously involved in structural and functional properties of hemoglobin. Appendix I at the end of this thesis is devoted to highlight this in finer detail.

1.1.D. Heterotropic ligands:

These nonheme ligands preferentially bind to the T structure and lower the oxygen affinity by stabilizing the molecule. The bound ligands are released during T $\longrightarrow$ R transition. On going from deoxy to oxy form, hemoglobin liberates protons. In fact, the released H^+ come from certain ionizable amino acids, which are actively involved in hydrogen bonding in T structure. The H-bonds break right at the onset of oxygenation, and the protons are discharged (see, for example, 52-56). Conversely, hemoglobin binds H^+ on release of oxygen. The dependence of oxygen affinity of hemoglobin on protons is called Bohr effect. Release of H^+ on ligation at heme above pH 6.0 is called alkaline Bohr effect, and proton uptake below pH 6.0 is referred to as acid Bohr effect.

While hemoglobin binds H^+ ions on deoxygenation, the equilibrium of the reaction of HCO_3^- formation is pushed to the right (see below). Also, as is true with other proteins, CO_2 forms carbamino compounds with hemoglobin. Carbamino adducts are bound to ϵ-amino group of lysine and NH_2-terminals of α and β chains (see 57).

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$
$$RNH_2 + CO_2 \rightleftharpoons RNH\text{-}COO^- + H^+$$
$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$$

Binding of chloride ions to deoxyhemoglobin causes a decrease in oxygen affinity and an increase in Bohr effect (58-60). Four residues, namely, ValNA1 and SerH14 of α chains and LysEF6 and HisH21 of β chains are involved in Cl^- binding (61-71). Chloride also binds to COOH-terminal residues of both α and β subunits (72). In fact, binding of Cl^- to T structure raises the pK's of ValNA1α and LysEF6β, which contribute to Bohr effect (3).

DPG, a major constituent of mammalian red cell, enters a cleft between the two β chains and forms H-bonds with ValNA1, HisNA2, LysEF6 and HisH21 of β chains (8). A schematic sketch of the interaction between DPG and deoxyhemoglobin is shown in Figure 1.3. By stabilizing the structure of deoxyhemoglobin, DPG lowers the oxygen affinity of mammalian hemoglobins. It increases both the alkaline and acid Bohr effect (53). In other forms of vertebrates this function is performed by different organic phosphates. A comparison of the binding sites for Cl^- and DPG shows that these two heterotropic ligands exert their effect competitively. This competitive functional effect is important in regulation of oxygen affinity of mammalian hemoglobins as we shall see later.

1.1.E. Nonphysiological heme ligands:

Ferrous hemoglobin binds two gaseous ligands, nitric oxide and carbonmonoxide, and mainly two nongaseous ligands, namely, isocyanides and nitrosoaromatic compounds at the heme iron (73). Complexes of hemoglobin with these nonphysiological heme ligands have been very useful to elucidate the structure and mechanism of hemoglobin. Studies on these complexes have enabled to test models for the kinetics and structure of HbO_2. Moreover, certain experimental techniques, not easily applicable to HbO_2 can be applied to them. For examnple, nitrosylhemoglobin (NOHb) is the only known paramagnetic derivative of hemoglobin which is amenable to electron spin resonance spectroscopy (74-79). Furthermore, ultrafast laser spectroscopy has been successfully applied to NOHb, COHb, and HbO_2 to observe transient reaction intermediates, their structure and pathway and mechanism of ligand binding (80-85).

1.1.F. Stability of R and T structures and free energy of cooperativity:

In terms of MWC model of allosteric theory (86), the low oxygen affinity of T structure relative to R arises from

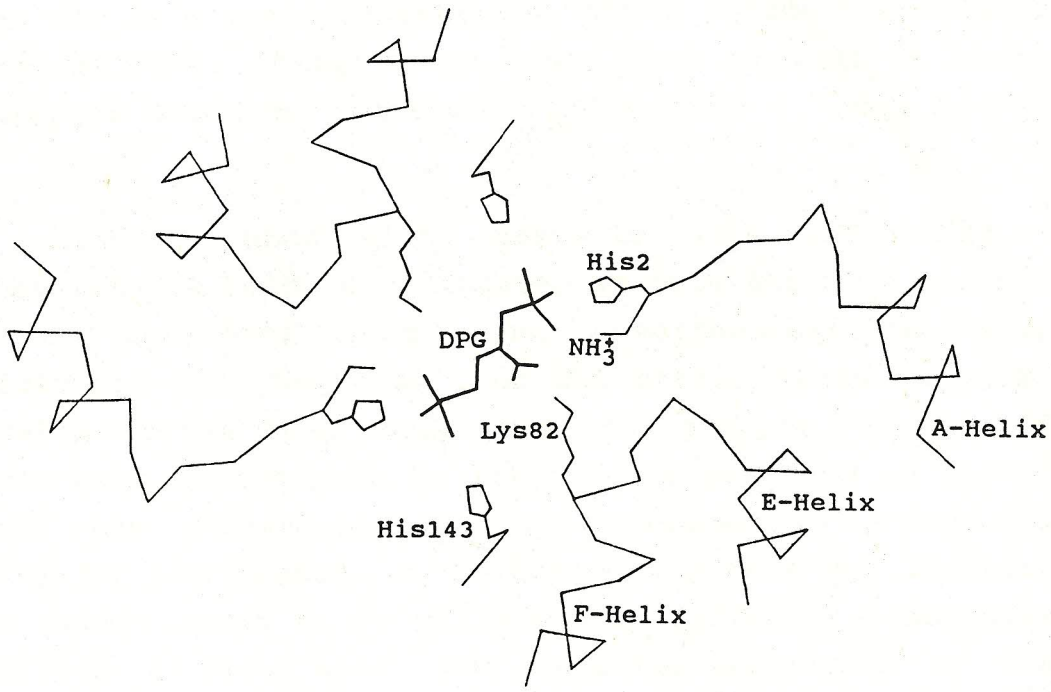


Figure 1.3. A stick diagram to show the cavity formed by the two β chains where DPG is positioned in deoxyhemoglobin. Stereochemical complementarity is notable. DPG forms salt bridges with ValNA1, HisNA2 and HisH21 of both β chains and with LysEF6 of one β chain. This sketch has been reproduced after modification from reference 8.

higher energy of the former. The additional energy comes from extra bonds between subunits in the quaternary T structure compared to those in R. The C terminal residues and DPG form 14 intersubunit salt bridges which are absent in the R structure (3). These additional salt bridges easily account for 14.4 kcal per tetramer, which is the free energy of cooperativity. Thus, it is clear that absence of these salt bridges would be compensated by a lower L and a higher K_T.

Although uncertainty prevails, the free energy of cooperativity is held to be located in both the heme and the globin in the form of strains. Nevertheless, extensive spectroscopic work suggests that the strain takes the form of stretch of the Fe-N bonds (see, for example, 87-95). If the iron atom cannot move to its optimum position on ligand binding, the strain induced is reflected in a reduced affinity for the ligand. It is noteworthy that the location of the strain seems to be different in different hemoglobin derivatives (3,96). Also, the relative strengths of the restraints of the globin and of Fe-N bonds appear to differ amongst different animal species (97-100).

1.2. BOVINE HEMOGLOBINS

Virtually all hemoglobins have the same chromophore, i.e., protoporphyrin IX, to which heme ligands bind. Yet there are diverse groups of hemoglobins which are structurally and functionally different. These differences originate from primary structural variations. In each of the four chains of vertebrate hemoglobins, there are seven invariable sites occupied by the same amino acids. These sites are mainly around the heme and in contacts between the subunits which are essential for allosteric switch between the T and R forms. Otherwise, hemoglobins from different species are not similar with respect to their primary

structure (see 101,102).

Bovine refers to a group of grazing animals which belong to the family Bovidae of the sub-order Ruminants of Artiodactyls. Bovine hemoglobins are functionally distinct and interesting. In fact, of the two group of mammalian hemoglobins, namely, intrinsically high and intrinsically low oxygen affinity hemoglobins, the bovine falls in the second category. Intrinsically low oxygen affinity hemoglobins respond little, or not at all, to the presence of organic phosphates. It was first observed by Bunn (103) that the oxygen affinity of cow hemoglobin is not lowered by 2,3 DPG. He pointed out that the red cells of ruminants have low concentration of DPG and their hemoglobins interact weakly with this allosteric effector. The reason for this weak interaction lies in a unique and common primary structure shared by all ruminant hemoglobins. A comparison of the amino acid sequences of human and ox hemoglobins is presented in Figures 1.4 and 1.5. There is a deletion of the N terminal residue in the β chains. This implies that bovine β chains have 145 amino acids instead of 146. Also, the next residue, which is histidine in the high oxygen affinity mammals, is methionine in bovine and in other ruminants (104). This has been known for quite sometime. Since both these N terminal residues form salt bridges with DPG, the deletion of the ultimate and replacement of the penultimate N- terminal amino acids have been assumed to be the reasons for diminished interaction.

However, even in the absence of organic phosphates bovine hemoglobins have markedly low oxygen affinity. What structural features regulate this behavior is not known. Certainly, it is the globin structure which plays the key role.

The genetic heterogeneity of bovine hemoglobins is rather divergent (105-108). Many breeds of cattle exhibit

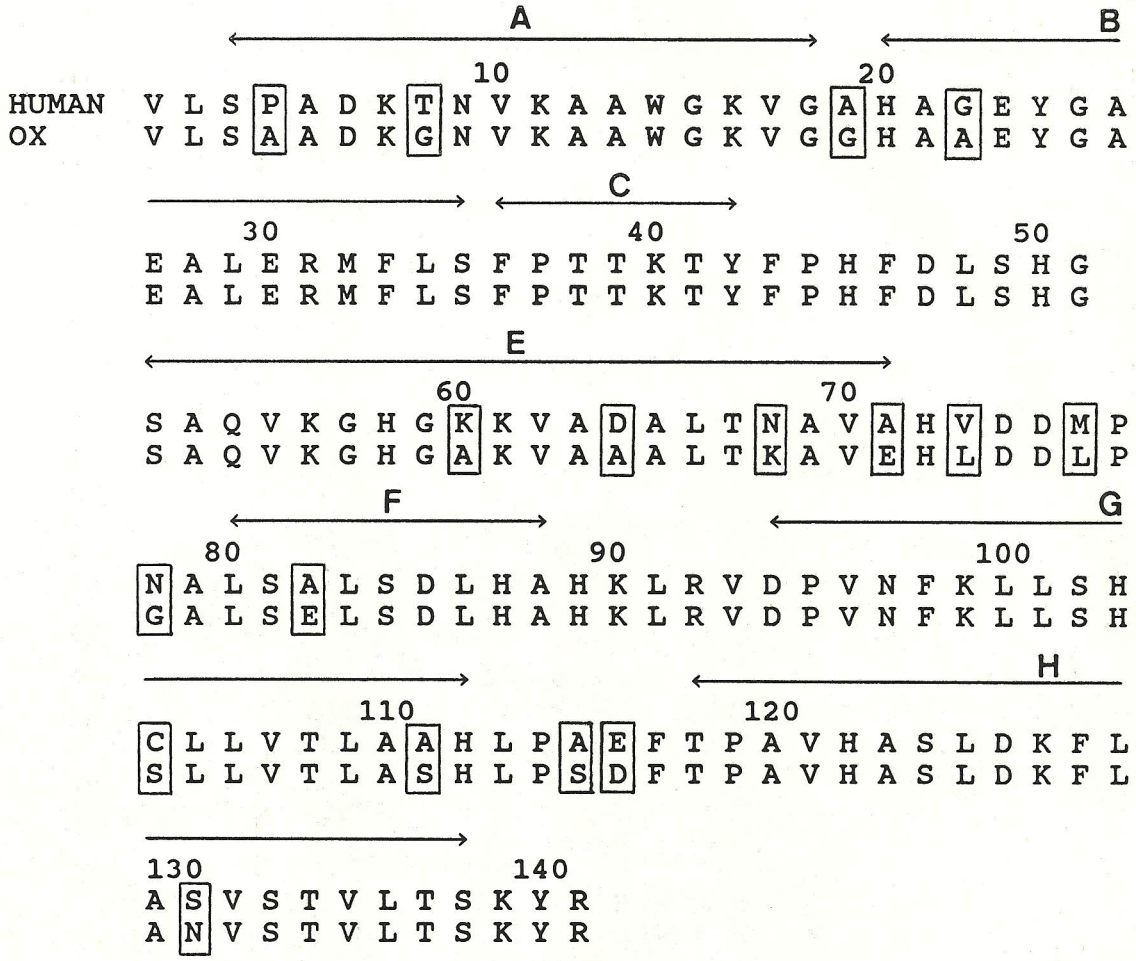


Figure 1.4. Comparison of amino acid sequence of α chains of human and bovine hemoglobins. α chains of both hemoglobins have 141 amino acids. Ox α chain differs from that of human at 17 different positions. Helical segments are marked with arrows above the human sequence. Helices are labelled from A through H. D-helix is absent in α chains.

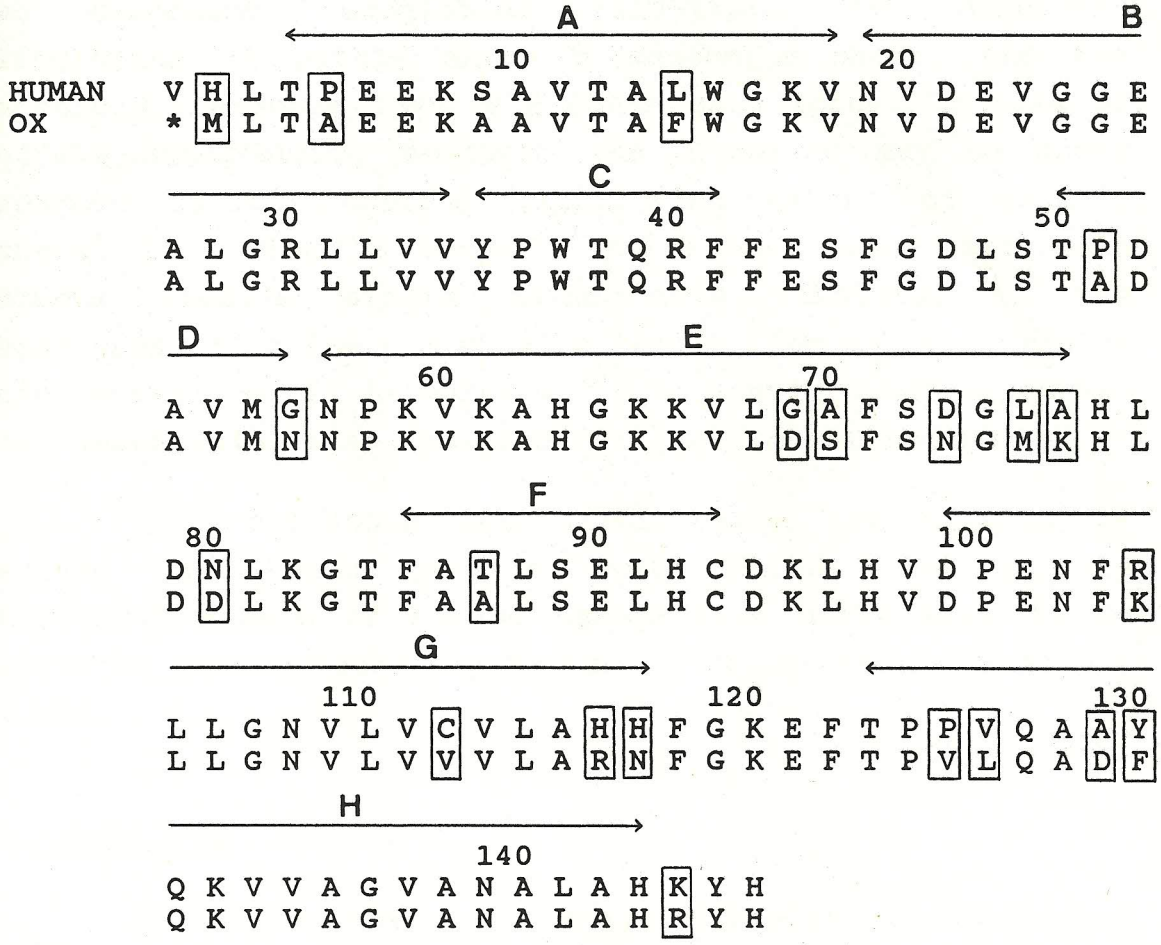


Figure 1.5. Comparison of amino acid sequence of β chains of human and bovine hemoglobins. Human β chain is composed of 146 amino acids. The N-terminal residue is deleted in all ruminants (indicated by *). The helical segments are marked from A through H.

polymorphism involving two hemoglobin types (109). Buffalo hemoglobins, on the other hand, exhibit monomorphism with two component hemoglobins (110-113). The component hemoglobins of cattle share a common α chain, and the genetic differences involve β chain gene locus. In case of buffalo hemoglobins, however, the α chains may be under separate genetic control (112). The nature of genetic control in bovine is unusual. While the human hemoglobin mutants involve single amino acid substitution, the phenotypes of bovine hemoglobins result from multiple random point mutations. It is worth while to mention that sheep and goat hemoglobins also show similar genetic heterogeneity.

It is not known what physiological advantage may be derived from such genetic constitution. The primary structural makeup of bovine hemoglobins might seem to be suited to other regulatory mechanism rather than the effect of DPG.

1.3. OBJECTIVE

Evidently, the underlying mechanism of functional regulation of bovine hemoglobins is puzzling. A number of important questions arise regarding this issue.

1. What is the status of the tertiary and quaternary conformation of the molecule? How stable are the T and the R structures relative to each other? In other words, does a unique $R \rightleftharpoons T$ equilibrium regulate the oxygen affinity?

2. How stable is the bovine tetramer towards acid and alkali, and other denaturation agents? What effects are exerted by the primary structural differences on the chemical nature of the molecule?

3. How do these hemoglobins respond to heterotropic or allosteric ligands? Is there any allosteric effector that is preferably bound to bovine hemoglobins?

4. What is the nature and basis of Bohr effect in bovine? Could an altered Bohr effect regulate the oxygen affinity?

5. Are there differences in functional properties of hemoglobins among different bovine species?

Hemoglobin literature has not repoprted any detailed investigation regarding these problems in proper perspective. An attempt has been made to give answers to some of these questions based on our experimental evidences. We have carried out studies on hemoglobins from two bovine species, namely, cow and buffalo, and from normal adult human. The human hemoglobin was chosen as a control to compare and contrast the results from the two bovine species. Our findings and the conclusions are presented in this thesis.

REFERENCES

1 Fermi, G. and Perutz, M.F. (1981) in Hemoglobin and Myoglobin. Atlas of Biological Structures; Phillips, D.C. and Richards, F.M., Eds.; Clarendon: Oxford

2 Dickerson, R.E. and Geis, I. (1983) Hemoglobin; Benjamin Cummings: Menlo Park, California

3 Perutz, M.F., Fermi,G., Luisi,B., Shaanan, B. and Liddington, R.C. (1987) Acc. Chem. Res. 20, 309

4 Baldwin, J.M. (1975) Progress in Biophys. Mol. Biol. 29, 227

5 Imai, K. (1982) Allosteric Effects in Hemoglobin; Cambridge University Press: Cambridge

6 Bunn, H.F. and Briehl, R.W. (1970) J. Clin. Invest. 49, 1088

7 Benesch, R.E., Benesch, R., Renthal, R.D. and Maeda, N. (1972) Biochemistry 11, 3576

8 Arnone, A. (1972) Nature 237,146

9 Perutz, M.F. (1970) Nature 228,726

10 Perutz, M.F. and Ten Eyck, L.F. (1971) Cold Spr. Harb. Symp. Quant. Biol. 36,295

11 Perutz, M.F. (1972) Nature 237,495

12 Muirhead, H., Cox, J.M. Mazzarella, L. and Perutz, M.F. (1967) J. Mol. Biol. 28,117

13 Perutz, M.F., Muirhead, H., Cox, J.M., Goaman, L.C.G., Mathews, F.S., McGandy, E.L. and Webb, L.E. (1968) Nature 219,29

14 Perutz, M.F., Muirhead, H., Cox, J.M. and Goaman, L.C.G. (1968) Nature 219,131

15 Perutz, M.F. (1969) Proc. Roy. Soc. B173,113

16 Perutz, M.F. (1969) Eur. J. Biochem. 8,455

17 Muirhead, H. and Greer, J. (1970) Nature 228,516

18 Bolton, W. and Perutz, M.F. (1970) Nature 228,551

19 British Medical Bulletin (1976) Vol. 32, 193-282 (Weatherall, D.J., Ed.), London: The British Council

20 Baldwin, J.M. and Chothia, C. (1979) J. Mol. Biol. 129, 175

21 Brzozowski, A., Derewenda, Z., Dodson, E., Dodson, G., Grabowski, M., Liddington, R.C., Skarzynski, T. and

Vallely, D. (1984) Nature 307,74
22 Shaanan, B. (1983) J. Mol. Biol. 171,31
23 Weissbluth, M. (1974) Hemoglobin:cooperativity and electronic properties; in Mol.Biol.Biochem.Biophys.15 (Kleinzeller, A., Springer, G.F. and Wittman, H.G., Eds); Springer-Verlag, Berlin
24 Gelin, B.R., Lee, A.W.-M. and Karplus,M. (1983) J. Mol. Biol. 171,489
25 Eisenberger, P., Shulman, R.G., Kincaid, B.M., Brown, G.S. and Ogawa, S. (1978) Nature 274,30
26 Perutz, M.F., Hasnain, S.S., Duke, P.J., Sessler, J.S. and Hahn, J.E. (1982) Nature 295,535
27 Shibayama, N., Morimoto, H. and Kitagawa, T (1986) J. Mol. Biol. 192,331
28 Bianconi, A., Congiu-Castellano, A., Dell'Aricia, M., Giovanelli, A., Morante, S., Burateini, E. and Durham, P.J. (1986) Proc. Natl. Acad. Sci. USA 83,7736
29 Lauher, R.G. and Ibers, J.A. (1974) J. Am. Chem. Soc. 96, 4447
30 Gerald, P.S. and Efron, M.L. (1961) Proc. Natl. Acad. Sci. USA 47.1758
31 Pulsinelli, P.D., Perutz, M.F. and Nagel, R.L. (1973) Proc. Natl. Acad. Sci.USA 70,3870
32 Nagai, K., Hori, H., Morimoto, H., Hayashi, A. and Taketa, F. (1979) Biochemistry 18,1304
33 Suzuki, T., Hayashi, A., Shimizu, A. and Yamamura, Y. (1966) Biochim. Biophys. Acta. 127,280
34 Perutz, M.F. (1979) Ann. Rev. Biochem. 48,327
35 Ladner, R.C., Heidner, E.J. and Perutz, M.F. (1977) J. Mol. Biol. 114,385
36 Dacie, J.V., Shinton, N.K., Gaffney, P.J., Carrell, R.W. and Lehmann, H. (1967) Nature 216,663
37 Bratu,V., Lorkin, P.A., Lehman, H. and Predescu, C. (1971) Biochim. Biophys. Acta. 251,1
38 Morimoto, H., Lehmann, H. and Perutz, M.F. (1971) Nature 232,408
39 Shelnutt, J.A., Rousseau, D.L., Friedman, J.M. and Simon, S.R. (1979) Proc. Natl. Acad. Sci. USA 76,4409
40 Fermi, G. and Perutz, M.F. (1977) J. Mol. Biol. 114,421

41 Shaanan, B. (1982) Nature 296.683
42 Shaanan, B. (1983) J. Mol. Biol. 171,39
43 Doster, W., Beece, D., Bowne, S.F., DiIorio, E.E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K.W. and Yue, K.T. (1982) Biochemistry 21,4831
44 Dlott, D.D., Frauenfelder, H., Langer, P., Roder, H. and DiIorio, E.E. (1983) Proc. Natl. Acad. Sci. USA 80,6239
45 Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14,5355
46 Eisenstein, L. and Frauenfelder, H. (1982) in Biological Events Probed by Ultrafast Laser Spectroscopy; Ed. Alfano, R.R., Academic Press, New York; pp 321
47 Perutz, M.F. and Mathews, F.S. (1966) J. Mol. Biol. 18, 547
48 Nagai, K. and Thogersen, H.C. (1984) Nature 309,810
49 Nagai, K., Perutz, M.F. and Poyart, C. (1985) Proc. Natl. Acad. Sci. USA 82,7252
50 Luisi, B. and Nagai, K. (1986) Nature 320,555
51 Lin, S.-H., Yu, N.-T., Tame, J., Shih, D., Renaud, J-P., Pagnier, J. and Nagai, K. (1990) Biochemistry 29,5562
52 Kilmartin, J.V. (1974) FEBS Lett. 38.147
53 Kilmartin, J.V., Fogg, J.H. and Perutz, M.F. (1980) Biochemistry 19,3189
54 Perutz, M.F., Kilmartin, J.V., Nishikura, K., Fogg, J.H., Butler, P.J.G. and Rollema, H.S. (1980) J. Mol. Biol. 138,649
55 Shih, D.-b. and Perutz, M.F. (1987) J. Mol. Biol. 195,419
56 Matsukawa, S., Itatani, Y., Mawatari, K., Shimokawa, Y. and Yoneyama, Y. (1984) J. Biol. Chem. 251,477
57 Morrow, J.S., Mathew, J.B., Wittebort, R.J. and Gurd, F.R.N. (1976) J. Biol Chem. 251,477
58 Antonini, E., Wyman, J., Rossi-Fanelli, A. and Caputo, A. (1962) J. Biol. Chem. 237,2773
59 Benesch, R.E., Benesch, R. and Yu, C.I. (1969) Biochemistry 8,2567
60 Bunn, H.F. and Guidotti, G. (1972) J. Biol. Chem. 247, 2345
61 Chiancone, E., Norne, J.E., Forsen, S., Antonini, E. and

Wyman, J. (1972) J. Mol. Biol. 70,675
62 Chiancone, E., Norne, J.E., Bonaventura, J., Bonaventura, C. and Forsen, S. (1974) Biochim. Biophys. Acta 336,403
63 Chiancone, E., Norne, J.E., Forsen, S., Bonaventura, J., Brunori, M., Antonini, E. and Wyman, J. (1975) Eur. J. Biochem. 55,385
64 O'Donnell, S., Mandaro, R., Schuster, T.M. and Arnone, A. (1979) J. Biol. Chem. 254,12204
65 Bonaventura, J., Bonaventura, C., Sullivan, B., Ferruzzi, G., McCurdy, P.R., Fox, J. and Moo-Penn, W.F. (1976) J. Biol. Chem. 251,7563
66 Bonaventura, C., Bonaventura, J., Amiconi, G., Tentori, L., Brunori, M. and Antonini, E. (1975) J. Biol. Chem. 250,6273
67 Bare, G.H., Alben, J.O., Bromerg, P.A., Jones, R.T., Brimhall, B. and Padilla, F. (1974) J. Biol. Chem. 249, 773
68 Kilmartin, J.V. and Wooton, J.F. (1970) Nature 228,766
69 Kilmartin, J.V., Breen, J.J., Roberts, C.K. and Ho, C. (1973) Proc. Natl. Acad. Sci. USA 70,1246
70 Nigen, A.M. and Manning, J.M. (1975) J. Biol.Chem. 250, 8248
71 Adachi, H., Asakura, T. and Adachi, K. (1983) J. Biol. Chem. 258,13422
72 Chiancone, E., Norne, J.E., Forsen, S., Mansouri, A. and Winterhalter, K.H. (1976) FEBS Lett. 63,309
73 Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, North-Holland, Amsterdam
74 Chien, J.C.W. (1969) J. Chem Phys. 51,4220
75 Scholler, D.M., Wang, M.R. and Hofman, B.M. (1979) J. Biol. Chem. 254,4072
76 Nagai, K., Hori, H., Toshida, S., Sakamoto, H. and Morimoto, H. (1978) Biochim. Biophys. Acta 532,17
77 Doetschmann, D.C. and Utterback, S.G. (1981) J. Am. Chem. Soc. 103,284
78 John, M.E., Lalthantluanga, R., Liljeqvist, G., Paleus, S. and Braunitzer, G. (1982) Z. Naturforsch 37b, 744
79 Bhuyan, A.K., Lemtur,A., Subramanian, J. and

Lalthantluanga, R. (1989) Biochim. Biophys. Acta 997,36
80 Greene, B.I., Hochstrasser, R.M., Weisman, R.B. and Eaton, W.A. (1978) Proc. Natl. Acad. Sci. USA 75,5255
81 Chernoff, D.A., Hochstrasser, R.M. and Steele, A.W. (1980) Proc. Natl. Acad. Sci. USA 77,5606
82 Cornelius, P.A., Hochstrasser, R.M. and Steele, A.W. (1983) J. Mol. Biol. 163,119
83 Martin, J.L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R. and Antonetti, A. (1983) Proc. Natl. Acad. Sci. USA 80,173
84 Hofrichter, J., Sommer, J.H., Henry, E.R. and Eaton, W.A. (1983) Proc. Natl. Acad. Sci. USA 80,2235
85 Murray, L.P., Hofrichter, J., Henry, E.R. and Eaton, W.A. (1988) Biophysical Chem. 29.63
86 Monod, J., Wyman, J. and Changeux, J.P. (1965) J. Mol. Biol. 12,88
87 Perutz, M.F., Heidner, E.J., Ladner, J.E., Beetlestone, J.G., Ho, C. and Slade, E.F. (1974) Biochemistry 13,2187
88 Sugita, Y. (1975) J. Biol. Chem. 250,1251
89 Wang, C.M. and Brinigar, W.S. (1979) Biochemistry 18,4960
90 Nagai, K. and Kitagawa, T (1980) Proc. Natl. Acad. Sci. USA 77,2033
91 Matsukawa, S., Mawatari, K., Yoneyama, Y. and Kitagawa, T. (1985) J. Am. Chem. Soc. 107,1108
92 Perutz, M.F., Kilmartin, J.V., Nagai, K., Szabo, A. and Simon, S.R. (1976) Biochemistry 15,378
93 Maxwell, J.C. and Caughey, W.S. (1976) Biochemistry 15, 388
94 Rousseau, D.L. and Ondrias, M.R. (1983) Ann. Rev. Biophys. Bioeng. 12,357
95 Friedman, J.M., Stepnoski, R.A. and Noble, R.W. (1982) FEBS Lett. 146,278
96 Philo, J.S. and Dreyer, U. (1985) Biochemistry 24,2985
97 Nagai, K. (1983) in Brussels Hemoglobin Symposium; Schnek, E.G. and Paul, C., Eds.; Editions de l'Universite de BRuxelles, Brussels
98 Friedman, J.M. (1985) Science 228,1273
99 Friedman, J.M., Scott, T.W., Fisanick, G.J., Simon, S.R., Findsen, E.W., Ondrias, M.R. and Macdonald, V.W.

(1985) Science 229,187
100 Findsen, E.W., Friedman, J.M., Ondrias,M.R. and Simon, S.R. (1985) Science 229,661
101 Dayhoff, M.O. (1972) Editor of Atlas of Protein Sequence and Structure, Vol.5, National Biomedical Research Foundation, Washington
102 Dayhoff, M.O. (1978) Editor of Atlas of Protein Sequence and Structure, Vol.5, suppl.3, National Biomedical Research Foundation, Washington
103 Bunn, H.F. (1971) Science 172,1049
104 Petruzzelli, R., Barra, D., Bossa, F., Condo, S.G., Brix, O., Nuutinen, M. and Giardina, B. (1991) Biochim. Biophys. Acta 1076,221
105 Cabannes, R and Serain, C (1955) C. R. Soc. Biol., Paris, 149,7
106 Bangham, A.D. (1957) Nature 179,467
107 Bangham, A.D. and Blumberg, B.S. (1958) Nature 181,1551
108 Braend, M. and Stormont, C. (1963) Nature 197,910
109 Gratzer, W.B. and Allison, A.C. (1960) Biol. Rev. 35,459
110 Giri, K.V. and Pillai, N.C. (1956) Nature 178,1057
111 Vella, F. (1958) Nature 181,564
112 Balani, A.S., Ranjekar, P.K. and Barnabas, J. (1968) Comp. Biochem. Physiol. 24,809
113 Balani, A.S. and Barnabas, J. (1965) Nature 205,1019