INTERACTION BETWEEN PROTEIN SUBUNITS FROM MODEL STUDIES

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ABSTRACT A molecular model of hemoglobin was constructed which made it possible to visualize the relation between various amino acid residues in the molecule. The model indicates that electrostatic forces might play a significant role in holding the subunits of hemoglobin together. This would explain why myoglobin does not form a tetramer while four β-chains, which are structurally similar to myoglobin, do assemble into a hemoglobin H molecule. Also, as far as the primary structures of hemoglobin chains of various species are known, the proposed ionic links between subunits are consistent with the fact that mammalian hemoglobins form stable tetramers while the peptide chains of lamprey hemoglobin are only weakly associated. The different behavior of hemoglobin H and of normal hemoglobin upon oxygen uptake is briefly discussed in terms of allosteric effects.

The work of Perutz and Kendrew on the structure of heme proteins has for the first time made possible a thorough study of relationships between structure and functionality (1–4). It can be expected that a full structural analysis of a group of closely related proteins will provide us with the clues necessary to understand the significance of the various molecular features. Of particular interest are the cooperative effects encountered in the binding and reactivity of substrates by proteins, as described by Monod, Wyman, and Changeux (5). These indirect interactions between sites, the so-called allosteric effects, occur usually in proteins consisting of a well defined number of subunits arranged in a symmetrical manner and are thought to be brought about by structural changes of the system. It will be possible to observe these changes directly only when high resolution X-ray diffraction data become available for the various forms (active and inhibited) of the protein. As exemplified by the work carried out at Cambridge University, this is a very difficult task. Perutz has recently described an atomic model of horse oxyhemoglobin based on the sequence of its amino acid residues, the X-ray data for hemoglobin (5.5 A resolution) and on analogies with the well studied myoglobin molecule (1.4 A resolution) (3). This study and a subsequent one by Perutz, Kendrew, and Watson (4) reveal important features of the polypeptide chain configuration, of the heme surroundings, and of interactions between subunits.

It can be anticipated that in the not too distant future low to medium resolution X-ray data on a number of proteins will become available. We therefore thought it worthwhile to see whether some useful information might not be obtained from less accurate structural data. First of all, it should be clear what kind of problems might be solved in this way, and which problems ought only be attacked by more refined methods.

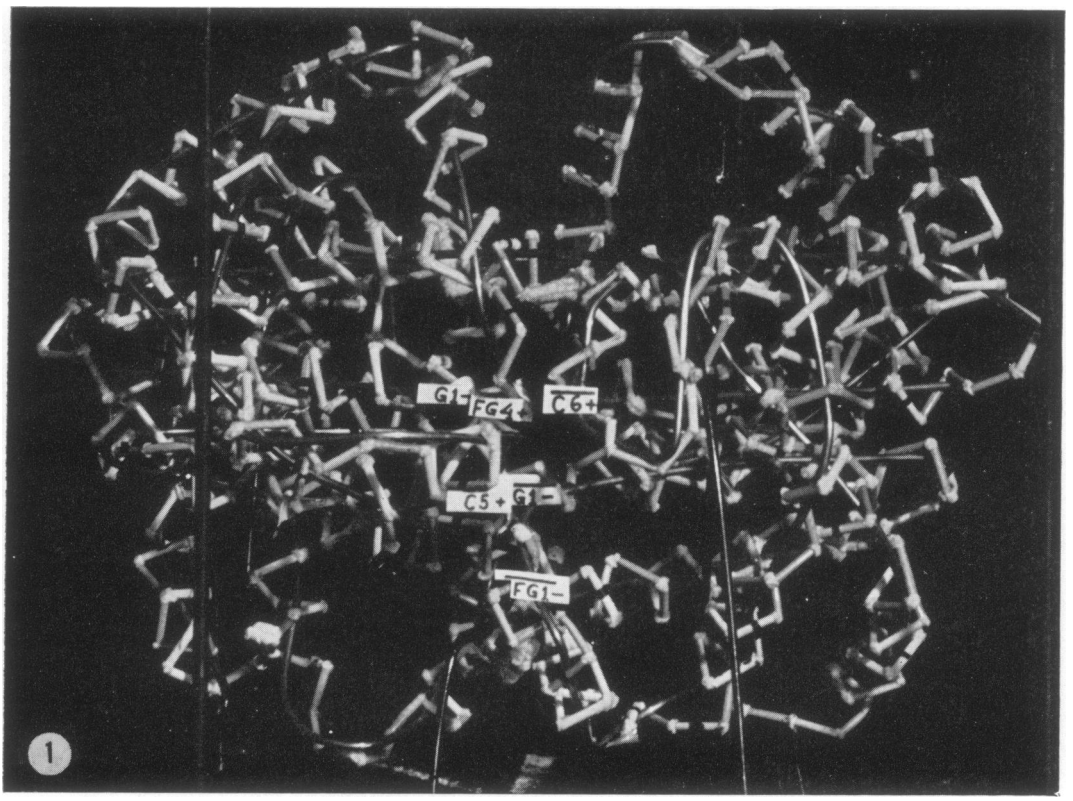

FIGURE 1 Model of hemoglobin showing the $\alpha_1\beta_2$-contact region. Segments represent amino acid residues. Heme groups are not shown. Plain labels indicate α-chain and the barred ones β-chain.

For example, X-ray data have revealed that the subunits of hemoglobin, which very much resemble myoglobin, do not undergo any gross distortions when being assembled into the protein. They are not covalently bound to each other but only seem to be hinged together at a number of points. Upon deoxygenation of horse oxyhemoglobin, some of these subunits move somewhat apart (6,7)—an effect which is clearly discernible by low resolution X-ray measurements. From this it appears that some information about the interactions between subunits might be

derived from simple model studies while interactions within the subunits would probably not be revealed without an accurate knowledge of the detailed structure.

In order to study the interactions between the subunits of hemoglobin a simple molecular model was constructed from the electron density data of Cullis, Muirhead, Perutz, Rossmann, and North (1).

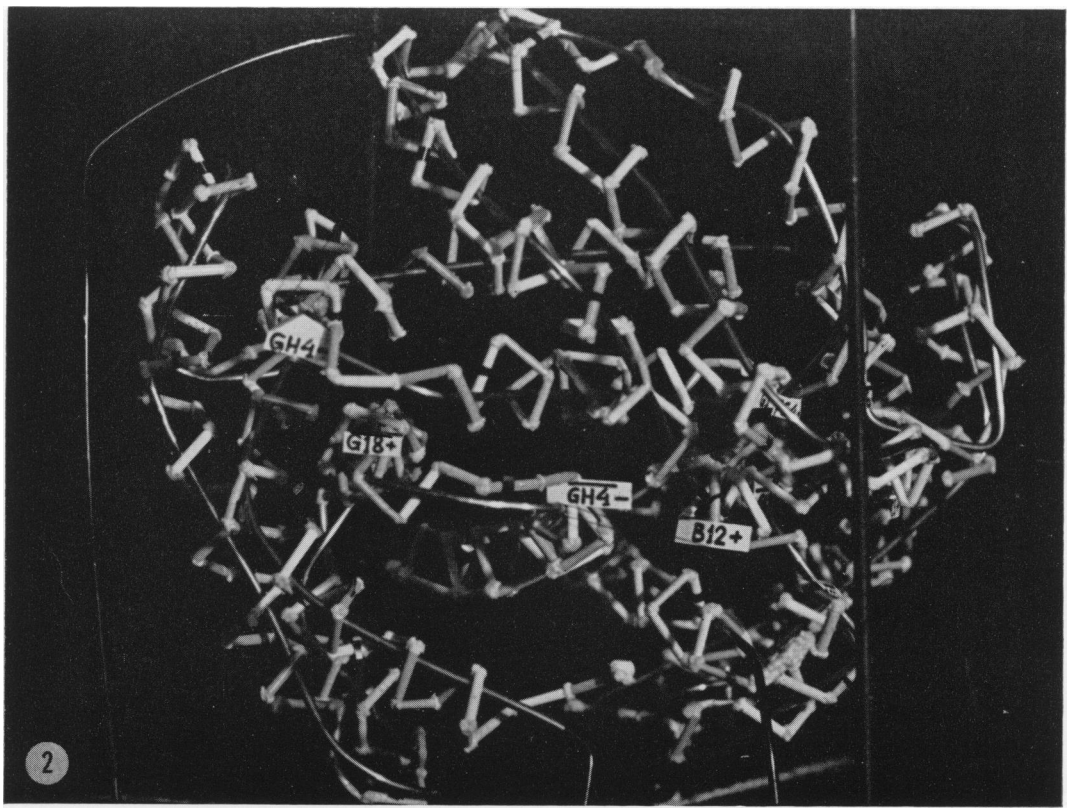

FIGURE 2 The $\alpha_1\beta_1$-contact region of hemoglobin. The second α-chain is removed from the back side of the model in order to obtain a clearer picture. Plain labels indicate α-chain and the barred ones β-chain.

The Model. The polypeptide chains were constructed on a scale of 0.5 cm per A unit. Pieces of plastic straw, 4 mm in diameter, 15 mm long, were strung onto a backbone of pipe cleaners at 3 mm intervals. Each amino acid was thus represented by a straight little stick 1.8 cm long. At the junctions of the amino acids, the backbone could be bent at will. The length of the amino acid residue backbone was taken to be 3.6 A, there being 7.2 amino acids for every two turns of the α-helix with a pitch of 5.4 A per turn of the helix. The straws were color coded so that the acidic, the basic, the bulky residues of valine, isoleucine, leucine and the aromatic amino acid residues could be distinguished from the rest. The hemoglobin was assembled by first constructing the primary structure and helical segments of each pep-

tide (8, 9, 3). These were then strung onto a copper wire (3 mm diameter) which had previously been bent into the shape of the peptide chain, according to the model of Cullis et al. (1). Finally the four peptides were brought into their proper mutual relation. The location of the amino acid residues in the various helical and nonhelical sections of the polypeptide chains were determined by using the well known properties of protein structure: helix breaks occur at the site of prolines and very often near aspartic acid, glutamic acid, and histidine residues (10); residues with ionized side chains are usually located on the (aqueous) outside of a subunit, bulky side chains often show up as bulges even in low resolution X-ray studies, and SH groups can be located by metal binding In fact a number of amino acid residues had already been located by the Cambridge group by such considerations (1) and these locations were then confirmed by comparison with myoglobin.

The model resulted in a rather loose structure which made it possible to observe the various parts of the molecule and their probable relation to each other more easily than in the electron density models of Cullis et al. We think that such a model represents adequately the degree of accuracy to be expected from a medium resolution ($\sim$5 A) X-ray diffraction study in that neither the side chains nor the location of the nonhelical parts of the molecule are exactly determined. Similar models could be constructed for other proteins of high helix content when their structural data become available.

The coordinates of the helices and helix breaks of our model were checked against the more accurate data presented by Perutz (3). They agreed within ± 1 A units. Though inaccuracies in the location of single residues might occur in the model, the features of the regions of subunit interaction are probably represented quite accurately. Figs. 1 and 2.

Perutz has described these regions in terms of interactions between nonpolar side chains. However, from our model it appears that a number of electrostatic interactions between subunits are also possible in solution near the outer protein surface. Since the ionized groups lie at the ends of flexible side chains they cannot be expected to show up in X-ray diffraction data. Two distinctly possible ionic links between peptide chains appear in the $\alpha_1\beta_1$-contact region (using Perutz's nomenclature). In the $\alpha_1\beta_2$-contact a variety of ionized groups could interact both in an inter- and intrapeptide fashion. Because of the proximity of the interacting chain sections we are unable to determine definite ion pairs. Instead we look upon the ionized regions as a kind of multipoles where the comparatively mobile side chains interact electrostatically and thus find their location of lowest free energy. The ionic bonds which seem possible to us are shown in Table I although intrapeptide interactions involving the same ionized groups cannot be excluded. It should be remembered that dissociation studies have revealed that at high and low pH values, as well as at high salt concentrations, the four unit hemoglobin molecule dissociates into dimers (11). As described in the discussion this behavior also indicates that electrostatic effects do play a part in subunit assembly.

We therefore think that although the multitude of nonpolar interactions is prob-

TABLE I

POSSIBLE IONIC BONDS BETWEEN α_1- AND β_1-PEPTIDES IN HEMOGLOBIN

The helix numbering is that appearing in the paper by Perutz (3). *N* indicates that the sequence at this location in the chain is not known.

Helix number	Myoglobin	Hemoglobin								Helix number	Hemoglobin						Myoglobin
		Lamprey	α-Chain					β-Chain*			β-Chain					Lamprey	
			Carp	Mouse	Pig	Horse	Human	Human			Human	Horse	Pig	Mouse	Carp		
GH4....	Asn	*N*	*N*	*N*	Asp	Asp	Glu	Glu		G18....	His	Arg	Arg	*N*	*N*	*N*	Ser
B12.....	Arg	Lys	Arg	Arg	Arg	Arg	Arg	Arg		GH4...	Glu	Asp	*N*	*N*	*N*	*N*	Asn

TABLE II

POSSIBLE IONIC BONDS BETWEEN α_1- AND β_2-PEPTIDES IN HEMOGLOBIN

The helix numbering is that appearing in the paper by Perutz (3). *N* indicates that the sequence at this location in the chain is not known.

Helix number	Myoglobin	Hemoglobin								Helix number	Hemoglobin						Myoglobin
		Lamprey	α-Chain					β-Chain*			β-Chain					Lamprey	
			Carp	Mouse	Pig	Horse	Human	Human			Human	Horse	Pig	Mouse	Carp		
FG2....	Lys	Ser	Lys	Lys	Lys	Lys	Lys	Lys									
FG4....	Lys	Gln	Arg	Arg	Arg	Arg	Arg	His		C6.....	Arg	Arg	*N*	*N*	*N*	Glu	Glu
G1.....	Pro	Asp	Asp	Asp	Asp	Asp	Asp	Asp		G1.....	Asp	Asp	*N*	*N*	*N*	Asp	Pro
C5.....	Leu	Gln	Lys	Lys	Lys	Lys	Lys	Glu									
C6.....								Arg		FG1....	Asp	Asp	*N*	*N*	*N*	Lys	Thr

* Human β–human β interaction represents hemoglobin H.

ably highly important in keeping the subunits of the protein assembled, a few ionic links might just be the deciding factor between the energetic pro and entropic contra of subunit association. That the free energy balance of association is indeed slight (only a few kilocalories/mole) has been shown by an estimate of Perutz (3).

DISCUSSION

Insight into the question of subunit interaction and the related problem of protein dissociation can be gained by a comparison of different but related subunits. If the above mentioned ionized side chains are indeed essential in subunit assembly we should find them at identical or similar locations in all the different hemoglobins which associate into tetramers. Conversely, those proteins which exist in the monomeric form should not form salt links in these critical regions.

In beginning our comparisons we could first ask why it is that myoglobin does not form a tetramer while four β-chains, which resemble the myoglobin structure closely, form the abnormal hemoglobin H. Hemoglobin H consists of four β-peptide chains and apparently it has a structure similar to that of reduced horse hemoglobin (12). This is an open structure where the chains have moved apart by about 7 A with respect to the oxygenated form. No contacts between diametrically opposed β-chains (corresponding to β-β–contacts in normal hemoglobin) should persist in this structure (6). Moreover, contacts between diametrically opposed chains, when present, seem mainly to consist of salt links between the terminal carboxyl (H-24) and amino groups (NA-1), and similar links could also be established by myoglobin molecules. We therefore think that contacts between adjacent subunits determine the tetramer stability. The composition of these contact regions with respect to both polar and nonpolar amino acid residues would change if myoglobin were substituted for β-chains in hemoglobin H. We do not see why the nonpolar contacts between hemoglobin H subunits should be much preferred to those between myoglobin molecules in establishing van der Waals bonds. On the other hand, there exists a great difference in ionic character between the two systems. In substituting four myoglobin molecules for hemoglobin H, the primary structure would change so that the ionic bonds indicated in Table I would disappear. Also, the protein would acquire a net positive charge of eight units which could cause repulsion between subunits.

Next let us compare hemoglobin H with the various normal hemoglobins which do exhibit heme-heme interactions and are supposed to form tetramers. Lately the amino acid sequences of hemoglobin peptide chains of various species have been determined and found to differ greatly from each other (13–16). Of the amino acid residues responsible for nonpolar contacts in the $\alpha_1\beta_1$- and the $\alpha_1\beta_2$-contact regions of normal hemoglobins, 40% are replaced by different groups at the equivalent sites of hemoglobin H. But both in hemoglobin H and in all cases where normal hemoglobin is thought to exist as tetramers, the sites necessary for ionic bond forma-

tion between subunits are indeed occupied by ionized residues which are able to form the proposed salt links.[1] This is shown in Tables I and II for hemoglobin chains of various species whose sequences have been established wholly or in part: human (α- and β-chains, reference 13), horse (α- and β-chains, references 8, 9, 3), pig (α, reference 14 and a few known residues of the β-chain, reference 15), mouse (α-chain reference 16), carp (α-chain, reference 13), and hemoglobin H.

On the other hand, lamprey hemoglobin exists mostly in the monomer form—at least when in the oxidized form (3, 13). Lamprey hemoglobin therefore seems to associate very weakly. In this case, the required acidic and basic amino acid residues have been replaced by other groups at all three proposed ionic bond sites equivalent to the $\alpha_1\beta_2$-juncture in other hemoglobins. The location of ionic groups in the $\alpha_1\beta_1$–contact region is unknown since the complete amino acid sequence has not yet been determined. The comparisons between myoglobin and hemoglobin chains, whose sequences are known, indicate strongly, therefore, that ionic bonds do indeed play an important role in the association of hemoglobin subunits.

Finally, we would like to consider briefly the structural changes accompanying uptake and loss of oxygen by hemoglobin. We see from Muirhead and Perutz' data that in normal mammalian hemoglobin the distance between the two peptides increases upon deoxygenation (6). The distance between the α_1- and β_2–chain heme groups remains constant and the α-chains do not change their positions. The effect looks similar to the opening of two opposing seams of a tetrahedron. Studies on dye binding also indicate that the molecular structure becomes more open upon deoxygenation (11). Our model shows that most of the close van der Waals contacts in the α_1-β_1–region must necessarily disappear or change partners in the process. Similarly no β-β–contacts should be possible in this conformation. The protein nevertheless does not dissociate, indicating that no critically important bonds have been broken. Again we see that the ionic contacts between β-chains do not determine the tetramer stability since deoxygenated hemoglobin dissociates even less readily than its oxygenated form (17). On the other hand, the GH4 Asp α_1–G18 Arg β_1 and the B12 α_1–GH4 Glu β_1 ionic bonds in the α_1-β_1–contact region need not necessarily be disrupted by these structure changes. The long side chains of these acidic and basic amino acids should be able to bridge the widening gap between the α_1- and β_1-chains. This again indicates the possible importance of ionic groups in subunit association.

On the other hand, from crystallographic work on hemoglobin H during uptake of oxygen, it appears that this protein has a similar structure both in the oxygen carrying and in the deoxygenated form. As mentioned before, this seems to be an open structure similar to that of normal human reduced hemoglobin. Also, its oxygen equilibrium curve does not show any heme-heme interaction. Whatever the detailed

[1] At site C5 of hemoglobin-H the basic Lys residue is replaced by Gln but the adjacent site C6 is occupied by Arg which might possibly form the salt link instead of Lys-C5.

mechanism of these interactions may be, the comparison between hemoglobin H and normal mammalian hemoglobins indicates that the theory of Monod, Wyman, and Changeux (5) might need some slight modification. According to this theory, it is the fully symmetrical hemoglobin H tetramer which should show cooperative effects, but such effects are not observed. It would seem that it is just the inequality of the α- and β-chains which confers homotropic interactions upon the normal mammalian hemoglobins. It would therefore become necessary to introduce as basic unit (protomer) an α-β–dimer. This unit would have to carry two stereospecific reactive sites contrary to one reactive site per protomer as postulated by the above mentioned authors. The only gross structural difference between the α- and the β-chains of hemoglobin lies in the region of helices C and D and the connecting section CD. It would be tempting to ascribe the cooperative effects to the structural differences of these regions. However, the detailed mechanism of heme-heme interactions is too complex to be investigated by crude model studies such as the ones described here.

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REFERENCES

1. CULLIS, F., H. MUIRHEAD, M. F. PERUTZ, M. G. ROSSMANN, and A. C. T. NORTH. 1962. *Proc. Roy. Soc. (London), Ser. A.* **265**:161.
2. KENDREW, J. C., H. C. WATSON, B. E. STRANDBERG, R. E. DICKERSON, D. C. PHILLIPS, and V. C. SHORE. 1961. *Nature.* **190**:663.
3. PERUTZ, M. F. 1965. *J. Mol. Biol.* **13**:646.
4. PERUTZ, M. F., J. C. KENDREW, and H. C. WATSON. 1965. *J. Mol. Biol.* **13**:66º
5. MONOD, J., J. WYMAN, and J. P. CHANGEUX. 1965. *J. Mol. Biol.* **12**:88.
6. MUIRHEAD, H., and M. F. PERUTZ. 1963. *Nature.* **199**:633.
7. PERUTZ, M. F., W. BOLTON, R. DIAMOND, H. MUIRHEAD, and H. C. WATSON. 1964. *Nature.* **203**: 687.
8. MATSUDA, G., R. GEHRING-MÜLLER, and G. BRAUNITZER. 1963. *Biochem. Z.* **338**:669.
9. SMITH, D. B. 1964. *Can. J. Biochem.* **42**:755.
10. GUZZO, A. 1965. *Biophys. J.* **5**:809.
11. FANELLI, A. R., E. ANTONINI, and A. CAPUTO. 1964. *Advan. Protein Chem.* **19**:73.
12. PERUTZ, M. F., and L. MAZZARELLA. 1963. *Nature* **199**:639.
13. BRAUNITZER, G. 1966. *J. Cellular Comp. Physiol.* **67** (suppl. 1):1.
14. YAMAGUCHI, Y., H. HORIE, A. MATSUO, SH. SASAKAIRO, and K. SATONE. 1965. *J. Biochem. (Tokyo)* **58**:186.
15. BRAUNITZER, G., and H. KOHLER. 1966. *Z. Physiol. Chem.* **343**:290.
16. POPP, R. A. 1965. *Fed. Proc.* **24**:1252.
17. WYMAN, J., JR. 1964. *Advances Protein Chem.* **19**:223.