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A STUDY ON THE QUATERNARY STRUCTURE CHANGE OF HEMOGLOBIN I. INTERMEDIATE STRUCTURES IN O₂ LIGATION PROCESS

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A STUDY ON THE QUATERNARY STRUCTURE CHANGE OF HEMOGLOBIN I. INTERMEDIATE STRUCTURES IN O₂ LIGATION PROCESS

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SUMMARY

Conformational interaction at the interfaces between subunits are investigated on the basis of the atomic coordinates of human deoxy and human oxy Hbs. Hypothetical intermediate structures are used, each of which is obtained from the procedure where one or more subunits in deoxy Hb are replaced by the corresponding O₂-liganded subunits in oxy Hb using the method of superimposition of two sets of atomic coordinates. When either α or β subunit is substituted with the corresponding subunit in oxy Hb, serious steric hindrances are produced between α_1 FG 4(92)Arg and β_2 C 3(37)Trp or between α_1 C 6(41)Thr and β_2 FG 4(97)His, all of which belong to the allosteric core affected directly by ligand binding. These steric hindrances become more serious when both $\alpha_1(\alpha_2)$ and $\beta_2(\beta_1)$ subunits are substituted. However, no steric hindrance can be seen between subunits when the subunits in oxy Hb are substituted with the corresponding subunits in deoxy Hb.

INTRODUCTION

In order to obtain knowledge about the coupling between tertiary and quaternary alteration, conformational studies are carried out by Arata et al.⁽¹⁾ from the point of subunit interface interaction, which is important for the understanding of the cooperative ligand binding of Hb. In their work, hypothetical intermediate structures are used, each of which is obtained by replacing one or more subunits in deoxy Hb with corresponding subunits having liganded tertiary structures in CO-Hb. Examinations concerning the appearance of steric hindrance between the subunits in these postulated structures are undertaken. When steric hindrance is found to occur at any pair of amino residues, other conformation of these residues is further examined by varying their internal rotation angles. Whether steric hindrance is avoidable or not is judged on the bases of the torsional energy of these residues and interaction energy of these residues with the other residues surrounding them. The quaternary structure of carbonmonoxy Hb is also investigated with the successive replacement of its subunits by the subunit with non-liganded tertiary structure in deoxy Hb. In addition to the stereo-chemical study of the hypothetical intermediate structures,

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calculations regarding interaction energy between subunits of the non-liganded (deoxy) Hb and carbonmonoxy Hb are also carried out with the same method as in the paper by Arata and Otsuka⁽²⁾. The calculation results are used as the criterion for judging the stability of the intermediate structures.

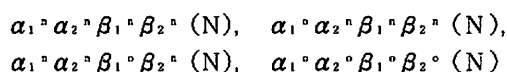
In the present work, atomic distances between subunits are calculated for the hypothetical intermediate structures defined in the previous paper⁽¹⁾ using the atomic coordinates of human O₂-Hb⁽³⁾ and deoxy Hb⁽⁴⁾ in order to see the differences between CO-Hb and O₂-Hb.

The atomic coordinates of human deoxy Hb⁽⁴⁾ and human oxy Hb⁽³⁾ used in the present calculation have been obtained through the sub-center of PDB⁽⁵⁾ in Japan (Institute for Protein Research, Osaka University).

METHOD

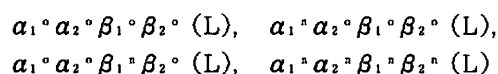
It is first assumed that the tertiary structure of each subunit is mainly determined by the state of heme; i.e., whether it is liganded or not. Accordingly, the authors regard the hypothetical intermediate structures as one in which one or more subunits in non-liganded (deoxy) Hb are replaced by the corresponding subunit(s) with O₂-liganded tertiary structure in O₂-Hb. All structures considered within the non-liganded quaternary structure are as follows.

Non-liganded Quaternary Structure:



The capital letter N in parentheses represents the non-liganded quaternary structure, and the superscript letters n and o represent the non-liganded and O₂-liganded tertiary structures, respectively. The first structure $\alpha_1^n \alpha_2^n \beta_1^n \beta_2^n (N)$ represents that of the non-liganded Hb determined by X-ray diffraction analyses. In the same way, we also consider the following structures within the quaternary structure of O₂-Hb.

Liganded Quaternary Structure :



Except for the structure $\alpha_1^o \alpha_2^o \beta_1^o \beta_2^o (L)$, which is the structure of O₂-Hb, the structures are obtained by the replacement of subunits in O₂-Hb with the corresponding ones in the non-liganded Hb.

(A) Procedure for Replacement of Subunits

In order to obtain the intermediate structures listed above, two sets of atomic coordinates of O₂-liganded and non-liganded subunits must be compared and made to coincide as closely as possible. The procedure for replacement of subunits is the same as the one in the previous

paper⁽¹⁾.

Our preliminary study reveals that only some special amino residues differ substantially in their positions when the corresponding subunits in O₂-liganded and non-liganded Hbs are superimposed. They are 1Val, 139Lys, 140Tyr and 141Arg in the α subunit, and 144Lys, 145Tyr and 146His in the β subunit. In the non-liganded Hb, these amino residues of N- and C-termini participate in the formation of salt-bridges, while in the fully liganded Hb, the bridges are broken and each of the C-terminal residues rotates freely⁽³⁾. The influence of the drastic changes in the termini mentioned above may extend to other residues in HC and NA regions. Therefore, these residues are excluded when superimposing the atomic coordinates of non-liganded and liganded subunits. For all atoms of the remaining amino residues as well as those of the heme, the corresponding subunits of non-liganded Hb and O₂-Hb can be superimposed to the extent that the value of root mean square difference is 0.7 Å for α subunit and 0.8 Å for β subunit respectively.

(B) Steric Hindrance

For each of the hypothetical intermediate structures, investigations are undertaken as to whether any steric hindrance appears between the subunit or not. The checking for steric

TABLE I
EMPIRICAL LOWER LIMITS FOR NONBONDED CONTACT DISTANCES

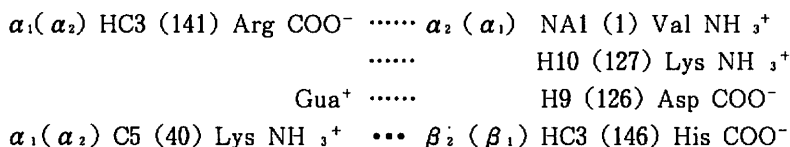
The values of "lower extreme limit" for nonbonded contact distance, which is used for each of steric hindrance between atoms, are shown for each pair of atoms. Those values of "lower normal limit" are also given in the parentheses. The values are given in units of angstrom.

	C	N	O
C	2.9 (3.0)	2.8 (2.9)	2.7 (2.8)
N		2.6 (2.7)	2.6 (2.7)
O			2.6 (2.7)

hindrance is first carried out according to the criterion of the "lower extreme limit" defined empirically for van der Waals contact distance by Ramachandran and Sasisekharan⁽⁶⁾. The values of "lower extreme limit" are listed in Table I as well as those of the "lower normal limit" as defined by the authors mentioned above. The differences between the values of "lower normal limit" and "lower extreme limit" are only 0.1 Å. The nonbonded atomic pairs with the distance of "lower extreme limit" have positive van der Waals interaction energy of less than 0.5kcal/mol by the estimation with the "6-12" potential function⁽⁷⁾. The slight repulsive interaction can be made to disappear by small deviations in bond and torsion angles as well as in bond length⁽⁸⁾. For our purpose, it is assumed to be unnecessary to consider such a small difference in atomic position.

RESULT

First, the main features of subunit interface contacts of the non-liganded and fully O₂-liganded Hb are summarized. The electrostatic energy arising from partial charges of amino residues is not a dominant factor when considering the difference between subunit interaction of the two types of Hb. Such a result obtained from the present calculations is also the same for horse deoxy and oxy Hbs⁽²⁾, and for human deoxy Hb and CO-Hb⁽¹⁾. The van der Waals interaction energy calculated with the same method as in the previous paper⁽²⁾ are listed in Table II for every pair of segments in the $\alpha_1\alpha_2$, $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\beta_1\beta_2$ interfaces. Although each energy value is somewhat different from that of the previous results on horse deoxy and oxy Hbs or human deoxy Hb and CO-Hb, the main features are almost analogous. In O₂-Hb, van der Waals interaction is diminished in the interface between α_1 and β_2 subunits while this interaction increases in $\alpha_1\alpha_2$, $\alpha_1\beta_1$ and $\beta_1\beta_2$ interfaces. With the increase in the van der Waals interaction in these pairs of subunits, the following salt-bridges which forms in the non-liganded Hb are ruptured in the O₂-Hb.



Furthermore, in β subunit, N⁺ atom of HC3(146)His is also in a short enough distance from COO⁻ of FG1(94)Asp to be able to form a salt-bridge between them. According to the results of superimposition of each subunit in O₂-Hb to the corresponding subunit in the non-liganded Hb, the rupture of these salt-bridges is attributable to the large displacement of the C-terminal residues ; 141Arg in α subunit and 146His in β subunit, whose atomic positions are different by 5Å or more between the non-liganded and fully O₂-liganded Hbs.

(A) Intermediate Structures within Non-liganded Quaternary Structure

The distinguishing feature of intermediate structures within the non-liganded quaternary structure is that unavoidable steric hindrance occurs between $\alpha_1(\alpha_2)$ and $\beta_2(\beta_1)$ subunits when the subunits in non-liganded (deoxy) Hb are replaced by those subunits having liganded tertiary structure.

(1) $\alpha_1 \circ \alpha_2 \circ \beta_1 \circ \beta_2 \circ$ (N)

A remarkable feature seen in this intermediate structure is that side chain of α_1 FG4(92)Arg have contacts with main chains of C2(36)Pro, C3(37)Trp and C5(39)Gln of β_2 subunit within the distance of "lower extreme limit" as shown in Table III. This is due to the large deviations of the side chain atoms of FG4(92)Arg in O₂-Hb by 2Å~4Å from those in non-liganded α_1 subunit when they are superimposed on each other. The FG region including the FG4(92)Arg has been noted as one where a relatively large change is indicated

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TABLE II

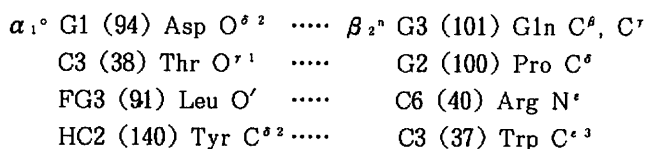
VAN DER WAALS INTERACTION ENERGY BETWEEN SUBUNITS OF HUMAN O₂-Hb AND HUMAN DEOXY Hb

The upper and lower value for each segment pair correspond to the one for O₂-Hb and DeoxyHb, respectively. The energy values are given in units of Kcal/mol.

(a) $\alpha_1 \alpha_2$ contact				(b) $\alpha_1 \beta_1$ contact						
NA	G	H	HC	$\beta \rightarrow B$	C	D	G	GH	H	
		-1.8	-7.0	B			-0.1	-2.2	-27.6	
		-0.3	-0.3				0.0	-1.6	-22.1	
A			-0.3	C			-0.1		-1.7	
			0.0				0.0		-1.3	
G	-0.1			G			-16.5	-5.3	-4.1	
	0.0						-14.0	-2.6	-4.0	
H		-0.1	-8.1	GH	-2.7			-6.9		
		0.0	-8.7		-2.9			-6.7		
HC			-0.3	H	-19.6	-1.2	-2.7	-1.4		
			0.0		-17.9	-1.9	-2.1	-1.6		
(c) $\beta_1 \beta_2$ contact				(d) $\alpha_1 \beta_2$ contact						
NA	H	HC		$\beta \rightarrow B$	C	CD	F	FG	G	HC
		-0.5		C	-6.0		0.0	-4.2	-1.1	-0.8
		0.0			-3.5		-0.2	-4.5	-6.3	-6.3
EF	-0.2			CD	-0.1			0.0		
	0.0				0.0			-2.6		
H		-0.9		F	0.0					
		0.0			-0.1					
				FG	-12.4	-0.1				
					-13.7	-0.9				
				G	-7.3				-4.7	
					-3.9				-8.4	
				HC	-0.0	-6.8				
					-2.4	-10.2				

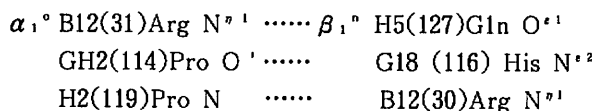
from the comparison of tertiary structures of non-liganded and fully CO-liganded Hbs⁽⁹⁾. The arginine indicated here is the nearest neighbor of FG5Val which is suggested to have the possibility of being pushed away by a change of the orientation of heme plane due to ligation⁽¹⁰⁾. The extremely close contacts between α_1 FG4(92)Arg and some residues of β_2 subunit listed in Table III, however, may not be a serious steric hindrance when considering the stability of the intermediate structure, as well as those steric hindrances in the previous paper⁽¹¹⁾.

The other atomic pairs which are within the distance of "lower extreme limit" are recognized in other area of the interface between α_1 and β_2 subunits. They are



The close contacts α HC2(140)Tyr and β C3(37)Trp, and α C3(38)Thr and β G2(100)Pro are not found in the previous work when atomic coordinates of CO-Hb are used as a liganded structure⁽¹¹⁾. These extremely close contacts may be easily avoided by slight modification of the respective internal rotation angles. Consequently, they are not important when considering steric hindrance between subunits in this intermediate structure.

Between the α_1 and β_1 subunits, atomic pairs within the distance of "lower extreme limit" are



These pairs are within a short distance even in the non-liganded Hb. The extremely close contacts indicated above may be also avoided by slight changes of the respective internal rotation angles.

Between α_1 and α_2 subunits in this $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N) structure, α_1 HC3(141)Arg is far from α_2 H9(126)Asp and α_2 H10(127)Lys. However, such a result does not necessarily mean that the salt-bridges of α_1 141Arg with α_2 126Asp and α_2 127Lys are broken by the conformational change of 141Arg upon the ligation to the α_1 subunit. According to the results of superimposition of atomic coordinates of the subunit in O₂-Hb to the corresponding subunit in non-liganded Hb, differences between the two atomic coordinates for all atoms of 126Asp and 127Lys are less than 2Å, while those differences for 141Arg is more than 4Å. Concerning these contacts, detailed discussions are given in section (B) of the previous paper⁽¹¹⁾. Other close contact in this structure is made between the atom O^γ of α_1° HC3(141)Arg and N of α_2° NA1(1)Val to be able to form salt-bridges between them, while no close contact is found between α_1° 1Val and α_2° 141Arg because of large deviation of α_1° 1Val in the O₂-liganded tertiary structure from that in the non-liganded one.

(2) $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N)

No atomic pair within the distance of "lower extreme limit" is found between the subunits α_2° and β_2° and between the β_1° and β_2° . On the other hand, β_2° FG4 (97) His is close to α_1° C6(41)Thr and four atomic pairs are found within the distance of "lower extreme limit". These are shown in Table IV. Since these atomic pairs occur between the main chain atoms of the respective residues, it is impossible to avoid the extremely close contacts without any conformational change of some amino residues in the non-helical FG region. Although the number of amino residues is somewhat different between α and β subunit, FG4(97)His in the β subunit occupies the position similar to that of FG4(92)Arg in the α subunit in the sense that they are in the vicinity of F8His which is liganded to the heme iron. Furthermore, the C region of C6(41)Thr is suggested to be affected by FG5Val upon ligation⁽¹⁰⁾. There is a probability that these extremely close contacts (steric hindrances) may be attributable to the direct effect of atomic displacement due to ligation. Therefore, when a β subunit is ligated strain energy may be stored in the non-helical FG region.

(3) $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N)

In the $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N) structure, the pairs of atoms within the distance of "lower extreme limit" are concentrated on the interface between α_1 and β_2 subunits. They are listed in Table V. Most of the atomic pairs appear between α_1 and β_2 subunits in either the structure $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N) or $\alpha_1^{\circ}\alpha_2^{\circ}\beta_1^{\circ}\beta_2^{\circ}$ (N). The distaces of those pairs are shortened in the structure which is examined in this section. Unavoidable steric hindrances are present between main chain atoms in pairs of residues, α_1 41Thr and β_2 97His, and α_1 92Arg and β_2 37Trp. The steric hindrances between the former pair of residues are recognized in $\alpha_1^{\circ}\beta_2^{\circ}$, while those between the latter pair appear only in the $\alpha_1^{\circ}\beta_2^{\circ}$. This is due to the fact that the atoms of α_1° 92Arg are shifted to the residues 36Pro and 37Trp of β_2° subunit in comparison with the corresponding arginine in α_1° subunit and that the main chain atoms of those residues in the β° subunit are deviated from those of the correspoding subunit in non-liganded Hb. The difference of the atomic coordinates of those residues in β subunit ranges from 0.6Å to 1.0Å, and is a relatively large deviation for a main chain atom.

No atomic pair within the distance of "lower extreme limit" is found between the subunits α_1° and β_1° . The essential difference of atomic contacts between α_1° and β_1° and that of α_1° and β_1° is not noticeable.

Between α_1° and α_2° subunits, no atomic pair within the distance of "lower extreme limit" appears and atoms forming the salt-bridges in non-liganded Hb separate from each other. Also α_2° 1ValNH₃⁺ separates from α_1° 141 ArgCOO⁻, although they are close to each other between the subunits α_1° and α_2° . No atomic pair within the distance of "lower extreme limit" is found between the β_1° and β_2° as well as between α_1° and α_2° .

(B) Intermediate Structures within Liganded Quaternary Structure

TABLE III

ATOMIC PAIRS WITHIN THE DISTANCE OF "LOWER EXTREME LIMIT" IN RELATION TO α FG4(92)Arg BETWEEN THE SUBUNITS α_1° AND β_2° WITHIN NON-LIGANDED QUATERNARY STRUCTURE

α_1°	β_2°	
FG4 (92) Arg C2 (36) Pro	$N^{\epsilon}-C^{\gamma}, N^{\epsilon}-O^{\gamma}, C^{\epsilon}-O^{\gamma}$
 C3 (37) Trp	$C^{\beta}-O^{\gamma}, C^{\gamma}-(C^{\alpha}, O^{\gamma})$
		$C^{\beta}-(C^{\alpha}, C^{\gamma}, C^{\beta}), N^{\epsilon}-C^{\alpha}$
 C5 (39) Gln	$N^{\eta 1}-C^{\beta}$

TABLE IV

ATOMIC PAIRS WITHIN THE DISTANCE OF "LOWER EXTREME LIMIT" BETWEEN THE SUBUNITS α_1° AND α_2° WITHIN NON-LIGANDED QUATERNARY STRUCTURE

α_1°	β_2°	
C6 (41) Thr FG4 (97) His	$O^{\gamma}-C^{\beta}, C^{\beta}-(O^{\gamma}, C^{\gamma}, C^{\beta})$

TABLE V

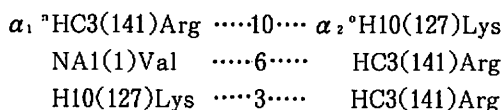
ATOMIC PAIRS WITHIN THE DISTANCE OF "LOWER EXTREME LIMIT" BETWEEN THE SUBUNITS α_1° AND β_2° WITHIN NON-LIGANDED QUATERNARY STRUCTURE

α_1°	β_2°	
C3 (38) Thr G2 (100) Pro	$O^{\gamma 1}-C^{\gamma}, C^{\gamma 2}-O^{\beta 1}$
C6 (41) Thr FG4 (97) His	$C^{\beta}-(O^{\gamma}, C^{\beta})$
	$O^{\gamma 1}-(C^{\alpha}, C^{\gamma}, O^{\gamma}, C^{\beta}), C^{\gamma 2}-O^{\gamma}$
FG4 (92)Arg C2 (36) Pro	$C^{\beta}-C^{\gamma}, N^{\epsilon}-(C^{\gamma}, O^{\gamma}), C^{\epsilon}-(C^{\gamma}, O^{\gamma})$
		$N^{\eta 1}-O^{\gamma}, N^{\eta 2}-O^{\gamma}$
 C3 (37) Trp	$C^{\alpha}-C^{\beta}, C^{\beta}-(C^{\alpha}, C^{\beta}), C^{\gamma}-(C^{\alpha}, C^{\gamma}, O^{\gamma}, C^{\beta})$
		$C^{\beta}-(N, C^{\alpha}, C^{\gamma}, C^{\beta}), N^{\epsilon}-N, N^{\epsilon}-C^{\alpha}$

In the quaternary structure of O₂-Hb the space between the α_1 and α_2 subunits is narrowed, and the C-terminal amino residue HC3(141)Arg of α subunit is pushed out, while in the non-liganded Hb, that amino residue forms salt-bridges with the residues H9(126)Asp and H10(127)Lys of the other α subunit. Because of the large conformational difference between the C-terminal residue HC3(141)Arg in liganded and that in non-liganded α subunit, the arginine is found in most of the atomic pairs within the distance of "lower extreme limit" in the intermediate structures within the liganded quaternary structure. When the α subunit of O₂-Hb is substituted with the corresponding subunit of non-liganded Hb, one way to avoid the extremely close contacts in relation to the arginine is to change the relative position of the two α subunit in order to make sufficient space between them⁽⁹⁾. In contrast to the non-liganded quaternary structure, except for α_1 - α_2 , no extremely close contacts between main chain atoms are found in the intermediate structures within the liganded quaternary structure.

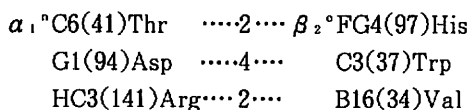
(1) $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (L)

Between the α_1° and α_2° subunits, the atoms within the distance of "lower extreme limit" are found in the following pairs of amino residues.



The number of atomic pairs within the distance of "lower extreme limit" is indicated on the dotted line. Several atoms in the main chain of α_1 HC3(141)Arg are participants in the extremely close contacts between the arginine and H10(127)Lys. The contacts between α_1 NA1(1)Val and α_2 HC3(141)Arg and between α_1 H10(127)Lys and α_2 HC3(141)Arg are not found when atomic coordinates of CO-Hb are used as a liganded structure.

Between the α_1° and β_2° subunits, atomic pairs within the distance of "lower extreme limit" are found between the following residues.

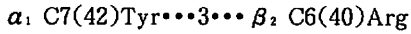


These close contacts may be easily avoided by slight change of internal rotation angles of side chains of the participants.

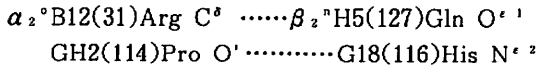
No atom within the distance of "lower extreme limit" is found between the α_1° and β_1° subunits.

(2) $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (L)

No atom is found within the distance of "lower extreme limit" between the two β subunits, when one of the β subunits is replaced by the corresponding subunit having non-liganded tertiary structure. Between the α_1° and β_2° subunits, the following pairs of atoms are within the distance of "lower extreme limit"



Between the α_2° and β_2° subunits, the following two pairs of atoms are within the distance of "lower extreme limit"



They are also found as the atomic pairs making extremely close contacts in the structure $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (N).

(3) $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (L)

Between the α_1° and α_2° subunits, $\alpha_1(\alpha_2)$ 141Arg comes close to 127Lys of $\alpha_2(\alpha_1)$ subunits within the distance of "lower extreme limit". The feature of the extremely close contacts associated with these amino residues is essentially the same as those contacts found in the structure $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (L). This is because there is little difference of atomic positions of 127Lys between the liganded and non-liganded tertiary structures. In the interface between the subunits α_1° and β_2° , three pairs of amino residues, α_1 141 Arg and β_2 34 Val, α_1 94 Asp and β_2 37 Trp, and α_1 C7(42)Tyr and β_2 C6(40)Arg make extremely close contacts within the distance of "lower extreme limit"; their features are also essentially the same as those in the structure $\alpha_1^{\circ} \alpha_2^{\circ} \beta_1^{\circ} \beta_2^{\circ}$ (L). Between the subunits α_1° and β_1° , no atom within the distance of "lower extreme limit" is found.

DISCUSSION

The purpose of this work is to see the influence of the inaccuracy of atomic coordinates on the conclusion obtained from the results calculated with the atomic coordinates of CO-Hb and deoxy Hb in the previous paper⁽¹⁾. In the present work, in order to see the differences between both results obtained with the atomic coordinates of human CO-Hb and human O₂-Hb, the atomic coordinates of O₂-Hb and deoxy Hb are used in the calculation.

The calculation results of both cases are almost the same as a whole, although few differences are found in the atomic distances and in the number of atomic pairs within the distance of "lower extreme limit" between subunits.

In the previous paper⁽¹⁾, steric hindrances between $\alpha_1(2)$ FG4(92)Arg and $\beta_2(1)$ C3(37) Trp, and between $\beta_1(2)$ FG4(97)His and $\alpha_2(1)$ C6(41)Thr are indicated to be especially important when considering the coupling between the tertiary and quaternary structure changes underlying the cooperative ligand binding in hemoglobin. These steric hindrances also appear in the present results and seem to be important as suggested in the previous paper. In the present results, the above steric hindrance between α_1 FG4(92)Arg and β_2 C3(37)Trp is more serious than that in the previous paper⁽¹⁾. The steric hindrance between α_1 C6(41)Thr and β_2 FG4(97)His is weakened in the present results than that in the previous paper⁽¹⁾.

However, the differences found between the results in the present work and those results in the previous paper is not so large to explode the conclusions offered in the previous paper.

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