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Evaluation of the (haem)Fe—$N^{\varepsilon 2}$(HisF8) bond distances from haemoglobin structures deposited in the Protein Data Bank

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Scientists working on the structure and function of proteins use haemoglobin as a model of allosteric proteins. In this molecule, the (haem)Fe—$N^{\varepsilon 2}$(His) bond is one of the most significant because the length of this bond provides relevant information about the mechanism of cooperativity and affinity for O_2. Thus, the aim of the present study was to evaluate the quality of the structural models of Hb deposited in the Protein Data Bank (PDB), in particular the reliability of the Fe—$N^{\varepsilon 2}$ bond distance. To achieve this, 329 Hb structures solved by X-ray diffraction were downloaded from the PDB. The Fe—$N^{\varepsilon 2}$ bond distance was computed and compared with the ideal value determined using the spectroscopic techniques of X-ray absorption and EXAFS. This investigation showed the presence of crystallographic structures of native haemoglobins deposited in the PDB in which the Fe—$N^{\varepsilon 2}$ bond distance was far beyond the ideal value found for this length, a fact that makes their use in studies that correlate haemoglobin structure and function questionable.

1. Introduction

In recent decades, rapid progress has been made in crystallographic techniques for X-ray diffraction data collection from crystals of biological macromolecules, as well as in the development of computer programs for X-ray data processing and structure determination. Such progress has enabled the rapid conversion of X-ray diffraction data captured on an image plate to a three-dimensional virtual structure. The most evident consequence of such progress is the large number of macromolecular structures that have been deposited in the Protein Data Bank (PDB; Bernstein *et al.*, 1977; Berman *et al.*, 2000).

The validation of archives containing the spatial coordinates of macromolecules to be deposited in the PDB follows a standard procedure set by the specific site. In such a procedure, the bond lengths and angles of the atoms that comprise the macromolecule are calculated and compared with ideal values pre-established in the data bank of validation software such as *PROCHECK* (Laskowski *et al.*, 1993). Such software evaluates the possible structural errors in angle, planarity and bond distances. However, owing to the lack of certain information in their topology dictionaries, those programs do not validate bond parameters between apoproteins and their prosthetic groups, such as, for example, the bond distance between the globin and the haem group in the haemoglobin molecule.

The PDB has a large number of crystallographic structures of haemoglobin (Hb) in its collection from many sources

including genetically modified organisms, animals and vegetables.

Despite the increasing number of Hb structures that have been deposited, there are still unresolved issues regarding the molecular basis of the cooperativity phenomenon in Hb (Barrick *et al.*, 1997; Perutz *et al.*, 1998). Understanding of this phenomenon necessarily depends on the correlation between functional properties and structural alterations observed in the crystallographic structures of Hb species.

The discussion of the molecular mechanisms of cooperativity in Hb still divides the opinions of researchers even four decades after the resolution of the first crystallographic structure of Hb by Perutz in the 1970s (Perutz, 1970; Colombo & Seixas, 1999; Yonetani *et al.*, 2002; Goldbeck *et al.*, 2004; Ackers *et al.*, 2004; Nagatomo *et al.*, 2005; Kavanaugh *et al.*, 2005).

It is known that well solved crystallographic structures may help in understanding the molecular mechanisms involved in the cooperativity process and in the affinity of the protein for the ligand. It is also known that many proteins reflect the changes generated in crystallization conditions in their crystallographic structures (Silva *et al.*, 1992) or show experimental artefacts in Hb, changing intramolecular or intermolecular bonds of the crystallized protein (Wilson *et al.*, 1996). The haemoglobin crystal structures deposited in the PDB provide an excellent system to investigate the mechanism that controls the protein reactivity within the haem group.

One of the most significant changes observed in the different species of Hb deposited in the PDB is the variation in the (haem)Fe—$N^{\varepsilon 2}$(HisF8) bond distance; in other words, the bond that joins the Fe atom of the haem group to the proximal histidine residue present in the globin chain. Scientific reports on the subject (Baldwin & Chothia, 1979; Gelin *et al.*, 1983; Sharonov *et al.*, 1989; Wu *et al.*, 2001) highlight the importance of the Fe—$N^{\varepsilon 2}$ bond in the cooperativity mechanism and in the affinity of haemoglobins for oxygen, as well as the importance of careful refinement of this bond (Jones, 1994; Liddington, 1994).

The aim of the present study was to evaluate the (haem)Fe—$N^{\varepsilon 2}$(HisF8) bond distances in the crystallographic structures of haemoglobins deposited in the PDB and to compare them with the ideal values for a bond of covalent character as previously established by scientific findings. The investigation was motivated by a need to evaluate the quality of Hb models deposited in the PDB, in relation to the Fe—$N^{\varepsilon 2}$ bond, refined using different software.

2. Materials and methods

The search for haemoglobin structures was performed at the Macromolecular Structure Database (MSD) using *MSDmotif* (Golovin *et al.*, 2005), which displayed 388 crystallographic structures containing the Fe—$N^{\varepsilon 2}$ bond. However, some of these structures did not conform to the haemoglobin pattern. Considering this fact and the necessity of identifying the experimental conditions of crystallization for each structure, a new search of the PDB was carried out, resulting in 373 files. The results of both searches were compared and 329 structures at several resolutions were found to be suitable. Of these, 106 files containing haemoglobin spatial coordinates with resolution better than 1.90 Å were downloaded and visualized using the *SPDBV* software (Guex & Peitsch, 1997). Furthermore, the haem pocket in these structures, the distances between the atoms (haem)Fe—$N^{\varepsilon 2}$(HisF8) and information about the *R* factor, the resolution, the software that was used in the refinement as well as the *B* factors of the related atoms were examined.

The distance values measured were compared with the theoretical ideal value, which was established according to the following criterion: the sum of the ideal distance of the (haem)Fe—$N^{\varepsilon 2}$(HisF8) bond from oxyhaemoglobin and deoxyhaemoglobin as determined by X-ray absorption spectroscopy, which is 1.99 Å (Eisenberger *et al.*, 1976), plus the average experimental error in the crystallographic structures, which is around 0.20 Å, as determined by the *SFCHECK* program (Vaguine *et al.*, 1999), plus the limit of 0.05 Å that represents the error established by the *PROCHECK* program, giving a value of 2.24 Å, which was used in our comparisons as a reference maximum distance for the (haem)Fe—$N^{\varepsilon 2}$(HisF8) bond. The decision to make use of structures with resolutions better than 1.90 Å was based on the assumption that distances over 1.99 Å are well defined at this resolution.

3. Results and discussion

The downloaded Hb structures with resolutions between 1.25 and 1.90 Å were analysed without taking into account the binding status of the haem group. The structures that presented Fe—$N^{\varepsilon 2}$ bond distances greater than 2.24 Å are listed in Tables 1 and 2.

Structural variations at the Hb quaternary and tertiary level depend on the status of the haem group. In the deoxygenated form (deoxyHb), the Fe atom moves out of the plane of the porphyrin ring towards HisF8 (proximal). When the deoxy form of haemoglobin binds oxygen, the Fe atom moves into the plane of the porphyrin ring, thus stretching the Fe—$N^{\varepsilon 2}$ bond (Perutz, 1970).

Of the 106 haemoglobin structures analysed, 21 (19.8%) presented one or more Fe—$N^{\varepsilon 2}$ bonds longer than 2.24 Å. Only for six (5.7%) of these could a structural justification for the larger distance be inferred. Within this group were structures crystallized in the T-conformation (deoxyHb) that had been exposed to oxygen after the crystal had been obtained. Such a procedure made the intermolecular bonds, which stabilize the crystalline structure, arrest the protein in the quaternary T-conformation, a typical deoxy behaviour.

With exposure of the deoxyHb crystal to oxygen, the haem pocket changes to the oxy state, thus showing structural changes at the tertiary level towards the R-conformation, which is typical of oxyHb, although the quaternary structure remains tied in the T-conformation. This artifice is used in Hb studies to investigate the molecular mechanisms of cooperativity in oxygen binding. It is expected that the Fe—$N^{\varepsilon 2}$ bond

Table 1
Hb structures deposited in the PDB with an Fe—$N^{\varepsilon 2}$ bond distance greater than 2.24 Å with justification.

PDB code	Distance (Å)	Refinement program	Resolution (Å)	*R* factor
1ibe	2.28	*PROLSQ*	1.8	0.179
1o1o	2.28, 2.26	*SHELXL*97	1.8	0.180
1qsh	2.30, 2.27	*PROLSQ*	1.7	0.175
1thb	2.28, 2.25, 2.26	*PROLSQ*	1.5	0.196
1uiw	2.26, 2.27, 2.26, 2.29, 2.25, 2.28, 2.27	*REFMAC*5.1.22	1.5	0.172
1vwt	2.30, 2.29	*X-PLOR*3.851	1.9	0.169

Table 2
Hb structures deposited in the PDB with an Fe—$N^{\varepsilon 2}$ bond distance greater than 2.24 Å without justification.

PDB code	Distance (Å)	Refinement program	Resolution (Å)	*R* factor
1a3n	2.26, 2.36	*REFMAC*	1.8	0.171
1bz1	2.26	*PROLSQ*	1.59	0.170
1dxt	2.25, 2.25	*PROLSQ*	1.7	0.160
1dxv	2.25	*PROLSQ*	1.7	0.156
1fhj	2.40	*X-PLOR*	1.8	0.194
1flp	2.31	*ARP/wARP/TNT/X-PLOR*	1.5	0.170
1hbg	2.37	*PROLSQ*	1.5	0.146
1jf3	2.31	*X-PLOR*3.851	1.4	0.189
1jf4	2.27	*X-PLOR*3.851	1.4	0.189
1jl6	2.26	*X-PLOR*3.851	1.4	0.179
1qi8	2.35	*REFMAC*	1.8	0.169
1qpw	2.75, 2.85, 2.87, 2.88	*X-PLOR*3.1	1.8	0.207
1xq5	2.30	*REFMAC*5.2	1.9	0.243
1xz7	2.35	*REFMAC*5	1.9	0.173
2h8d	2.25, 2.26	*SHELXL*97	1.78	0.182

in these proteins is under strain, which might lead to lengthening of the bond (Liddington *et al.*, 1988; Paoli *et al.*, 1997).

Variations in this bond distance are also expected when the iron of the haem group is replaced by another metal (Miyazaki *et al.*, 1999).

On the other hand, in 15 (14.2%) of the 21 structures no apparent structural or experimental justification could be found for the longer bond length.

Fig. 1 shows the frequency distribution of Fe—$N^{\varepsilon 2}$ bond distances (above 2.24 Å) from the structures with and without experimental justification.

Fig. 1 also shows that the structures where there is no justification appear at higher distances with uniform frequency when compared with structures with justification, which are clustered at high frequency in an distance interval of 2.25–2.30 Å. Statistical details of the analysis are shown in Table 3.

The fact that there is a significant deviation from the average distance of the Fe—$N^{\varepsilon 2}$ bond in the justifiable and non-justifiable structures compared by t-test for independent samples, without any artifice arising from structural changes in the haem pocket, has led investigators to quote an average distance for the Fe—$N^{\varepsilon 2}$ bond appropriate for the value found in structures crystallized under conditions that lead to structural changes in this pocket. Table 4 shows the statistics of structures that present Fe—$N^{\varepsilon 2}$ bond distances within the parameters established. The comparison of the results shown in the two tables indicates a discrepancy in the distances from non-justifiable structures.

A similar analysis was also carried out on Hb structures at all resolutions (Table 5). The similarity between the means displayed in Table 3 seems to be influenced by the high *B* factors of the low-resolution structures. Nevertheless, considering only the structures with highest resolution (Table 3), the redundant data set on the basis of the resolution is no longer a significant factor in this type of analysis. Thus, the difference between Fe—$N^{\varepsilon 2}$ bond distances in justifiable and non-justifiable high-resolution structures (as shown in Table 3) remains significant.

The comparison between the results shown in Tables 3, 4 and 5 suggests that the resolution of the structure is not the main reason for the increase in Fe—$N^{\varepsilon 2}$ bond distance in non-justifiable structures.

Finding a justification for the fact that a covalent bond has a distance above 2.24 Å is difficult because when the bond between two atoms is stretched beyond the ideal value, it becomes unstable and tends to break (Lee, 2004). For 14.2% of the Hb structures deposited in the PDB and analysed in the present study, this distance is over 2.25 Å without any structural or experimental justification.

Determining the causes of such an increase in bond length is not the aim of the present investigation, because each protein structure has its own features. Nevertheless, it is possible to wonder about the probable causes of such an increase. Among the possible causes are the incorrect use of computer programs in the refinement; for example, the use of *X-PLOR* (Brünger, 1992) or *CNS* programs (Brünger *et al.*, 1998), which, despite having in their internal archives all the topology parameters for the haem group and for its binding status, do not have the bond distances that connect the Fe atom to the ligand and also to the protein (Fe—O_2 and Fe—$N^{\varepsilon 2}$, respectively), thus leading the program user to manually set the parameters for these bonds. In the language of those programs, such parameters are defined as 'patches'. If the patches are not inserted, the program will not consider them in

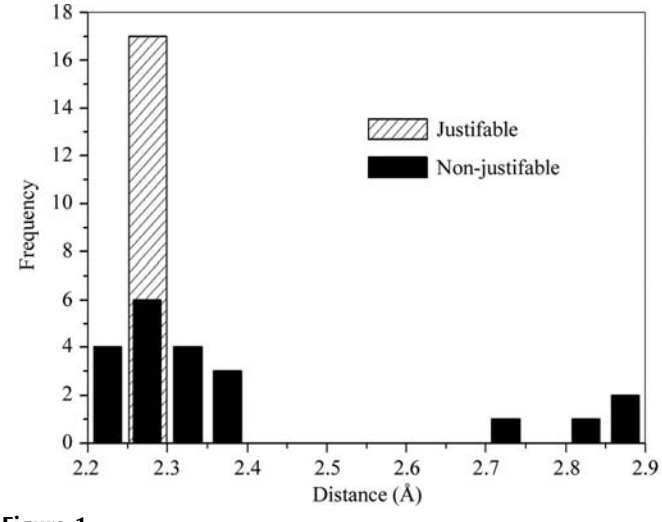

Figure 1
Frequency of Fe—$N^{\varepsilon 2}$ bond distances (above 2.24 Å) found in high-resolution Hb structures.

Table 3
Statistics of Fe—$N^{\varepsilon 2}$ bond distances (above 2.24 Å) in high-resolution Hb structures.

	n†	Mean‡ (Å)	S.d. (Å)	Min. (Å)	Max. (Å)	Median (Å)	Variance (Å)	Fe mean B factor (Å^2)	$N^{\varepsilon 2}$ mean B factor (Å^2)
Justifiable	17	2.274	0.0158	2.25	2.30	2.27	0.00025	13.236	12.329
Non-justifiable	21	2.400	0.2231	2.25	2.88	2.31	0.04976	17.959	16.699

† The asymmetric unit in the PDB file may contain more than one haem group. ‡ The two means are significantly different ($p < 0.05$).

Table 4
Statistics of Fe—$N^{\varepsilon 2}$ bond distances (less than 2.24 Å) in Hb structures with resolution from 1.25 to 1.80 Å.

	n†	Mean‡ (Å)	S.d. (Å)	Min. (Å)	Max. (Å)	Median (Å)	Variance (Å)	Fe mean B factor (Å^2)	$N^{\varepsilon 2}$ mean B factor (Å^2)
α chain	27	2.095	0.1065	1.78	2.24	2.09	0.01133	14.40	12.92
β chain	27	2.127	0.0737	1.98	2.23	2.13	0.00543	14.89	13.67

† Only Hb structures with α-type and β-type chains were considered. ‡ The two means are not significantly different ($p > 0.05$).

Table 5
Statistics of Fe—$N^{\varepsilon 2}$ bond distances (above 2.24 Å) from Hb structures at all resolutions.

	n†	Mean‡ (Å)	S.d. (Å)	Min. (Å)	Max. (Å)	Median (Å)	Variance (Å)	Fe mean B factor (Å^2)	$N^{\varepsilon 2}$ mean B factor (Å^2)
Justifiable	55	2.457	0.4794	2.25	2.60	2.30	0.22981	19.870	28.189
Non-justifiable	58	2.363	0.1768	2.25	2.88	2.31	0.03126	22.550	18.832

† The asymmetric unit in the PDB file may contain more than one haem group. ‡ The two means are not significantly different ($p > 0.05$).

Table 6
Fe—$N^{\varepsilon 2}$ distances (Å) from refinement experiments.

	Software used in simulation/PDB code				
	X-PLOR, 1kd2		*CNS*, 1hbb		
Chain	Distance without patch	Distance with patch	Distance without patch	Distance with patch	1hbb original file
A	2.66	2.12	2.55	2.12	2.20
B	2.65	2.10	2.30	2.11	2.21
C	2.62	2.17	2.48	2.12	2.20
D	2.67	2.12	2.39	2.11	2.21
Mean	2.65†	2.13†	2.43†	2.12†	2.21†
Deviation‡ (%)	18.3	—	8.5	—	—

† Mediumly significantly different from each other ($p < 0.01$). ‡ Percentage deviation from 2.24 Å.

the refinement process of the structure, which could explain the variation found in certain archives deposited in the PDB.

In other refinement programs, such as *REFMAC*5 (Murshudov *et al.*, 1997), the parameters of such bonds are not easily accessible to the user; however, the bond parameters are already set as defaults in the software.

Some experiments were performed in order to test the hypothesis that errors in the specification of parameters for Fe—$N^{\varepsilon 2}$ bonds in the structure-refinement programs may affect the final value for this bond. Electron density for deoxyHb was obtained (Seixas *et al.*, 1999) and the refinement of the Hb structure using the *X-PLOR* program performed with and without the insertion of patches.

When the patches were inserted into the script of the refinement program, the Fe—$N^{\varepsilon 2}$ bond distances were within the distance limit accepted in the present study, with an average value of 2.13 Å (see Table 6).

When the patches were not inserted into the script of the refinement program, the values of this bond distance were about 2.65 Å, thus exceeding the distance value of 2.24 Å by more than 18% (Table 6).

Some of the structures listed in Table 2 show bond distances that are close to the values found in the simulations carried out without the insertion of patches. For instance, the structure with PDB code 1qpw (Lu *et al.*, 2000) showed an average distance of 2.84 Å. According to the information provided in the PDB file of this structure, *X-PLOR* was the refinement program used by the authors.

Other low-resolution structures deposited in the PDB showed similar large bond distances. Examples are the structures with PDB identifiers 1hbs (Padlan & Love, 1985) and 1hds.

Several simulations were made in order to reinforce these findings. To achieve this, archives of Hb that provided structure factors and spatial coordinates were downloaded from the PDB. The simulation was about the spatial coordinates of human deoxyHb crystallized under low-salt conditions (Kavanaugh *et al.*, 1992) with PDB code 1hbb.

Two simulations from these archives were made by using the program *CNS*, in which the 1hbb structure of haemoglobin was once again refined. In the first simulation, the patches connecting the Fe—$N^{\varepsilon 2}$ bond were not set. The results showed that the average distance for this bond was 2.43 Å, thus exceeding the limit value adopted in the present investigation by more than 8.5% and also exceeding the average value of the distance found in the original archive by almost 10%. In the second simulation, the patches connecting the Fe—$N^{\varepsilon 2}$ bond were used. The results showed that the average distance of the bond was 2.12 Å, *i.e.* a value within the limits adopted in the present study. Details regarding the Fe—$N^{\varepsilon 2}$ bond found in the simulations are shown in Table 6.

In the test simulations, once patches were included as constraints the Fe—$N^{\varepsilon 2}$ distances moved closer to the ideal values, affecting the structure in two different ways. The proximal HisF8 residue as well as the F7 and F9 residues were

affected, a local effect that may be seen by calculating the r.m.s.d. of the main-chain atoms of the residues spread around F8 (Fig. 2) after superposing the pyrrolic N atom of haem-group structures with and without the strain.

The major differences in r.m.s.d. are found in the α chains, essentially in the A chain (0.171 Å). As revealed by *PROCHECK*, the local effect of strain does not modify the HisF8 geometry. The small deviation in the F8 geometry does not account for the variation of 0.43 Å found between A chains (Table 6).

The major effect of the strain seems to be distributed all along the structure; in the β chain, the r.m.s.d.s of the main-chain atoms of HisF8 are not above the r.m.s.d.s of the residues around F8 (Fig. 2). This is a consequence of the use of the patch, which stipulates the strain before positional refinement. No change in Fe position in relation to the plane of the porphyrin ring was observed between structures with and without strain.

Nevertheless, Fe—$N^{\varepsilon 2}$ bond distances above 2.50 Å may imply that such bonds are seriously weakened. Such an assumption is also confirmed by EXAFS experiments, which showed that the Fe—$N^{\varepsilon 2}$ bond distance in oxyHb is 1.98 ± 0.01 Å, whereas in deoxyHb it is 2.055 ± 0.01 Å (Eisenberger *et al.*, 1978; Perutz *et al.*, 1982). It is also important to point out that the oxidation state of the iron does not significantly affect the Fe—$N^{\varepsilon 2}$ bond distance (Korszun *et al.*, 1982), but it can affect the ligand (O_2 or CO) affinity. The strain on the Fe—$N^{\varepsilon 2}$ bond applied during refinement is based on the ideal parameters of angle and distance for this bond independent of the oxidation state of the haem.

The observations reported here suggest that some of the Hb structures deposited in the PDB might not have been obtained using appropriate constraints for the Fe—$N^{\varepsilon 2}$ bond, a fact that does not contribute to the understanding of the issues that correlate haemoglobin structure and function.

The results of the present investigation suggest that researchers working with crystallographic structure refinement of haemoglobins should pay special attention to the refinement of the Fe—$N^{\varepsilon 2}$ bond.

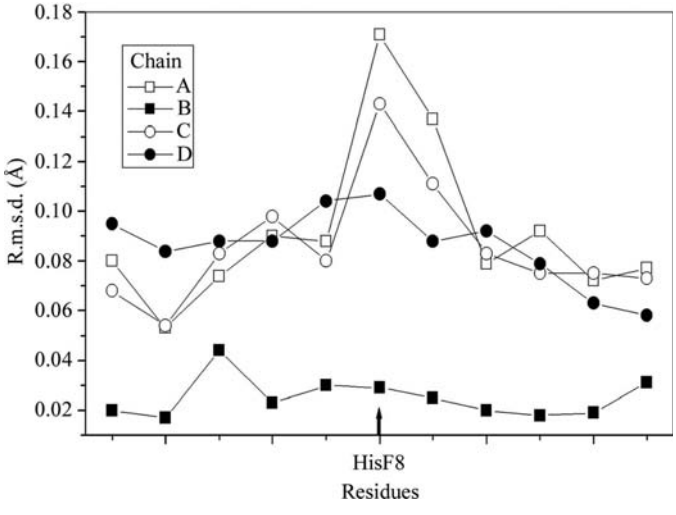

Figure 2
R.m.s.d. of the main-chain atoms of residues surrounding HisF8 calculated after the superimposition of pyrrolic N atoms of the haem group from structures with Fe—$N^{\varepsilon 2}$ strain patch, in the same reference frame as the structure without strain. Open symbols, α chains; filled symbols, β chains.

4. Conclusion

The Fe—$N^{\varepsilon 2}$ bond distance is of fundamental importance in studies focusing on the stereochemical mechanisms of co-operativity in haemoglobins, thus demanding precaution in the refinement of crystallographic structures of haemoglobins.

There are structures of hemoglobin in the native state deposited in the PDB in which the Fe—$N^{\varepsilon 2}$ bond distance is far from the ideal distance as observed by EXAFS and X-ray absorption spectroscopy experiments, even considering the experimental errors inherent to the crystallography techniques, a fact that makes the utilization of these structures questionable, especially in studies that correlate the structure and the function of haemoglobins.

The results of the present investigation suggest that regardless of the crystallographic structure, haemoglobin shows an Fe—$N^{\varepsilon 2}$ bond distance, with or without justification, that differs from the ideal values; thus, special care must be taken in the refinement of this bond since the bond is not evaluated by the structure-validation programs distributed with the usual program packages and also adopted by the PDB. The findings of this communication alert researchers about possible errors in their structural model.

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