EFFECT OF LIGAND AND HEME ON CONFORMATIONAL STABILITY (INTRAMOLECULAR CONFORMATIONAL MOTILITY) OF HEMOGLOBIN AS REVEALED BY HYDROGEN EXCHANGE

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1. Introduction

Recently published crystallographic data show that functionally important events are frequently accompanied by small, in absolute magnitude, but widespread perturbation of protein structure [1–5]. These conformational rearrangements are still hardly enlightened because they do not usually affect the content of regular structure, the fraction of solvent, exposed aromatic residues or the shape of molecule and therefore could not be detected by optical or hydrodynamic methods. To clarify the physical basis and the functional meaning of such subtle conformational changes it is necessary to know whether they lead to any changes in conformational energy of the protein.

To study this we have chosen two derivatives of Hb – MetHbH$_2$O and MetHbCN – as a model because recently the above-mentioned conformational differences between these two liganded Hbs have been well documented by a sensitive difference Fourier technique [5]. Hydrogen exchange was used to estimate the difference in conformational energy of Hb-derivatives because the conformational stability and intramolecular motility registered by this method seems to be governed by functionally-linked conformational energy [6].

The results presented here demonstrate that replacement of one ligand – H$_2$O – by another one – CN – markedly slows all of the observable exchange rates of peptide hydrogens and hence leads to increased conformational stability and decreased intramolecular motility of a considerable part of a protein molecule. This fact suggests that fine structural perturbations revealed by X-ray analysis of MetHbCN and MetHbH$_2$O as well as of other proteins might be one of the ways of conformational energy storage in a delocalized form [7], affecting the probability of conformational transitions under non-denaturing conditions. Comparison of the exchange behaviour of globin and Hb-derivatives shows that the amount of conformational energy gained by the globin moiety from binding of a sizable cofactor – heme – may be halved on subsequent binding of a small heme ligand. Such significant changes in the protein conformational energy upon ligation can markedly affect the observed binding energy of the ligand as such [8].

Abbreviations: Hb, hemoglobin A; HbO$_2$ and HbCO, different ligand states of ferrohemoglobin A; deoxy Hb, unliganded ferrohemoglobin A; MetHbH$_2$O, MetHbOH and MetHbCN, different ligand states of ferrihemoglobin A (MetHb)

2. Materials and methods

Stripped human HbO_2 and MetHb were obtained by standard procedures [9,10]. The rate of hydrogen–deuterium ($^1H \rightarrow {}^2H$) exchange in peptide NH-groups was monitored by infrared spectroscopy [6,11]. The protein concentration was usually ~ 1 mM, to eliminate the denaturation of MetHb at higher temperatures [12] the concentration of protein was raised to ~ 5 mM. The pH of all solutions in D_2O are reported as pH* without adding 0.4 pH-units to the pH-meter reading.

The exchange data are presented as the X and ratio A (amide II)/A (amide I) versus time of exchange. The ratio of peak intensities (A) of amide II and amide I bands is proportional to the fraction of unexchanged peptide hydrogens – X (proportionality coefficient $\omega = 1/0.5$). The deviation of parallel experimental curves from the mean in figs 1 and 2 does not exceed ±0.005 in units of the ratio A (amide II)/A (amide I).

3. Results and discussion

3.1. *Differences between MetHbCN and MetHbH$_2$O*

Data of figs 1 and 2 show that replacement of H_2O by CN decreases by 3–5-times the rate of $^1H \rightarrow {}^2H$ exchange of all 70% of peptide hydrogens in which exchange can be followed by our procedure.

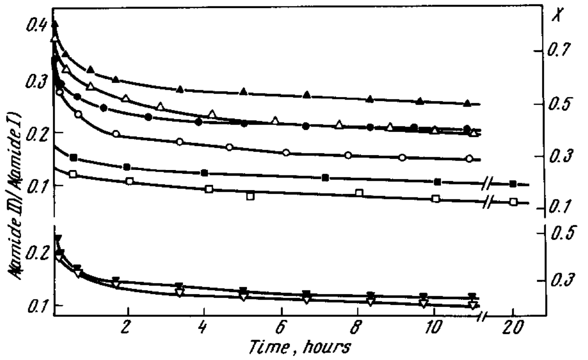

Fig.1. Hydrogen–deuterium exchange of the peptide hydrogens of MetHbCN (closed symbols) and MetHb (open symbols). pH* 5.1, 20°C, 0.05 M acetate buffer (▲△); pH* 6.4, 20°C, 0.05 M bis–Tris buffer (●○); pH* 7.1, 48°C, 0.1 M phosphate buffer (■□); pH* 9.0, 20°C, 0.05 M borate buffer (▼▽).

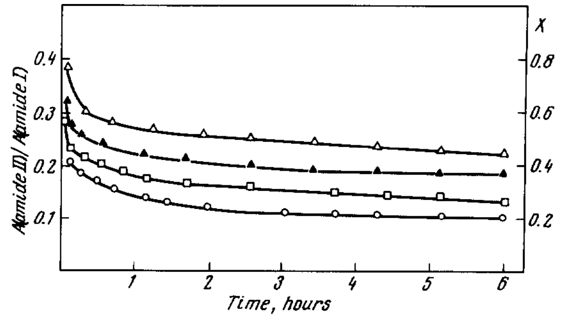

Fig.2. Hydrogen–deuterium exchange of the peptide hydrogens of deoxy-Hb (△), MetHbCN (▼), MetHb (□) and globin (○) at pH* 7.1, 20°C, 0.2 M bis–Tris buffer. The exchange in deoxy-Hb was studied in the presence of dithionite (2 mg/ml solution).

This concerns both the most labile hydrogens in which exchange is partly observable at pH* 5.1, 20°C and the hard-to-exchange hydrogens in which exchange is accessible to registration only at pH* 7.1 after raising the temperature. Such a marked difference in the exchange rate is observed only between the high-spin MetHbH$_2$O and low-spin MetHbCN. In alkaline medium at pH* 9.0 where the iron spin is lowered due to the replacement of H_2O by OH the retardation of exchange rate in MetHbCN as compared to MetHbOH becomes negligible (fig.1).

The observed change in the exchange rate of such a large fraction of peptide hydrogens upon replacement of H_2O by CN is in accord with the X-ray data. The cyanide Met – aquo Met difference Fourier map shows that ligand replacement results in a small motion of a great number of amino acid residues belonging to different helices and non-helical segments as well as in a linked displacement of some helical segments [5]. These widespread perturbations of tertiary structure inevitably accompanied by the distortions of dihedral angles may be supposed to cause the observed changes in exchange rate of many peptide hydrogens. Such conformational rearrangement may occur within the same secondary or quaternary protein structure as is the case with MetHb as well as in other cases [1–5]. It appears reasonable to assume that the similar structural perturbations are reflected in the observed alterations of the exchange rate on coenzyme binding [13], on oxidation of ferrocytochrome *c* [14], on formation of soybean or pancreatic trypsin inhibitor–trypsin complexes [15,16], on formation of inter-subunit contacts in ligand hemoglobin [6], on inhibitor binding to lysozyme [17], etc. [18–20].

The analysis of experimental curves (figs 1 and 2) in terms of exchange 2 mechanism [21] shows that upon replacement of H_2O by CN the free energy of local** (segmental) *trans*-conformations exposing internal peptide hydrogens to solvent, ΔG°_L, increases by 0.6–1 kcal/mol.

3.2. *Differences between globin and various derivatives of Hb*

Hydrogen exchange rates in globin and various derivatives of Hb are compared in fig.2. The exchange rate in MetHbCN at pH* 7.1 and 20°C is similar to that in HbO_2 [6] and HbCO. Data for globin were reported previously [6,12]. The difference between the $^1H \rightarrow {}^2H$ exchange rates in deoxy Hb and ligand Hb (except $MetHbH_2O$) is in a reasonable agreement with differences reported by others between the $^3H \rightarrow {}^1H$ exchange rates under similar experimental conditions [22,23]. Analysis of experimental curves indicates that attachment of non-ligand heme to globin (formation of deoxy-Hb) gives rise to the highest (more than 100-fold) retardation of the exchange, corresponding to an increase of ΔG°_L by 3 kcal/mol, i.e., about 2-fold. The binding of diatomic CO, O_2 or CN ligands by ferro- or ferri-heme of Hb affects the conformation of both heme and globin [5,24]. These conformational changes result in 5-fold acceleration of exchange with ΔG°_L decreasing by ~ 1 kcal/mol. Replacement of CN by H_2O also affects the conformations of heme and globin [5] with ΔG°_L decreasing by one additional kcal.

Thus in going from non-ligand high-spin ferroheme of deoxy-Hb to ligand high-spin ferriheme of $MetHbH_2O$ the conformational energy gained by the globin moiety from its interaction with the heme–ligand group is decreased more than twice. This finding reveals that the amount of conformational energy accumulated in protein due to interaction with a heme–ligand group is strongly dependent on the stereochemistry of the heme and on the pattern of the heme–ligand interactions with globin framework, varying significantly without destruction of the most of the numerous heme–protein contacts.

4. Conclusions

Our present and previous [6,12,25–28] data show that a large number of functionally important events are accompanied by the change in conformational stability and intramolecular motility of Hb, which are registered by hydrogen exchange. These events involve a binding of heme and low mol. wt ligands (see also ref. [22,23]) a formation of inter-subunit contacts, an ionisation of single protein groups and 'inactivation' of protein, i.e., its transformation into hemichrome. At pH about 7, Hb and its constituent parts can be arranged according to their increased conformational stability and hence the conformational energy of globin moiety as follows:

$$\text{globin} \ll \text{ligand } \alpha\text{SH-chains} < \text{MetHbH}_2\text{O} \leqslant \text{ligand } \beta\text{SH-chains} < \text{ligand low spin Hbs} < \text{deoxy-Hb}.$$

As we have shown the rate of proteolytic destruction also decreased in the same order [28]. An important feature of conformational changes affecting the conformational stability of these proteins is, as a rule, the absence of any changes both in the content of α-helical segments and of the shape of macromolecule. At present for a protein in solution the overall pattern of these conformational rearrangements can be established only by the application of hydrogen exchange. It seems also of importance that such, at first sight, different events as the splitting of inter-subunit contacts, removal of the heme, replacement of ligand or ionization of single protein groups give rise to comparable changes in protein stability. All these cases as many others [14–20,22] reveal the 'cooperative' nature of local *trans*-conformations, which is manifested by simultaneous changes in a conformational stability and intramolecular motility of a considerable part of the protein molecule [19]. One of the molecular bases for the cooperative behaviour of local *trans*-conformations may be the same widespread perturbations of the static structure,

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**The definition 'local' is used here only as a conventional one, as the physical nature of local *trans*-conformations is yet unclear and is now under investigation in our, as well as in other, laboratories.

which have been made 'visible' by the X-ray difference Fourier technique.

It is to be noted that hydrogen exchange permits the study of only the conformational energy which affects the probability of the conformational transitions exposing to the solvent internal sterically hindered peptide hydrogens [21,29]. Choosing the different Hb-derivatives as a model we were able to demonstrate that the probability of just such transitions is influenced by various functionally important events. These findings support our suggestion [6], that such a probability is strongly dependent on the functionally-linked conformational energy of protein and that the ΔG°_{L} as determined from hydrogen exchange data is closely related to this energy.

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