EFFECT OF INTER-SUBUNIT CONTACT ON INTRAMOLECULAR CONFORMATIONAL MOTILITY (CONFORMATIONAL STABILITY) OF HEMOGLOBIN AS REVEALED BY HYDROGEN EXCHANGE

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1. Introduction

Structural-functional studies of various proteins and particularly of hemoglobin reveal the essential difficulties in elucidating the mechanism of biological functioning without knowledge of ways of storage and transformation of conformational energy [1,2]. To solve the problem we need in principle to follow the conformational stability and intramolecular motility whose alterations are governed by functionally-linked conformational energy. One of possible experimental approaches is a study of hydrogen exchange as the rate of hydrogen exchange increases with increased intramolecular motility (decreased conformational stability) of the protein [3].

Taking hemoglobin as a model we have attempted to find an interrelation between the change in a functional state of the protein and its intramolecular motility, detected by hydrogen exchange. It is known that the separation of hemoglobin into native subunits results in the disappearance of cooperativity in ligand binding as well as of specific pH-dependence of ligand affinity (the alkaline Bohreffect) [4]. The present report demonstrates that this cleavage of inter-subunit contacts in ligand hemoglobin leads not only to an increased exchange rate, i.e. to higher intramolecular motility, but also to a simultaneous disappearance of pH-dependent change of intramolecular motility.

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Abbreviations: HbO_2 oxyhemoglobin A; αSH, βSH, isolated subunits with free thiol groups; PHMB *p*-hydroxymercuribenzoate.

2. Materials and methods

Non-stripped human HbO_2, globin and αSH and βSH chains in oxy-form were obtained by standard procedures [4–7]. The rate of hydrogen – deuterium ($^1H \rightarrow {}^2H$) exchange in peptide NH groups was monitored by infrared spectroscopy [8] with the use of UR-10 and UR-20 spectrophotometers (Carl Zeiss, Iena, DDR). The exchange reaction was initiated by gel filtration through a short column of Sephadex G-25 (fine), equilibrated with a heavy water buffer. The pH of all solutions in ^2H_2O are reported as pH* without adding 0.4 pH unit to the pH-meter reading.

Exchange experiments in αSH and βSH chains were carried out using 2–3 preparations which were obtained independently from different samples of HbO_2. Nativity and purity of the proteins under study were checked by absorption spectra in the visible and ultraviolet regions (for oxygenated and deoxygenated derivatives), by electrophoresis on polyacrylamide gel, starch gel and cellulose acetate strips ('Millipore') at pH 7.4, 8.3 and 8.6, as well as by PHMB titration of SH groups and by hemoglobin recombination from αSH and βSH chains and from globin and heme. The extent of the HbO_2 recombination from the separated

chains was monitored by starch gel electrophoresis and PHMB titration of free SH groups. On the studies of methemoglobin recombination from hemin and globin the unbonded hemin was separated on DEAE Sephadex A-25.

3. Results

Kinetic curves of $^1H \rightarrow {}^2H$ exchange in peptide NH groups are shown in fig.1. The ratio of peak intensities (A) of amide II and amide I bands is proportional to the fraction of unexchanged peptide hydrogens (proportionality coefficient $\omega = 1/0.5$). The deviation of parallel experimental curves from the mean in fig.1 does not exceed ± 0.005 in units of the ratio $A_{\text{amide II}}/A_{\text{amide I}}$. It follows from these data, that the maximum acceleration of exchange reaction (about 40 X) results from the splitting off of the heme, i.e. from globin formation (dimer apo ($\alpha\beta$)). The exchange rate in isolated chains also exceeds that in intact HbO_2 tetramer, but to a lesser extent. In isolated αSH chains (monomers) the rate of exchange is about 11 X and in βSH chains (tetramers β_4) about 5 X higher than that in tetrameric molecules of intact HbO_2.

A strong increase of the exchange rate upon cleavage of inter-subunit contacts cannot be accounted for by irreversible changes in protein structure, since the exchange rate in HbO_2 reconstructed from isolated

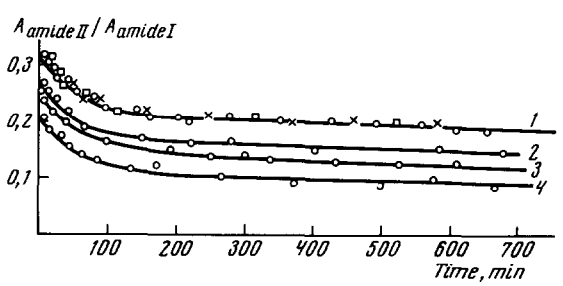

Fig.1. Hydrogen–deuterium exchange of the peptide hydrogens of oxyhemoglobin (1), oxy-βSH chains (2), oxy-αSH chains (3) and globin (4) at pH* 7.0 20°C, 0.1 M phosphate buffer. The lines are plotted for samples, prepared from total human hemoglobin. The data for proteins prepared from A_0 component of hemoglobin are represented by circles. Curve I has additional experimental points for $^1H \rightarrow {}^2H$ exchange in oxyhemoglobin reconstituted from isolated chains (□) and in the sperm-whale metmyoglobin (×).

chains is practically the same as that in the parent tetramers.

One can suggest that the higher exchange rate in αSH chains is due to the smaller size of the macromolecule, αSH chains being in solution essentially as monomers of mol. wt ~ 16 500, whereas HbO_2 and βSH chains are tetramers of mol. wt 65 000. But the exchange rate in whale-sperm metmyoglobin, whose molecular weight is about the same as that of αSH chains and its tertiary structure resembles that of hemoglobin subunits, is practically the same as the exchange rate in tetrameric HbO_2 and lower than in isolated chains (see fig.1 and [9]). Therefore the main reason for increased exchange rate is the higher intramolecular motility of the protein matrix as a result of the cleavage of inter-subunit contacts (in the case of αSH chains) or the contact of substitution of one by another type (in the case of βSH chains).

The study of an acid-denatured protein, at pH 3.0, as well as the kinetic curve prediction (according to [10]) shows that exchange rates of peptide hydrogens of both chains coincide when their peptide groups freely contact the solvent. The 2-fold difference in exchange rate between the native chains is thus determined by higher intramolecular motility of αSH chains. One cannot exclude that it is these differences that are associated with different functional properties of αSH and βSH chains both in the isolated state and within tetramer, particularly at pH below 8.0 [11–14].

It should be noted that increased intramolecular motility (lowered conformational stability) in the order $HbO_2 \rightarrow$ βSH chain → αSH chain → globin is confirmed by the data on proteolytic degradation of these proteins [15].

Another difference between isolated chains and HbO_2 as well as globin is seen from the analysis of data on the pH dependence of the exchange rate. According to the generally accepted EX-2 type of exchange mechanism [3], experimental points for the pH range over which the static and dynamic protein structures remain unchanged should form in the coordinates of fig.2 one continuous exchange curve, individual for a protein [16]. Such continuous "generalized" curves whose fragments are shown in fig.2 are actually obtained for isolated αSH and βSH chains. But the experimental points for ligand HbO_2 (fig.2) and globin (data not shown) over pH 7.0–8.0 do not fit continuous lines. Experiments on raising

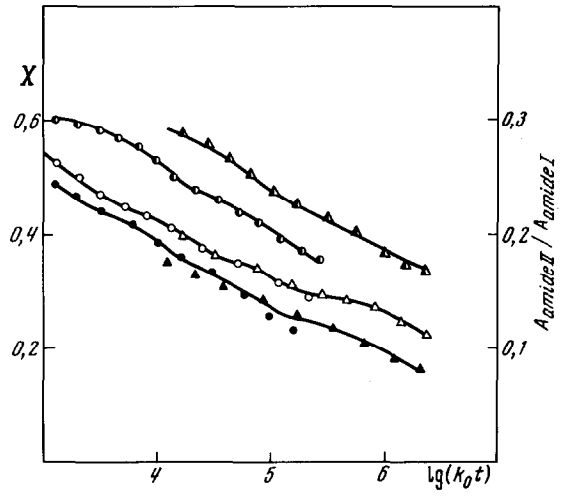

Fig.2. Experimental data on the hydrogen–deuterium exchange of the peptide hydrogens at pH* 7.0 (○●●) and pH* 8.0 (△▲▲), 20°C plotted as X and $\frac{A_{\text{amide I}}}{A_{\text{amide II}}}$ versus log (k_0t). X is the fraction of unexchanged hydrogens at time t and k_0 is the pH-dependent rate constant for solvent exposed peptide hydrogens. Oxyhemoglobin half-filled symbols, oxy-αSH chains closed symbols, oxy-βSH chains open symbols.

the pH in the course of exchange (procedure in ref. [17]) show that the break origin cannot be explained by shielding some solvent-exposed peptide hydrogens as a result of the local structural rearrangement on changing pH from 7.0 to 8.0. Therefore the main cause of the break is the lowered intramolecular motility on pH increase from 7.0 to 8.0, that is 5.5 X for the ligand HbO_2 and about 3 X for globin.

4. Discussion

The data presented here demonstrate that formation of $\alpha_1\beta_1(\alpha_2\beta_2)$ and $\alpha_1\beta_2(\alpha_2\beta_1)$ inter-subunit contacts in ligand HbO_2 tetramer lead not only to lowered intramolecular motility but also to the appearance of a qualitatively new property of the intramolecular motility, namely the possibility for the latter to be regulated by a small 'physiological' change in the pH value. Intramolecular motility of HbO_2 alters in the same pH–range where the changes in ligand affinity (alkaline Bohr effect) and ligand dissociation rates are observed [4,14,18]. On the other hand these functional properties [4,14] as well as intramolecular motility are pH-independent for the isolated αSH chains. All these facts permit us to consider that the intramolecular motility and conformational stability of protein detected by hydrogen exchange are dependent on the functionally important conformational energy changes being responsible for the ligand affinity alterations. The physical nature of dynamic short-lived exchangeable conformers is possibly close to that of transient key states providing the functioning of the protein. The observed changes in intramolecular motility are caused by a small perturbation of static structure on inter-subunit contact formation and on ionization of single protein groups. These perturbations of structure seem to be similar to those revealed by X-ray analysis of mutant human hemoglobins. The widespread changes involve the shift of atoms by several Å, the change in size of internal cavities and mechanical strains in the protein matrix [19–21].

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