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Site-directed mutagenesis in hemoglobin

Effect of some mutations at protein interfaces

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The role of selected amino acid residues in the monomer–monomer contacts of Hb A has been studied by site-directed mutagenesis of the α chain bearing substitutions in the subunit surfaces. Mutation α38Thr→Trp induced a stabilization of tetrameric Hb-CO with a decrease of the K_d for the equilibrium $\alpha_2\beta_2 \Leftrightarrow 2\alpha\beta$, but had no effect on ligand binding. Mutation α40Thr→Arg resulted in a complete loss of cooperativity in ligand binding. Mutation α103His→Val had no noticeable effect. We also studied the behaviour of isolated, mutated α chains with respect to self association: compared to wt α chains, mutant α38Thr→Trp showed stabilization of the dimeric state and (at high protein concentration) a detectable amount of tetramers Mutant α103His→Val showed only a minor stabilization of the α_2 dimer.

Hemoglobin mutant; Subunit contact; Protein engineering

1. INTRODUCTION

The allosteric behaviour of Hb, whereby the quaternary T→R conformational transition modulates the oxygen affinity of the heme iron, depends on its assembly into an $\alpha_2\beta_2$ heteroteramer [1,2]. Oligomeric vertebrate hemoglobins evolved from an ancestral monomeric globin via gene duplication and selection of complementary surfaces to promote specific aggregation. Other proteins acquire their characteristic biological activity only when associated into oligomers stabilized by weak forces between complementary surfaces, where packing of side chains is as tight as the interior of a protein [3].

Theories for the molecular basis of cooperative effects and allosteric control in the binding of O_2 to Hb have been tested in the past by experiments carried out with natural mutants. In the last few years site directed mutagenesis has been successfully applied to the same problem [4–6], with original contributions concerning the role of distal site residues in controlling ligand affinity of the heme, and the significance of critical amino acid residues in the evolution of hemoglobins.

In the assembly of a functional tetramer, the α and β chains interact with each other *via* two types of contacts called α1β1 and α1β2, involving different amino acid side chains on each monomer [2,7,8]. Interface α1β1 is rather rigid, since it involves a larger molecular contact (~ 2,000 Å^2) and is unchanged in the T→R quaternary transition. Our attention was focussed on the α1β2 interface (~ 1,200 Å^2 in R and ~ 1,500 Å^2 in T), where the transition from T to R is associated to large conformational changes; moreover it is known that dissociation of Hb into αβ dimers involves cleavage along this interface [9]. Here two positions of the β chains (β37 and β40) establish extensive contacts with the partner α chains both in Hb A and Hb H (the β_4 tetramer, see [10]); in the α chains the corresponding topological positions are occupied by Thr. The side chains β37Trp and β40Arg play a key role in keeping the α and β chains associated in the tetrameric state, since two natural mutants at these positions, Hb Hirose (β37Trp→Ser) and Hb Austin (β40Arg→Ser), display an increased dissociation into dimers [11,12]. Moreover it is known that Trp at position β37 is an useful optical probe to follow the quaternary state of Hb A, and therefore the type of contact at the α1β2 interface; the ligand induced change of the narrow-banded difference spectrum around 290 nm, has been assigned mostly to a change in the environment of β37Trp as a result of the quaternary transition [13,14].

Using information from structural data [2] and from natural mutants we have engineered four mutations in the α chains of Hb A to introduce new amino acid side chains which may enhance the stability of Hb heterotetramer and, more generally, help understanding the rules governing surface complementarity and recognition. Site directed mutagenesis of amino acid residues at the surfaces which mediate αβ aggregation may in

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addition provide a clue to understand why the α and β chains, though homologous in sequence and structure, display substantial differences in their association behaviour, given that isolated β chains can form a tetramer, while α chains cannot [15,16].

2. MATERIAL AND METHODS

The Hb mutants were produced with the technique described by Kunkel et al. [17] using the gene of the α chains, synthetized and expressed by Hoffman et al. [18]. This expression system allows the production in *E. coli* of fully functional tetrameric Hb. We used three 21-mers with a 3 bases mismatch for the single mutant, and a 62-mer with two 3 bases mismatches for the double mutant. No additional mutation was found in all mutants by resequencing the entire coding sequence.

Mutant α103His→Val was denatured by exposure to the detergents used to disrupt the cells, and the other three mutants contained a significant fraction of met Hb Iron oxidation and denaturation were avoided by growing the cells in a medium containing traces of carbon monoxide (CO) and breaking the cells by sonication. The bacterial extract was then centrifuged and the supernatant was passed through a CM-Sepharose column equilibrated with 10 mM phosphate buffer at pH 6.0; elution with a gradient from 10 mM phosphate at pH 6.0 to 70 mM phosphate at pH 7.0 resulted in purification of Hb as verified by SDS-PAGE. All the buffers and colums used in the purification contained traces of CO.

Splitting the $\alpha_2\beta_2$ Hb tetramer was obtained by reaction with pHMB [19], purified chains were regenerated by incubation with β-mercaptoethanol

Rapid mixing experiments were carried with a Gibson-Durrum stopped-flow apparatus; flash photolysis experiments with the apparatus described by Brunori and Giacometti [20] The fraction of slowly reacting (SRF) and quickly reacting form (QRF) with CO were determined after flash photolysis at different hemoglobin concentrations [21].

3. RESULTS AND DISCUSSION

3.1. *Mutant hemoglobins*

3.1.1. Molecular modelling

At the α1β2 interface, residues β37Trp and β40Arg play a very significant role in the stabilization of the tetramer; the corresponding topological positions on the α chains are occupied by two Thr. Computations indicate that (respectively) 21% and 19% of the total non-bonded interactions with the facing a monomer can be attributed to these two residues (β37Trp and β40Arg). As shown in Fig. 1, panel a, β37Trp makes contacts with 4 residues on the partner a chain, and its role in the the stability of the α1β2 contact is suggested by its invariance in all vertebrate hemoglobins [22]. On the contrary Thr in the corresponding position of the α chains, although in a similar environment, establishes fewer contacts (Fig. 1, panel b). Simulation of the mutations: α38Thr→Trp, α41Thr→Arg and of the double mutant α38Thr→Trp/α41Thr→Arg using the program BRUGEL [23], indicated that these substitutions could all be accomodated in the α1β2 interface of both oxy and deoxy Hb; moreover mutation α38Thr→Trp (Fig. 1, panel c) was associated to a substantial stabilization of the interface and to an increase in the buried surface area (of 69 Å^2 per interface). Thus a Trp in this position can mimick the role of the Trp in the homologous site of the β chains, due to the pseudo-symmetry of the interface and to sequence homology between α and β chains.

Mutation α103His→Val (interface α1β1) was produced to test the prediction by Chothia and Lesk, based on molecular modelling (personal communication), that this substitution could facilitate aggregation of isolated α chains (see below).

3.1.2. Ligand binding

Table I contains a list of the α chain Hb mutants prepared and characterized in this work, as well as information on the presence of heme-heme interactions and on the tetramer-dimer dissociation constant.

The time course of combination of deoxy Hb with CO is shown in Fig. 2 for the four Hb mutants. Mutant α41Thr→Arg and α38Thr→Trp/α41Thr→Arg are characterized by a monofasic, fast combination with CO ($k_{on} = 2.5 \times 10^6$ M^{-1}·s^{-1}, like the isolated Hb chains, [1]), indicating that introduction of an Arg at position α41 abolishes cooperativity in ligand binding (either by destabilizing the T state or by stabilizing the R state). On the other hand Hb α38Thr→Trp and Hb α103His→Val both display a slow combination with CO, with an autocatalytic time course similar (but not identical) to that characteristic of Hb A (see Fig. 2). The kinetic time courses were analyzed with a 4-step irreversible Adair scheme, which allows to obtain a reliable estimate of the CO combination rate constants for the T state (k_T) and for the R-state (k_R); the result is reported in Table I in terms of the ratio (k_R/k_T), which is a quantitative estimate of the kinetic cooperativity for CO [24, 25].

The oxygen binding isotherm was determined for the mutant Hb α38Thr→Trp; the result shows that oxygen binding is cooperative ($n_{1/2} = 2.2$ and $p_{1/2}(O_2) = 3.4$ mmHg), in agreement with the conclusion drawn from the kinetic data.

3.1.3. Measurement of tetramer–dimer dissociation constant

The finding that Hb α38Thr→Trp and Hb α103His→Val are both cooperative allows to estimate the equilibrium dissociation constant (K_d) for the dimerization reaction ($\alpha_2\beta_2 \Leftrightarrow 2\alpha\beta$) of the CO-derivative of these mutants, using the photolysis method. It is known that after photodissociation of the Hb–CO complex with a long flash ($\tau > \mu$s) CO rebinds to Hb following a bifasic kinetics [1]. The relative amplitude of the two phases is protein concentration dependent [21], and the quickly reacting (QRF) and slowly reacting (SRF) forms correspond respectively to dimeric and tetrameric Hb. As shown in Fig. 3, HbCO α38Thr→Trp clearly

displays a tendency to aggregate into tetramers greater than wt HbCO, while the mutant Hb α103His→Val is indistinguishable from wt HbCO. The absolute values of the dissociation constants are reported in Table I.

These results on the stability of the liganded tetramers upon modification of the two αβ contact surfaces (i.e. α1β1 and α1β2) are in complete agreement with the literature [9]. In fact mutation (Thr→Trp) at the α1β2

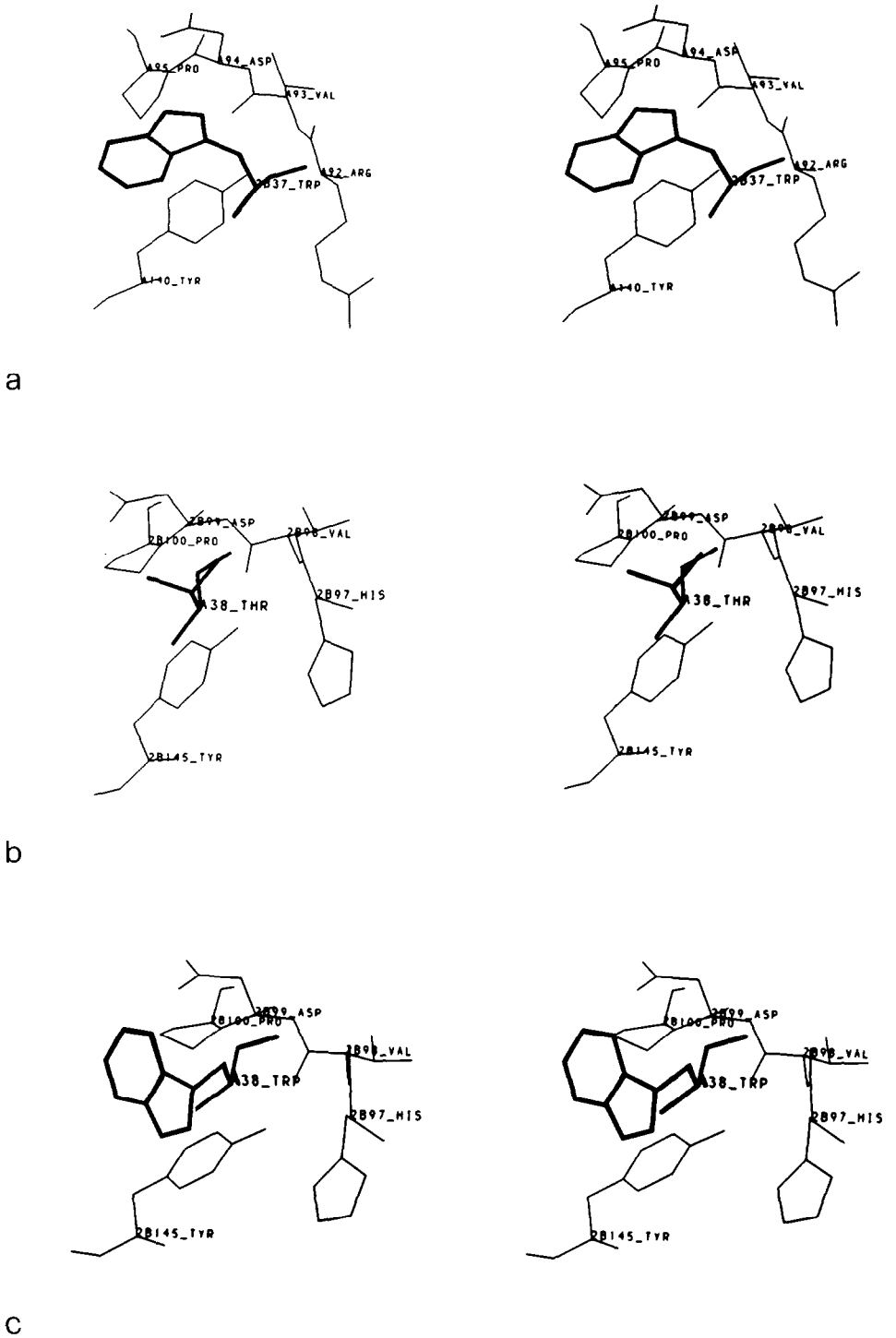

Fig. 1. Stereo pictures of β37Trp, α38Thr and α38Trp with their respective neighbours on the facing monomer in the following structures: (a and b) Human hemoglobin wt, R state. (c) Model of human Hb (α38Thr→Trp), R state. Trp and Thr are drawn with a thick line and their neighbours on the facing monomer with a thin line. These models, like all computations employed in this work, have been performed with the program BRUGEL, running in VAX/VMS environment and with an Evans & Sutherland PS390 station [23].

contact, known to be cleaved upon dissociation into dimers, is shown to induce a stabilization of the tetramer consistent with an increase in the number of molecular contacts established (in the mutant) by α38Trp with the complementary residues on the partner β chain (see Fig. 1, panel c). Mutation α103His→Val at the α1β1 contact, on the other hand, has no effect on dimerization of Hb-CO.

3.1.4. UV difference spectra

Information on the reorganization of α1β2 interface can be obtained by UV spectroscopy, since a narrow band around 290 nm was shown to reflect a perturbation of the environment of aromatics, particularly β37Trp [13,14].

On the basis of the molecular modelling illustrated above (see Fig. 1), we expected the ligand-linked T→R quaternary transition to be associated to a perturbation of all Trp at α1β2 interfaces (a total of 4 per tetramer) in mutant Hb α38Thr→Trp. Detemination of the derivative of the oxy and deoxy UV spectra of Hb α38Thr→Trp and comparison with the spectra obtained for wt Hb, indeed shows that the amplitude of the fine structure around 290 nm (one maximum at 289 nm and two minima at 285 and 292.5 nm) is approximately doubled in the mutant Hb (see Fig. 4). This is consistent with expectation, and shows that the additional Trp at the α1β2 interface is a second optical probe of the quaternary change, which may be exploited for investigations of the interface dynamics.

3.2. *Mutant α chains*

The isolated mutant α chains were studied to obtain information on the interface involved in α chains dimerization, and on amino acid substitutions which may enhance association of the α chains, estimated by analytical ultracentrifugation. As shown in Fig. 5, sedimentation coefficients were fully consistent with the literature for horse myoglobin [1] (monomeric $s_{20,w} = 1.8$), and for *Scapharca inequivalvis* Hb (dimeric $s_{20,w} = 2.8$) [26]. Sedimentation experiments with wt α chains confirm some tendency to dimerize; the estimated dissociation constant is however 5 times larger than the value reported by Valdes and Ackers [16]. More interesting is the finding that mutant α38Thr→Trp has a considerably greater tendency to dimerize, with a K_d at least 10 times smaller than that of wt α chains; moreover at the highest concentrations (i.e. 13.5 mg/ml) we observed a sedimentation coefficient for this mutant significantly higher than the value expected for a dimer, suggesting a fraction of α_4 homotetramers.

On the other hand, the double mutant α38Thr→Trp/α41Thr→Arg and the single mutant α103His→Val (Fig. 5), are only slightly more associated than the wt α chains; the single mutant α41Thr→Arg is like wt α chains. Therefore, the mutation α38Thr→Trp at the α1β2 interface is the one that has a clearcut positive

Table I

Synopsis of the properties determined for the hemoglobins containing various mutant α chains. Column labeled 'interface' indicates the surface region in which the mutation is situated. Ratio (k_R/k_T) determined from the time course of combination with CO (see text). Tetramer - dimer dissociation equilibrium constants (K_d), expressed on heme basis.

Mutant	Interface	k_R/k_T	K_d for $\alpha_2\beta_2 \Leftrightarrow 2\alpha\beta$
α38Thr → Trp	α1β2	30	0.65 μM
α41Thr → Arg	α1β2	1	n.d.
α103His → Val	α1β1	44	2.1 μM
α38Thr → Trp/α41Thr → Arg	α1β2	1	n.d.
wt Hemoglobin	–	47	2.1 μM

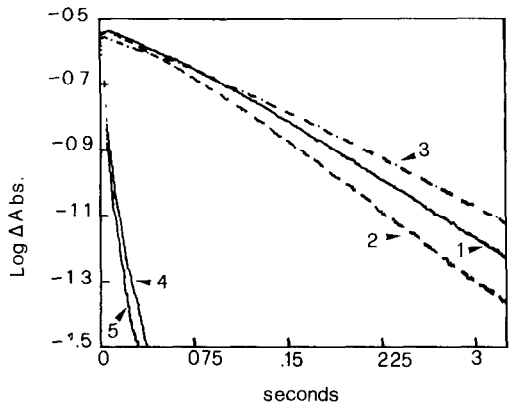

Fig. 2 Time course of the combination with CO of wt and mutant deoxy hemoglobins. Conditions: 0.1 M Bistris-Cl, pH 7.0, 20°C, CO=25 μM after mixing, Hb 2 μM after mixing. Trace 1, wt Hb A; trace 2, Hb α38Thr→Trp; trace 3, Hb α103His→Val; trace 4, Hb αThr41→Arg; trace 5, Hb α38Thr→Trp/α41Thr→Arg. The dashed part of the trace in 4 and 5 represents the part of the reaction lost in the dead time of the instrument, given the fast combination with CO of the these two Hb mutants.

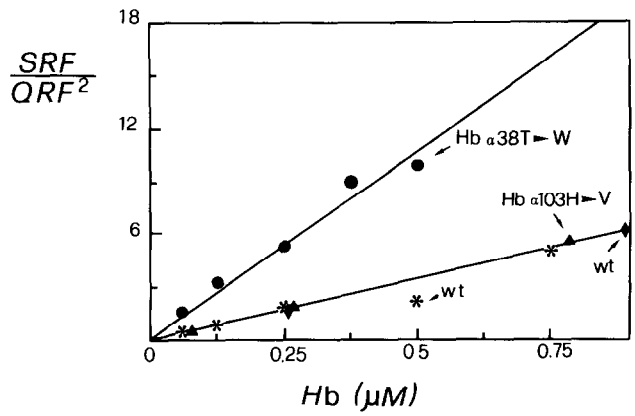

Fig. 3. Plot of the fraction SRF/QRF^2 as a function of tetrameric Hb A concentration; the slope of the straight line is equal to $4/K_d$ for the dimerization reaction. Conditions: 0.1 M Bistris-Cl, pH 7.0, 20°C, CO = 50μM. Symbols (mutants): ●, Hb α38Thr→Trp; *, wt Hb; ▲, Hb α103His→Val; ◆, wt Hb produced in *E coli*.

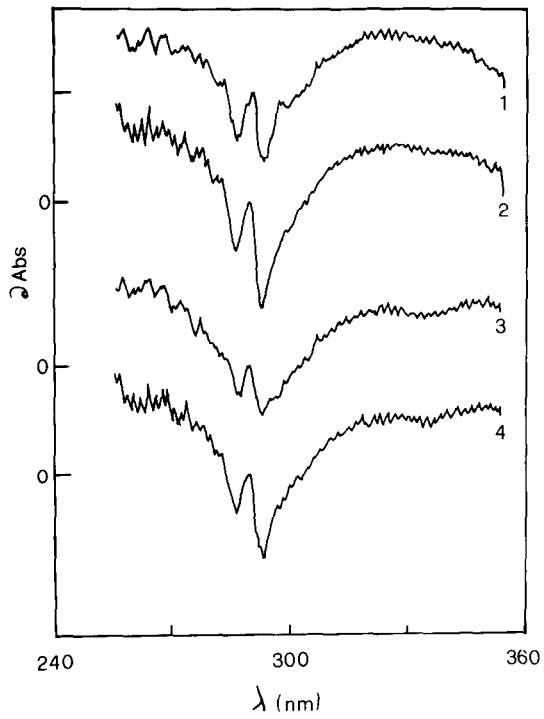

Fig. 4. UV region derivative spectra of oxy- and deoxyhemoglobins. Experimental conditions: 55 μM Hb in 0.05 M Bistris-Cl, pH 7.4, t = 20°C. Derivative of the oxy and deoxy spectra were calculated using the first-derivative mode of the data manipulation program of the spectrophotometer (Olis Instruments modified Cary-14). 1, HbA-O2; 2, Hb (α38Thr→Trp)-O2; 3, deoxy Hb A; 4, deoxy Hb (α38Thr→Trp).

effect on association of the isolated α chains, although some effect is also seen with the mutant α103His→Val.

These observations may suggest the following interpretation: wt α chain dimers are formed via a subunit contact homologous to the α1β1 contact of Hb A, and mutation α103His→Val has a slight effect by eliminating an unfavourable contact at this interface. Introducing the mutation α38Thr→Trp, the contact α1β2 is also partially stabilized, and a fraction of α_4 tetramers is populated at high protein concentration. Arg in α41 as in the double mutant α38Thr→Trp/α41Thr→Arg, partially counterbalances the aggregating effect of Trp in α38, indicating that this interface is destabilized, consistently with the loss of cooperativity of the Hb carrying this mutation. Dimerization due to an α1β1-like contact should not be affected by substitution α41Thr→Arg, and in fact the single mutant α chains bearing an Arg in α41 shows the same behaviour as wt α chains as far as dimerization is concerned.

This interpretation is in agreement with the modeling carried out by Vallone B., Schaad O. and Edelstein S.J. (unpublished data) who simulated an α_4 tetramer on the basis of the geometry of HbA, and found that the α1β1-like contact involves more interacting surface than the interface topologically corresponding to α1β2. Therefore they formulated the hypothesis that α chains dimerization was mediated by the contacts corresponding to interface α1β1 in Hb A; simulation of mutation α38Thr→Trp in the α_4 tetramer, seemed to stabilize the α1β2-like contact.

4. CONCLUSIONS

We have shown that it is possible to introduce a Trp at the α1β2 interface of human Hb at position α38; this side chain is compatible with the overall thermodynamic stability of the T and R conformations, and does not alter the mechanism of molecular switch, since cooperativity in ligand binding is retained. The molecular contacts of Trp in α38 seem analogous to those of β37Trp, as predicted by simulation and confirmed by the observation that (similarly to β37Trp) the optical spectrum of α38Trp is perturbed upon the T→R transition.

Mutation α38Thr→Trp is associated to stabilization of tetrameric HbCO, compared to wt Hb by 0.65 kcal/mol. Determination of the 3D structure of this mutant will allow a correlation of the measured stabilization free energy with the number of molecular contacts established by the indol side chain. It is very interesting that the isolated α chains carrying this mutation are more tightly associated not only with the partner β

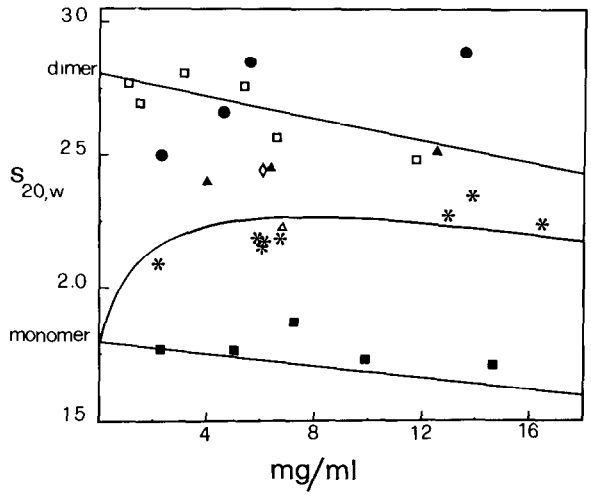

Fig. 5. Sedimentation coefficients $s_{20,w}$ for hemoglobin α^{SH} chains (wt and mutants) as the CO derivative, plotted as a function of the protein concentration. Sedimentation experiments were carried out on a Spinco Model E ultracentrifuge equipped with a RTIC unit at 52,000 rpm Conditions: Tris-Cl 0.1 M + 0.1 M NaCl, pH 7.4, t = 7–15°C. The lines labeled 'monomer' and 'dimer' correspond to the values expected for dimeric and monomeric globins [15], corrected for the increased viscosity of the solution as the protein concentration increases, and are in agreement with values reported for horse Mb [1] and the dimeric hemoglobin from *Scapharca inequivalvis* [26]. The curve represents the expected $s_{20,w}$ for a K_d = 0.49 mM for the equilibrium $\alpha_2 \Leftrightarrow 2\alpha$. Symbols: ■, horse Mb; □, *Scapharca inequivalvis* dimeric Hb; *, wt Hb α^{SH} chains; ●, α^{SH} chains α38Thr→Trp; △, α^{SH} chains α41Thr→Arg; ▲, α^{SH} chains α103His→Val; ◆, α^{SH} chains α38Thr→Trp/α41Thr→Arg.

chains (across the $\alpha 1\beta 2$ interface), but also with other α chains, given that full α_2 dimers and a fraction of α_4 tetramers are detected by ultracentrifugation.

A dramatic effect on the properties of Hb was observed upon introduction of a second Arg at position C6 of interface $\alpha 1\beta 2$, as in mutant α41Thr→Arg which completely loses coperativity in ligand binding. This effect was not predicted by modelling, possibly due to underestimation of the electrostatic component of the nonbonded energy in the potential energy function used. Interestingly also Hb H (i.e. the β tetramer), that has two Arg at position C6, is noncooperative in ligand binding [1], suggesting that the T conformation is not allowed in the presence of two Arg in the $\alpha 1\beta 2$ interface, possibly because α41Arg (or β40Arg) makes unfavourable contacts with β145Tyr across the $\alpha 1\beta 2$ interface.

In summary we obtained novel information on the role of some amino acid side chains at the molecular contacts between hemoglobin subunits, and engineered improved protein-protein surfaces, which lead to enhanced association of one mutant α chain (α38Thr→Trp) with another α chain or with its partner β chain. This is yet another example that point mutations at a physiologically important interface promote association, leading to evolution of complementary surfaces mediated by a few mutational events, and permitting fine tuning of biological function.

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