Signal Transmission Between Subunits in the Hemoglobin T-state

Joan J. Englander*, Jon N. Rumbley and S. Walter Englander

Introduction

Hemoglobin provides the most accessible model for study of the intramolecular signalling processes that underlie biological regulation. Contrary to the impression promoted by mass circulation textbooks, the pathway for signal transduction within the hemoglobin (Hb) molecule is not at all clear. In attempts to unravel the principles of allostery, fundamental alternatives have generally been phrased in terms of simplified global-level models (Monod et al., 1965; Koshland et al., 1966; Wyman & Gill, 1990). One wants to understand these processes mechanismically at a detailed structural level. The insights of Perutz and his co-workers, derived from crystallographic studies of the liganded and deoxy end states of hemoglobin, provide important guidelines. Comparison of the end state structures suggests that the liganding of heme groups is translated into a physical displacement at the heme iron “trigger”, which levers the motion of an attached helix, breaking certain non-covalent bonds and promoting a quaternary structural transition. The quaternary T-state (deoxy) to R-state (oxy) transition switches the affinity of remote hemes for the binding of subsequent ligands (Perutz, 1970; Baldwin & Chothia, 1979; Fermi et al., 1984; Perutz, 1989, 1990; Paoli et al., 1996).

To dissect intramolecular signalling pathways, structural information is essential but it will also be necessary to measure signal transmission in a quantitative way at a detailed structural level. Quantification must be couched in terms of the true currency of these interactions, namely structural free energy and changes therein. This is so because Hb and other regulatory proteins function as energy-interconverting machines. When Hb binds its initial ligands, some of the binding energy is sacrificed and transduced into structure-change energy, carried by way of structure changes to remote heme sites, and transduced back into binding energy. Thus early ligands are bound with reduced energy and later ones with enhanced energy, generating the functionally important S-shaped binding curve.

The measurement of structural free energy requires the measurement of a structure-dependent equilibrium. An important example measures changes in Hb’s tetramer-to-dimer equilibrium, which can be shown to couple directly to the global change in allosteric free energy (Ackers & Halvorson, 1974; Smith & Ackers, 1985; Ackers, 1998). In that work, site resolution was achieved...
by studying the effects of defined chemical and mutational modifications (Ackers & Smith, 1985; Turner et al., 1992). The work reported here exploits the naturally occurring hydrogen exchange (HX) behavior of protein molecules which couples to the free energy of structural stabilization through its dependence on transient local unfolding equilibria (Hvidt & Neilson, 1966; Englelnder & Kallenbach, 1984). HX methods have the unique ability to quantify the influence of known modifications on other site-resolved positions, both locally and remotely, as shown in the accompanying paper (Englander et al., 1998). HX measured in small proteins by NMR can be used to read out structural and free energy changes to the level of individual amino acid residues (Wagner & Wuthrich, 1982; Wand & Englelnder, 1996). In work with the large Hb molecule, we use alternative medium-resolution methods that can approach this level in favorable cases (Rosa & Richards, 1979; Englander et al., 1985; Mallikarachchi et al., 1989; Englander & Englelnder, 1994; Englander et al., 1998).

The present work measures the effects of partial Hb liganding on structural (de)stabilization at known local and remote sites, within the liganded and non-ligated subunits, while Hb remains in the T-state. Results obtained bear on the 35 year long debate concerning the concerted symmetry-conserving nature of allosteric change (Monod et al., 1965; Koshland et al., 1966; Szabo & Karplus, 1972; Gelin et al., 1983; Lee & Karplus, 1983; Wyman & Gill, 1990) and approach the issue of more detailed structural mechanism.

Results

These experiments use an HX functional labeling method to selectively place tritium label on allosterically sensitive positions. Figure 1 shows HX data for the allosterically sensitive sites studied here. A set of five NH groups placed near the beginning of the a-chain was isolated and measured on the fragment a1-29 (Ray & Englelnder, 1986). (Only four hydrogen atoms were measured due to incomplete labeling in the 35 minute exchange-in.) Four allosterically sensitive NH groups within the last six residues of the b-chain were isolated and measured on the fragment b130-146 (Louie et al., 1988b). Within each set, all the hydrogen atoms exchange at similar rates in deoxy Hb and they move as a group to a new, faster rate in oxy Hb. This group behavior is evidently due to the transient concerted unfolding of the end turns of the helix holding them.

The NH groups at the a-chain N terminus exchange faster by ninefold in oxy Hb, indicating a loss in stabilization free energy at that position of 1.2 kcal/mol in the T-state to R-state transition. The 750-fold HX acceleration at the b-chain C terminus reflects a loss of 3.6 kcal/mol. The total, 9.6 kcal/tetramer, provides a lower estimate of the overall allosteric free energy of the Hb molecule (Englander et al., 1998) under the present conditions (pH 7.4, 0°C, with pyrophosphate effector).

We have previously studied some of the peripheral allosterically sensitive links that stabilize these segments by eliminating one or more of them and then determining the resulting changes in HX rate of the probe hydrogen atoms (Louie et al., 1988a;

Figure 1. HX of two sets of allosterically sensitive hydrogen atoms in HbA. Two sets of amino acid residues at the N terminus of the a-chain and at the C terminus of the b-chain were isolated and measured on the fragments a1-29 and b130-146, respectively. The exchange of either set of hydrogen atoms is not monoexponential because the component NH groups have somewhat different intrinsic chemical HX rates. These results define a standard curve shape for each set which is then fit to subsequent data for the same hydrogen atoms in different experiments. In this and subsequent figures, the small background contribution of allosterically insensitive NH groups has been subtracted. The difference in HX rate between the oxy (R-state) and deoxy (T-state) forms gives the change in stabilization free energy felt at the segment holding the NH groups. The differences shown (ΔΔG) are on a per tetramer basis. For details see the accompanying paper (Englander et al., 1998). The exchange-out conditions here and in subsequent Figures were 100 mM sodium phosphate (pH 7.4), 100 mM NaCl, 20 mM ferrous pyrophosphate, 0°C.
Englander et al., 1998). Those data evaluate the stabilization free energy due to individual bonds and show how they interact. Here, we observe the effects of liganding at the central heme “trigger”. Partial liganding was accomplished by separating the subunits, liganding either the $\alpha$-chain or the $\beta$-chain hemes in the cyanomet (CN-met) form (oxidized heme iron plus cyanide ligation), and then reconstituting the symmetric hybrid tetramer. CN-met ligation has been widely studied as a model for partially ligated Hb states.

**Local and cross-subunit effects of liganding**

Figure 2 shows HX results for Hb with either the $\alpha$-chain hemes or the $\beta$-chain hemes liganded in the cyanomet form while the opposite chains are in the deoxy form. Liganding of the $\alpha$-chains speeds the exchange of the probe NH sites at the $\alpha$-chain N terminus by fourfold, indicating an energetic destabilization of 0.75 kcal/mol (1.5 kcal/tetramer). When the $\alpha$-chains are liganded, the probe sites at the $\beta$-chain C terminus are also accelerated, by tenfold, indicating an energetic destabilization of 1.24 kcal/mol (2.5 kcal/tetramer) at that position. Figure 2 shows that liganding the $\beta$-chain hemes produces the very same result.

These data demonstrate two interesting results. Liganding on one type of subunit produces effects within that subunit but also within the opposite subunit type, apparently within the T-state. The effects seen at either subunit are the same independently of which hemes are liganded. These are the central results of this work.

Further experiments were done to test for possible artifacts that might explain these results. Tests for ligand migration and Hb integrity

A possible explanation of the result found is that the cyanomet ligation may artifactually redistribute among the chains during handling. This has been considered especially by Shibayama & colleagues (Shibayama et al., 1997, 1998) who found CN-met ligand migration at 21.5°C on a time scale of ~30 hours. Our CN-met Hb experiments were done at 0°C and were completed within five hours. The amount of cyanomet heme present in each preparation was measured spectrophotometrically before and after each HX experiment and found to be close to 50% (total range 45 to 57%). To test whether Hb was damaged during subunit separation and reconstitution, the hybrid Hb preparations were returned to the fully deoxy form by brief exposure to dithionite (50–100 mM, 0°C, five minutes) and their HX behavior was measured. The results reproduce the HX of untreated deoxy HbA (Figure 2).

Parallel experiments were done with a mutant recombinant Hb, rHb $\beta$Val67Glu, in which the $\beta$-chain hemes are covalently held in the oxidized form by liganding to the carboxylate side chains of Glu67 (the recombinant equivalent of Hb M Milwaukee). HX experiments used the recombinant prepared by us and also a mutant prepared and reconstituted in the laboratories of Drs A. Arnone and R. Noble. The oxidized $\beta$-chain hemes were liganded with cyanide by maintaining KCN at 20 to 100 mM in all solutions ($K_d$ is $5.3 \times 10^{-4} \text{ M}$ under our conditions). HX results for both mutant preparations (Figure 3) duplicate the results found before for hybrid CN-met HbA (Figure 2).

The total CN-met fraction measured spectrophotometrically after each experiment was always close to 50%, ruling out any significant ligation on

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**Figure 2.** HX behavior in HbA at the allosterically sensitive chain termini when both $\alpha$-chains or both $\beta$-chains are liganded in the cyanomet form. Conditions are as for Figure 1 with 2 mM KCN and 1 mM IHP added to the deoxy exchange-out buffer. Dotted lines mark the position of the HX curves in oxy and deoxy HbA, from Figure 1. The ligation condition of each subunit is tabulated where the dash (—) symbolizes a reduced deoxy heme group, here and in the subsequent Figures. The same effects are seen when either the two $\alpha$-chains or the two $\beta$-chains are liganded. Control experiments checked the integrity of the partially liganded Hb preparations by returning them to the fully reduced form (open triangles).
the \( \alpha \)-chains. HX data measured after reduction of both of these preparations with dithionite reproduce the standard curve for deoxy HbA (Figure 3). These experiments unequivocally rule out ligand migration and also demonstrate the integrity of the preparations used. These results further show that the \( \beta \)Val67Glu mutation by itself does not affect the HX behavior of the probe NH groups in deoxy T-state Hb.

**Tests for R-state population**

A possible explanation of the cross-subunit effects is that liganding produces some equilibrium occupation of the R-state. HbA half liganded in the CN-met form adopts the R-state at room temperature in the absence of phosphate effector (Ogawa et al., 1972; Ackers, 1998). A small fractional occupation of the R-state can multiply the HX rate for the \( \beta \)-chain hydrogen atoms impressively since they are faster in R-state Hb by 750-fold (Figure 1). For example, an equilibrium R-state contamination of 1/750 would double the rate measured. The \( \alpha \)-chain hydrogen atoms experience only a ninefold increase in HX rate in the R-state, thus are much less sensitive and require a 12% R-state contamination to double the HX rate. (Previous work showed that unliganded Hb in the R-state exchanges like liganded R-state Hb (Englander et al., 1998).)

The fourfold increase in rate seen in the \( \alpha \)-chain when either heme pair is liganded would require an R-state population of 40% (equations given by McKinnie et al., 1991). However, this would accelerate the \( \beta \)-chain exchange rate by 300-fold, inconsistent with the tenfold increase actually seen. The observed tenfold increase in rate of the \( \beta \)-chain hydrogen atoms could be explained by an R-state occupation of 1%, but the \( \alpha \)-chain would then show no measurable response, inconsistent with the fourfold acceleration seen.

In another test for the presence of R-state Hb, inositol hexaphosphate (IHP) was removed (Figure 4). The experiments in Figures 2 and 3 were done in the presence of 1 mM IHP, an allosteric effector that strongly favors the T-state. If some level of R-state occupation contributes significantly to these HX effects, then removal of the strong IHP effector would further increase the R-state occupation and the measurable HX rate. The removal of IHP has no effect on the \( \alpha \)-chain N terminus when either the \( \alpha \)-hemes or the \( \beta \)-hemes are liganded, and no effect on the \( \beta \)-chain C terminus when the \( \alpha \)-hemes are liganded. The \( \beta \)-chain shows a small effect when the \( \beta \)-chain hemes are liganded; HX is made faster by another twofold (Figure 4(b)). One possibility is that IHP removal raises the R-state population of the \( \beta \)-heme-liganded Hb to ~1%. Another possibility is a local effect due to the fact that His143, which is within the set of sensitive \( \beta \)-chain residues, directly participates in IHP binding. In either case it is clear that the observed cross-subunit effects of cyanomet ligation do not depend on some equilibrium R-state population.

It should be noted that the T-state is much more stable under our conditions than at room temperature without phosphate effector, the conditions that have often been used by other workers. All of our solutions were at 0°C and contained the weak allosteric effector sodium pyrophosphate (PPi), with or without IHP. (We use PPi-chelated ferrous ion (20 mM PPi, 5 mM ferrous ammonium sulfate) to reduce free oxygen and ensure deoxy conditions.) Under these conditions the total cooperative free energy is more than 9.6 kcal/tetramer (compare 6.3 kcal/tetramer at 20°C without phosphate), and the T-state to R-state switch point is
evidently moved well beyond the twice-liganded condition.

The heme trigger

Figure 5 shows results for rHB (βVal67Glu without added cyanide so that the oxidized (met) β-chain hemes remain liganded to the Glu67 carboxylate side chain. An HX acceleration of fivefold is seen locally, at the C terminus of the mutated β-chain (0.9 kcal/site destabilization), slightly smaller than the tenfold change (1.2 kcal/site) for the cyanide-liganded molecule. More importantly, no cross subunit effect is seen at the α-chain.

The carboxylate side-chain of the mutant glutamate residue is a weak field ligand and produces a high spin complex with the heme iron still out of plane by 0.2 Å (A. Arnone, personal communication). Cyanide used in the prior experiments is a strong field ligand and moves the heme iron into plane, pulling the Perutz “trigger”. The results suggest that pulling the heme iron trigger may be necessary in order to obtain the cross-subunit effect. An alternative possibility is that the complexation of Glu67(E11) to the heme hinders a required movement of the E helix (A. Arnone, personal communication).

Discussion

The hydrogen exchange probe

The amide hydrogen atoms of Hb exchange with solvent hydrogen atoms over a range of rates that covers more than ten orders of magnitude. A fraction of these hydrogen atoms, about one quarter, are sensitive to allosteric structure change (Malin & Englander, 1980). All of the allosterically sensitive hydrogen atoms appear to be faster exchanging in the R-state form of Hb and slower in the T-state form (Malin & Englander, 1980), indicating that the protein moiety of R-state Hb is a destabilized, higher-energy form. The fact that change in HX

Figure 4. HX behavior in half liganded HbA when IHP is removed, a test for R-state contamination. Broken lines indicate the previously found curves. (a) HX at the α N terminus is unchanged. (b) HX at the β C terminus is slightly changed only when liganding is on the β-chain heme. Symbols are as for Figure 3.

Figure 5. HX behavior in recombinant HbM Milwaukee (βVal67Glu) when the oxidized β-chain heme is liganded by the carboxylate side-chain of Glu67. Here no KCN was present. Symbols are as for Figure 3.
rate keys to change in structure stabilization free energy ensures that the sensitive hydrogen atoms mark positions that participate in energetic structure change.

The functional labeling method used here selectively labels allosterically sensitive positions with exchangeable tritium by exploiting the fact that they change their HX rates in the T to R transition. In the present experiments two sets of allosterically sensitive hydrogen atoms were labeled and studied; a set of five hydrogen atoms at the \( \alpha \)-chain N terminus and a set of four at the \( \beta \)-chain C terminus. The exchange of these hydrogen atoms with solvent, followed by a fragment-separation method, was used to detect and quantify structure and energy change at these positions when the heme iron atoms at both \( \alpha \)-chains or both \( \beta \)-chains are liganded.

**The cyanomet (CN-net) system**

For partial heme liganding we used the strong field CN-net ligation with either the \( \alpha \)-chain hemes or the \( \beta \)-chain hemes oxidized and liganded with cyanide anion. This system has certain benefits and some dangers. Shibayama *et al.* (1997, 1998) noted the danger of CN-net migration to other non-intended heme sites, which occurred in their hands during long incubation times (1 ~ 30 hours) at 21.5 °C. Our experiments were done at 0 °C and required only ~five hours for completion. Control experiments showed that ligand migration does not contribute to the effects seen. Another potential problem, the possibility that significant R-state population might contribute to the effects seen (Ogawa *et al.*, 1972; Mukerji & Spiro, 1994; Ackers, 1998) was tested for and ruled out under the stable T-state conditions used here (0 °C, with phosphate effector).

Another issue concerns the fact that the effects of CN-net liganding are not quantitatively identical with O\( _2 \) liganding. CN-net in the \( \alpha \text{,} \beta \)-liganded form is less destabilizing than the O\( _2 \) analog by 2 kcal (Ackers *et al.*, 1992; Huang *et al.*, 1996). It is slightly less destabilizing than O\( _2 \) in the \( \alpha \text{,} \beta \)- and the \( \beta \text{,} \beta \)-liganded conditions that we have used and also in the \( \alpha \text{,} \beta \)-liganded condition (Ackers, 1998). This difference is beneficial for the present experiments insofar as it makes partially liganded CN-net Hb less likely to adopt the R-state, which would tend to conceal the intra-T-state effects that the present experiments reveal.

In previous work we found that some of Hb’s allosterically important bonding interactions tend to break together while in the T-state while other bonds can break independently (Englander *et al.*, 1998). Such experiments provide information about structural intermediates, their free energy level, and their cooperativity. Partially liganded systems can provide analogous information about effects that originate at the liganded heme. Different ligands such as CN-net may modify the relationships typical for O\( _2 \) but it seems most unlikely that they could create, *de novo*, phenomena such as the cross-subunit pathways detected here.

**Cross-subunit signaling in the T-state**

The results found here demonstrate energetic cross-subunit signaling in the absence of the T to R transition. Liganding the \( \alpha \)-chain hemes exerts destabilizing effects in both the \( \alpha \) and the \( \beta \) subunits even though the protein maintains the fully T-state. Similarly, both subunit types are destabilized when only the \( \beta \)-chain hemes are liganded. It is striking that these effects are quantitatively identical, independently of which heme pair is liganded (\( \Delta AG = 0.75 \text{ kcal/site at the } \alpha \text{-chain N terminus and 1.25 kcal/site at the } \beta \text{-chain C terminus} \)).

These observations demonstrate that ligand-induced structure changes can carry an energetic signal between the \( \alpha \) and \( \beta \) subunits prior to quaternary T-state to R-state switching, as initially proposed by Daugherty *et al.* (1991). Structure changes that may contribute have been detailed (Perutz *et al.*, 1987; Luisi & Shibayama, 1989; Luisi *et al.*, 1990; Kavanaugh *et al.*, 1995; Paoli *et al.*, 1996; Bettati *et al.*, 1997; Kavanaugh *et al.*, 1998) and some of these have been characterized energetically (Holt & Ackers, 1995; Huang & Ackers, 1995; Huang *et al.*, 1996; Ackers *et al.*, 1997; Ackers, 1998; Kiger *et al.*, 1998) and functionally (Kwiatkowski *et al.*, 1998; Peterson & Friedman, 1998).

When the oxidized heme iron in the mutant \( \beta \text{-Val67Glu} \) binds to the weak field carboxylate ligand of Glu 67, no cross-subunit effect is seen, unlike the result for the strong field CN\( ^- \) anion. This suggests that the cross-subunit effect may depend on pulling the heme trigger of Perutz, i.e., moving the heme iron into the heme plane. An alternative possibility is that the cross-subunit effect requires movement of the \( \beta \)-chain E helix (containing Val67(E11)), since the E helix is constrained by the Glu67 to heme link (suggested by A. Arnone).

**Molecular switching code: the intra-dimer interface**

The cross-subunit effects seen here can be considered in light of the “molecular code mechanism” of Ackers and colleagues. In addition to the CN-net system, Ackers’ group has evaluated the energetic effects of heme liganding, using an extensive series of other oxygenation analogs with the ligand in each system placed at all combinatorial heme positions (Smith & Ackers, 1985; Ackers *et al.*, 1992; Turner *et al.*, 1992; Huang & Ackers, 1996; Ackers, 1998). The results show that the Hb T-state is destabilized equally when either the \( \alpha \) heme or the \( \beta \) heme or both together are liganded. Aspects of the molecular code proposal have been the subject of spirited debate (Edelstein, 1996; Ackers *et al.*, 1997; Henry *et al.*, 1997; Huang *et al.*, 1997; Shibayama *et al.*, 1997; Ackers, 1998; Shibayama *et al.*, 1998).
Our results show the very same effects, both the fact of cross-subunit energy transfer in the T-state and the fact of equivalent destabilization when either the a-hemes or the b-hemes are liganded. However, the HX results were obtained with ligand on both a-chain hemes or on both b-chain hemes. Thus they do not by themselves distinguish whether the cross-subunit effects are transmitted across the a1b2 or the a2b1 interface. The Ackers’ “molecular code mechanism” points to transfer across the a1b2 interface at the initial stages of lig-ation (favorable intra-dimer coupling) and to subsequent unfavorable transfer across the a2b1 interface (dimer-dimer anti-cooperativity for the T to R switch; Ackers et al., 1992).

Molecular switching code: the dimer-dimer interface

The work of Ackers and his group further showed that when Hb is liganded across the a1b1/ a2b2 interface, it switches preferentially to the R-state. Similar results have long been known (e.g., Ogawa et al., 1972; Mukerji & Spiro, 1994). However, this is not what we find; liganding at a1 and a2 or at b1 and b2 does not switch Hb to the R-state.

The discrepancy presumably stems from the different conditions used. Ackers’ work was done at 21.5°C with no phosphate effector. In that case the overall cooperative free energy (ΔG for the T to R transition) is 6.3 kcal/tetramer. Under these conditions, CN-met liganding of either one or both of the hemes on an a1b2 dimer was measured to destabilize the T-state by 3.2 kcal/tetramer. A similar result was found with the metal hybrid Mn3+ and Co2+ systems. Liganding on the opposite dimer then imposes a cross-dimer destabilization that is sufficient to drive the protein to the R-state. Our experiments were done at 0°C with phosphate effector where the cooperative free energy is over 9.6 kcal/tetramer. The results show that liganding both a-chains or both b-chains is then insufficient to drive the T to R transition and Hb remains in the T-state. These considerations emphasize that the quaternary switching model of Hb must be understood relative to the energies involved rather than in terms of absolute structure-based rules, as has been stressed previously (Ackers et al., 1992).

The fact that allosteric stability is large under our conditions (>9.6 kcal/tetramer) allows the exploration of the higher-energy destabilization space. When Hb is CN-met liganded at all four hemes, it does switch to the R-state, even in the presence of 1 mM IHP at 0°C (data not shown). When all four sites are liganded, a different balance of destabilization energies may come into play under various conditions. One option is that liganding specifically across the a1b2 interface imposes some additional destabilization that does not enter when liganding is across the a1a2 or the b1b2 interface. This effect would have been largely masked in the less stable system (21.5°C, no phosphate) by transition to the R-state in all cases.

Conclusions

This work focusses on the energy dimension of the allosteric process. The accompanying paper (Englander et al., 1998) deals with two sets of non-covalent bonds that help to stabilize the Hb T-state. These bonds are found to interact in surprising ways, with some near neighbor bonds showing independent additivity and some remote bonds showing interactions across subunit interfaces while Hb remains in the T-state. This paper describes effects due to heme ligation. Within the T-state, intra-subunit and cross-subunit destabiliz- ing effects occur. These results point to the role of specific pathways for information transfer within the T-state and emphasize the importance of quan-titative energetic measurements in their study.

Materials and Methods

Cyanomet hybrids

Hb purified from fresh red cells was equilibrated with CO and treated overnight with a tenfold excess of p-chloromercuribenzoic acid (PCMB; Bucci & Fronticelli, 1965). The a and β chains were separated by ion exchange chromatography (DEAE cellulose for the a-chains; CM cellulose for the β-chains (Riggs & Gibson, 1973)). PCMB was removed by treatment with N-acetyl-dl-penicillamine in the dark (a-chains—pH 8.0, 150 mM penicillamine, 0°C, 20 minutes; β-chains—pH 7.4, 200 mM penicillamine, four hours, 15°C; both in 10 mM sodium phosphate, 1 mM EDTA; R. Benesch, personal communication). Penicillamine was removed by gel filtration. The number of free SH groups per chain was determined by the absorbance change (250 nm) produced by titrating with PCMB previously calibrated with glutathione that was standardized with Ellman’s reagent (Boyer, 1954). Chain purity was checked by native gel electrophoresis. Bound CO ligand was removed by stir-ring at 0°C in air under a flood lamp (20 minutes).

To produce the cyanide-liganded form of the oxidized heme iron (cyanomet ligation), a 1.2-fold molar excess of K3Fe(CN)6 was added to solutions of the designated chain containing 2 mM KCN and 1 M glycine as a scavenger for free radicals (Cassoly, 1981). The reaction was allowed to proceed at room temperature for six to ten lifetimes (k = 70 M−1 second−1). Excess K3Fe(CN)6 was removed by a gel-filtration column equilibrated at pH 7.4 with 2 mM KCN and 500 mM glycine.

To form tetramers, a and β chains were mixed (one in the cyanomet form, with 1.2-fold excess β-chain) in a reaction mixture containing 2 mM KCN, 500 mM glycine, 100 mM phosphate at pH 7.4 (held overnight at 5°C). Excess β-chains were removed by DEAE cellulose chromatography (10 mM phosphate, 2 mM KCN (pH 6.9)). Purity was checked by native gel electrophoresis. To document the cyanomet content, the hybrid Hb was diluted into a CO-saturated buffer (pH 7.4), the spectrum was recorded, and dithionite was added to reduce the cyanomet and ligand these sites with CO. The change in absorbance at 418 nm was translated into...
percent cyanomet heme using the known extinction coefficients.

Samples were stored frozen in small aliquots at −80°C until needed.

**Recombinant Hb (rHb) βVal67Glu**

The adult human hemoglobin (HbA) gene construct, with both chains in the Val1Met form, was obtained from S. Sligar (Herman & Sligar, 1995), ligated into a pUC18 vector, and grown in Escherichia coli strain BL21 (Studier & Moffatt, 1986). The recombinant Hb mutant βVal67Glu was prepared using PCR and the SOEing method (Horton & Mood, 1989).

E. coli carrying the rHb βVal67Glu gene was grown in terrific broth (Tartof & Hobbs, 1988) at 37°C with shaking for up to 20 hours. Cells were pelleted and resuspended in pH 8.0 buffer containing DNase, phenylmethylsulfonyl fluoride and benzamidine (protease inhibitors), equilibrated with CO gas to stably ligand the Hb and avoid heme oxidation, and broken in a French press. After pelleting the cell debris, the supernate was diluted 1:1 with water, adjusted to pH 6.0, and Hb was isolated by CM cellulose chromatography (Herman & Sligar, 1995). Up to 50 mg of Hb per liter was obtained. CO ligand was removed as above. Due to the reducing environment in the E. coli host, β-chains were obtained in the reduced state and required two hours of incubation at 10°C for conversion to the carbonylmet form (Glu67 carboxylate side-chain ligated to the oxidized heme of the β-chain).

Native electrophoresis showed rHb βVal67Glu as a single band running with HbA. A biphasic azide binding curve reproduced the results found by Hayashi et al. (1969) for HbM Milwaukee (βVal67Glu). The reconstituted preparation of rHb βVal67Glu obtained from A. Arnone and R. Noble was prepared by them by combining the mutant β-chain expressed in E. coli with normal α-chains and heme, as described by Herman et al. (1992).

To convert the β-chain to the cyanomet form, rHb βVal67Glu was incubated overnight at 5°C in 20 mM KCN (Kp = 5.3 × 10⁻⁴). In order to maintain the cyanomet ligation all subsequent HX labeling and exchange was done in the presence of 20 mM KCN. The percent cyanomet was measured before and after HX experiments by spectrophotometric methods, as above.

**Hydrogen exchange**

HX procedures and data calculations were as described in the accompanying paper (Englander et al., 1998). A functional labeling method was used to selectively place tritium label on allosterically sensitive sites. The HX behavior of sites at the α-chain N terminus and the β-chain C terminus was structurally resolved by a fragmentation-separation method.

For exchange-out of the hybrid Hb molecules with one chain type in the heme-oxidized state and one in the deoxy heme-reduced state, rapid deoxygenation was accomplished by addition of dithionite at only 3.5 times the total oxygen concentration (free + bound) before immediate passage through a deoxygenated gel-filtration column (~30 seconds, 0°C) to avoid reduction of the oxidized heme iron. The column was washed with 0.1 M sodium phosphate (pH 7.4), 100 mM NaCl, 20 mM ferrocyanide, 2 mM KCN ± 1 mM IPTG. In the case of rHb βVal67Glu, 20 mM KCN was present in the exchange-in and exchange-out buffers. In control HX experiments done to check the integrity of the hybrid Hbs by returning the Hbs to a completely deoxy state for exchange-out, the chains were reduced using 50 to 100 mM dithionite prior to the initiation of exchange-out.

**Acknowledgments**

This work was supported by research grant DK11295 from the National Institutes of Health. We thank Drs A. Arnone and R. Noble for gifts of mutant hemoglobins, and G. Ackers, A. Arnone, J. Friedman, and R Noble for helpful comments on this work and on the manuscript.

**References**


*Edited by K. Nagai*

(Received 9 April 1998; received in revised form 24 September 1998; accepted 29 September 1998)