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Energetic Components of the Allosteric Machinery in Hemoglobin Measured by Hydrogen Exchange

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A hydrogen exchange (HX) functional labeling method was used to study allosterically active segments in human hemoglobin (Hb) at the α-chain N terminus and the β-chain C terminus. Allosterically important interactions that contact these segments were removed one or more at a time by mutation (Hbs Cowtown, Bunbury, Barcelona, Kariya), proteolysis (desArg141α, desHis146β), chemical modification (*N*-ethylsuccinimidyl-Cys93β), and the withdrawal of extrinsic effectors (phosphate groups, chloride). The effects of each modification on HX rate at the local and the remote position were measured in the deoxy Hb T-state and translated into change in structural free energy at each position.

The removal of individual salt links destabilizes local structure by 0.4 to 0.75 kcal/mol (pH 7.4, 0°C, 0.35 M ionic strength) and often produces cross-subunit effects while hemoglobin remains in the T-state. In doubly modified hemoglobins, different changes that break the same links produce identical destabilization, changes that are structurally independent show energetic additivity, and changes that intersect show energetic overlap. For the overall T-state to R-state transition and for some but not all modifications within the T-state, the summed loss in stabilization free energy measured at the two chain termini matches the total loss in allosteric free energy measured by global methods. These observations illustrate the importance of evaluating the detailed energetics and the modes of energy transfer that define the allosteric machinery.

Keywords: allostery; hemoglobin; hydrogen exchange; structure change; free energy

Introduction

Regulatory structure change in proteins is fundamentally a process of energy transduction. For example, hemoglobin binds its initial oxygen atoms with reduced energy. Some of the binding energy is converted into structure-change energy, carried through the protein by way of structure changes, and transduced back into binding energy for the later oxygen atoms. The initial low affinity and the later high affinity produce the S-shaped oxygen-binding curve. This kind of energy trade is likely to be a general aspect of protein function. To pursue the principles that govern these fundamental processes, we must learn to identify the working parts of the protein machinery and measure how the parts interact to perform energy transduction and transmission steps.

Hemoglobin (Hb) provides an excellent system for this kind of study. Oxygen binding changes the allosteric form of Hb from the deoxy T-state to the oxy R-state because liganding breaks interactions that selectively stabilize the T-state (Perutz, 1970; Baldwin & Chothia, 1979; Gelin *et al.*, 1983; Perutz, 1989). In this process, certain segments in Hb change their hydrogen exchange (HX) rates by factors of up to 10,000 (Liem *et al.*, 1980). This coupling occurs because the same bonding interactions that stabilize against equilibrium allosteric tran-

Abbreviations used: Hb, hemoglobin; met Hb, Hb with the heme iron oxidized; CN-met Hb, the cyanomet modification, with met heme bound by cyanide; NES-Hb, the *N*-ethylsuccinimidyl derivative at Cys93β; desArg Hb, Arg141α proteolytically removed; PPi, sodium pyrophosphate, used at 20 mM; DPG, diphosphoglycerate; IHP, inositol hexaphosphate, used at 1 mM; stripped, all phosphate effectors removed; HX, hydrogen exchange; MWC, Monod-Wyman-Changeaux.

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sitions also stabilize against the transient equilibrium unfolding reactions that determine HX behavior (Englander & Kallenbach, 1984). Thus the allosteric value of given interactions may be defined in free energy terms by HX measurements (Englander & Englander, 1994; Bai *et al.*, 1995).

Using specially designed HX methods, we approach the Hb problem from two directions. HX has identified two of the allosterically sensitive local unfolding units in Hb, placed at the N terminus of the α-chain and the C terminus of the β-chain (Ray & Englander, 1986). Allosterically important linkages at these positions are well known (see Figure 1). To evaluate the different interactions and their local and long-range cooperativity, we have perturbed individual known interactions using defined mutants, chemical modifications, and the addition and withdrawal of allosteric effectors, and then measured energy changes both locally and remotely. Here, we describe the results obtained. Also we have pressed on the functional heme trigger using partially liganded Hb molecules and then measured the energetic consequences at defined positions out in the protein. The accompanying paper describes that work. The results demonstrate the capabilities of the HX functional labeling approach and provide some insight into allosteric energy transduction and signal transmission in Hb.

Theory

The stabilization free energy due to any bonding interaction will show up in all equilibria that are coupled to the loss of the interaction. In Hb, one can determine the contribution of a given bond to the overall allosteric free energy by breaking the bond and measuring the change in the oxygen-binding equilibrium (Wyman & Gill, 1990; Imai, 1994) or in the molecular dimer-to-tetramer equilibrium (Smith & Ackers, 1985). The same changes can be measured by HX insofar as HX rates depend on dynamic structural unfolding equilibria (Englander & Kallenbach, 1984; Englander *et al.*, 1992; Wand & Englander, 1996).

Linderstrøm-Lang and his colleagues (Linderstrøm-Lang & Schellman, 1959) postulated that a peptide group NH hydrogen atom can exchange with solvent hydrogen atoms only when the H-bond protecting it is transiently broken, as in equation (1):

$$>\mathrm{C{=}O\cdots H-N<} \overset{K_{op}}{\longleftrightarrow} >\mathrm{C{=}O}$$

$$+\,\mathrm{H-N<} \overset{k_{ch}}{\longrightarrow} >\mathrm{C{=}O} + {}^{*}\mathrm{H-N<} \qquad (1)$$

$$k_{ex} \approx K_{op} k_{ch} \qquad (2)$$

$$\Delta G_{op} = -RT \ln K_{op} \approx -RT \ln k_{ex}/k_{ch} \qquad (3)$$

In the usually observed EX2 condition (Hvidt & Neilson, 1966), the HX rate for any given hydrogen atom is equal to the chemical exchange rate of the freely exposed NH (k_{ch}) reduced by the fraction of time the protecting H-bond is broken, essentially the K_{op} for H-bond breakage (equation (2)). In this case, the HX rate connects with the free energy for breaking the H-bond (equation (3)).

The H-bonds that protect the amide NH groups measured here break in small cooperative sets of four or five hydrogen atoms. The local unfolding model based on this view (Englander, 1975) leads to expressions identical with equations (2) and (3), with ΔG_{op} representing the sum of all the interactions that are broken in the opening reaction. When one of these stabilizing interactions is broken, for example in an allosteric transition or by some experimental modification, this will promote K_{op} and therefore the k_{ex} of all the hydrogen atoms exposed in the local unfolding reaction. The measured change in k_{ex} leads to the change in structural stabilization free energy felt at the position measured (equation (4)):

$$\Delta\Delta G_{op} = -RT\ \Delta \ln K_{op} = -RT \ln(k_{ex}/k'_{ex}) \qquad (4)$$

If the same bond is broken in the T to R transition, then equation (5) holds:

$$\Delta\Delta G_{op} = \Delta\Delta G_{R/T} \qquad (5)$$

Here, we measure changes in HX rate (k_{ex}/k'_{ex}) due to various allosterically involved bonding interactions in the deoxy T-form of Hb to determine their contribution to the stability of the Hb T-state.

Results

The experiments described here probe individual linkages at the α-chain N terminus and the β-chain C terminus (Figure 1) and their local and remote interactions. These interactions are centrally important in Hb's allosteric machinery (Perutz, 1970; Baldwin & Chothia, 1979; Gelin *et al.*, 1983; Perutz, 1989) and also account for most of the alkaline Bohr effect, the chloride effect, and part of the diphosphoglycerate (DPG) effect.

HX functional labeling and data analysis

Figure 2 shows HX data for the allosterically sensitive NH groups at these positions in unmodified HbA. These NH groups were selectively labeled with exchangeable tritium by the HX functional labeling method. Their exchange-out in various forms of Hb was then measured by a fragmentation-separation method that isolates and measures them on the fragments α1-29 and β130-146 (see Materials and Methods). This approach selectively labels and measures the exchange of four of the five allosterically sensitive NH groups that occur on α1-29 and all four that occur on β130-146. Earlier subfragmentation experiments localized these sensitive NH groups to the region

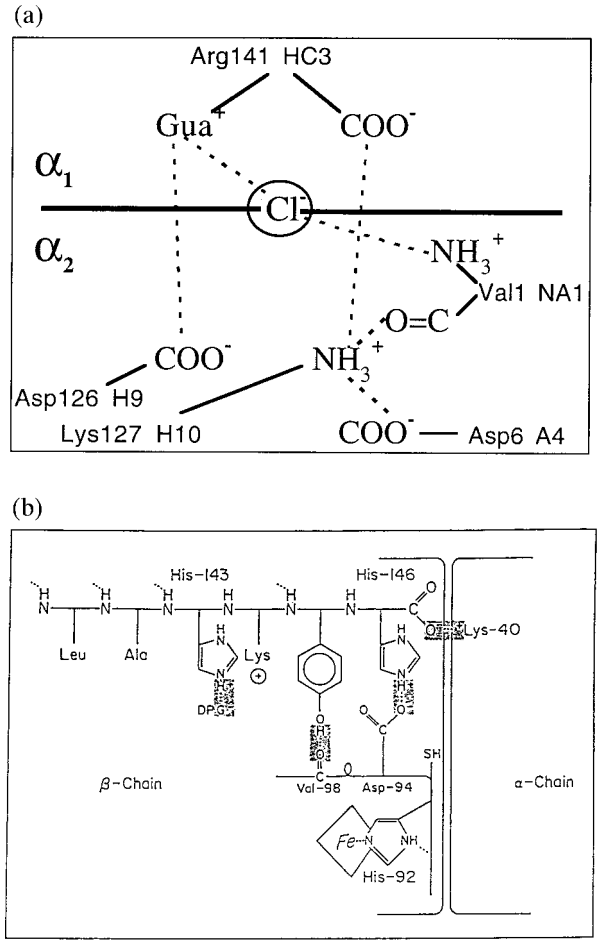

Figure 1. Some allosterically sensitive interactions in deoxy Hb at (a) the $\alpha_1\alpha_2$ interface and (b) the $\alpha_1\beta_2$ interface. The two sets of allosterically sensitive amide NH groups measured here are at the α-subunit N terminus and the β-subunit C terminus. The C-terminal Arg HC3(141) in each α-subunit links to groups that are at or near the N terminus and the C terminus of the opposed α-subunit. One link to the α-chain N terminus is mediated by disordered chloride anion in the central cavity. These links are drawn in the same aspect as Figure 3 of Kavanaugh *et al.* (1995). Interactions at the β C terminus involve a β_2 to α_1 cross-subunit interaction, a β to β cross-subunit interaction mediated by DPG (or PPi or IHP), and the intra-β interactions shown. Modifications studied remove the C-terminal Arg141α, the intermediating chloride ion, the individual inter and intra-subunit salt links to the C-terminal His146β, and the phosphate (DPG) interaction.

between residues 1 and 12 on the α-chain (Ray & Englander, 1986) and between residues 140 and 146 on the β-chain (Louie *et al.*, 1988a).

All the NH groups in a set exchange at roughly the same rate. When any of the linkages that stabilize the deoxy T-state (Figure 1) is experimentally broken, the HX rate of the whole set is accelerated as a unit. Evidently these two sets of NH groups are exposed to exchange with solvent hydrogen atoms by a dynamic cooperative unfolding-refolding behavior of the outer turn of the terminal helices. (Pre-breakage of the linkages tested here has no measurable effect on HX rates in oxy Hb, as expected (Turner *et al.*, 1992).)

The curve shape determined in Figure. 2(c) and (d) was used to fit HX data for the modified Hbs by multiplying the HX time scale by a fitting factor. The fitting factor provides the ratio, k_{ex}/k'_{ex} in equation (4), and thus the change in stabilization free energy, $\Delta\Delta G$, due to the modification tested. This approach is dictated by the fact that the curves are not monoexponential because the different peptide NH groups have somewhat different intrinsic chemical exchange rates (Bai *et al.*, 1993).

The results obtained are described below and summarized in Table 1.

The T-state to R-state transition

When T-state deoxy Hb is switched to the R-state, the sensitive NH groups at the α-chain N terminus respond by exchanging nine times faster (Figure 2(c)), indicating a loss of 1.2 kcal of stabilization free energy at each α-chain N terminus. The sensitive NH groups at the β-chain C terminus (Figure 2(d)) exchange faster by 190-fold (in the absence of phosphate effector; see Figure 6(b) below) pointing to a loss of 2.8 kcal at each β-chain C terminus. The summed change is 8.0 kcal/mol of Hb tetramer (pH 7.4, 0°C, with no phosphate effector). The summed allosteric free energy measured at these same conditions by subunit dissociation (G. J. Turner & G. K. Ackers) and by oxygen binding (J. A. Westrick & S. J. Gill) indicate a free energy difference between the T- and R-states of 8.3 kcal/mol (Englander *et al.*, 1992), in agreement with the HX result.

When phosphate effector is present (sodium pyrophosphate (PPi), diphosphoglycerate (DPG), or inositol hexaphosphate (IHP)), HX is made slower by fourfold at the β-chain C terminus in the T-state (see below). The T to R transition then registers the larger total $\Delta\Delta G$ of 9.6 kcal/mol tetramer (2×1.2 kcal at each α N terminus, as before, and 2×3.6 kcal at each β C terminus). This provides a lower estimate for the global cooperative free energy since the unfolding reaction at the β C terminus does not fully expel the phosphate effector (see below).

The internal ion pair: Hbs Cowtown, Bunbury, and Barcelona

An internal salt link joining His HC3(146)β to Asp FG1(94)β (Figure 1(b)) is removed in the mutant Hbs Cowtown (His146Leu), Bunbury (Asp94Asn), and Barcelona (Asp94His) (Wajcman *et al.*, 1982; Como *et al.*, 1983; Gelin *et al.*, 1983; Phillips *et al.*, 1983; Perutz *et al.*, 1984, 1985; Shih & Perutz, 1987). The HX results are shown in Figure 3. The curves for unmodified oxy and deoxy HbA, from Figure 1, are shown as broken lines in this and subsequent Figures.

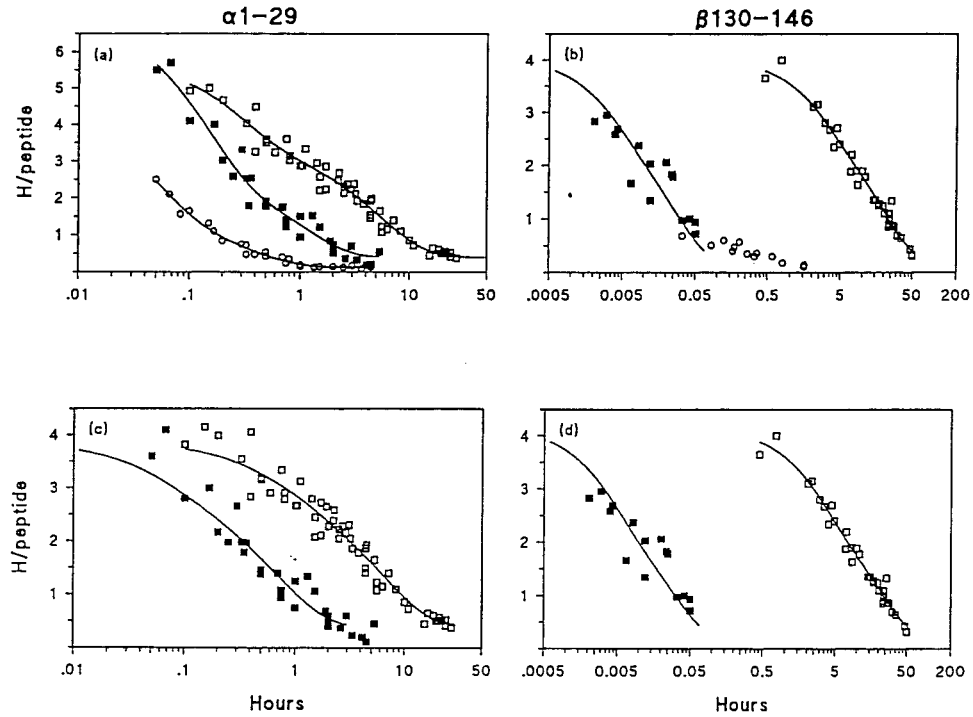

Figure 2. Functional labeling of unmodified HbA. (a) and (b) show raw data obtained by separating and analyzing the fragments α1-29 and β130-146 to determine the HX behavior of the amide NH groups on the allosterically sensitive segments at the α-subunit N terminus and the β-subunit C terminus, respectively. Oxy Hb was labeled by tritium using the functional labeling method (see Materials and Methods), then exchanged-out in either the deoxy (open squares) or liganded (filled squares) forms. These HX curves are enriched in allosterically sensitive sites but still contain some insensitive NH groups. The background curves (open circles), obtained by reversing the exchange-in/exchange-out protocol, are depleted of allosterically sensitive sites and portray the contribution of insensitive sites to the curves above. (c) and (d) show the data after subtracting the background contribution. The T to R transition destabilizes the α N terminus by 1.2 kcal (9× in HX rate) and the β C terminus by 3.6 kcal (750× in HX rate). Conditions used were pH 7.4, 0°C, 0.1 M NaCl, 0.1 M phosphate, 0.02 M PPi, 5 mM ferrous sulfate, unless otherwise indicated.

The allosterically sensitive NH groups at the β-chain C terminus exchange faster than in unmodified HbA by 2.7-fold in deoxy Hbs Cowtown and Bunbury, indicating a loss of 0.55 kcal/bond in stabilization free energy (equation (4)). In deoxy Hb Barcelona the sensitive NH groups are four times faster, indicating the somewhat larger destabilization of 0.75 kcal/substitution, perhaps

Table 1. Free energy loss in T-state hemoglobin due to the modifications studied

	α-Chain		β-Chain		Tetramer	Global
	k_{ex}/k'_{ex}	kcal/site	k_{ex}/k'_{ex}	kcal/site	kcal/tetramer	measurement (kcal)
T → R (+PPi)	9	1.2	750	3.6	9.6	
T → R (−PPi)	9	1.2	190	2.8	8.0	8.3
Cowtown			2.7	0.55	1.1	
Bunbury			2.7	0.55	1.1	
Barcelona			4	0.75	1.5	
NES	2.1	0.4	8	1.1	3.0	3
desHis	2.1	0.4	8	1.1	3.0	
NES desHis	2.1	0.4	8	1.1	3.0	
Kariya ±IHP	3	0.6	30	1.85	5	6
NES Kariya			30	1.85		
NES Kariya −PPi	60	2.25				
HbA −PPi	1	0	4	0.75	1.5	
HbA $-Cl^-$	3.6	0.7	1	0	1.2	
desArg $\pm Cl^-$	3.6	0.7	1.4	0.2	1.8	4.2
NES desArg	9	1.2	25	1.7	6.0	6.8
NES desArg −PPi	9	1.2	200	2.9	8.2	

HX measurements were at 0°C, pH 7.4, 0.35 M ionic strength. Free energy values were computed using the equations in the Theory section. The global values (last column) average measurements made under nearly the same conditions (5°C) in subunit dissociation (G. J. Turner & G. K. Ackers) and oxygen binding (J. A. Westrick & S. J. Gill) experiments, quoted by Englander *et al.* (1992). The global value for desArg Hb was measured at 21.5°C by subunit dissociation (Turner *et al.*, 1992). For spaces left blank, information is not available.

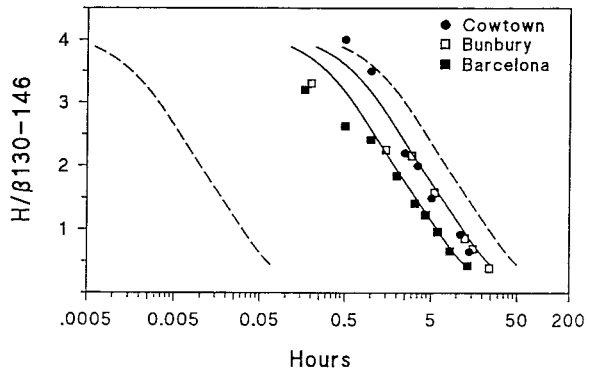

Figure 3. The β-chain intra-subunit salt link. The mutants studied destabilize the β C terminus by 0.55 kcal (Hbs Cowtown and Bunbury) and 0.75 kcal (Hb Barcelona), about half the effect seen for breaking both links at this position (see Figure 4). Here, and in subsequent Figures, the symbols represent the effect of modifications on the T-state deoxy form; the outer broken curves are for unmodified deoxy HbA (right) and oxy HbA (left) from Figure 1.

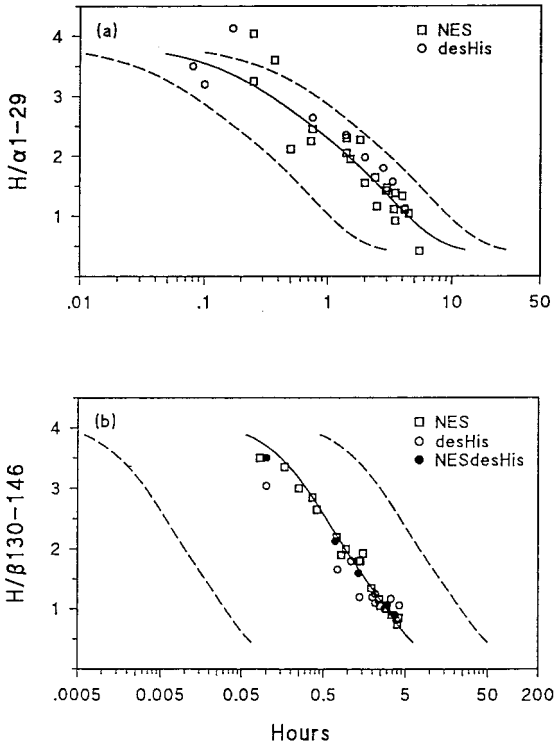

Figure 4. The His HC3(146)β salt links. The NES and desHis modifications and the two together destabilize the β C terminus by 1.1 kcal (8x in HX), evidently by removing both of the His146 salt links. Breaking both of these links also destabilizes the remote α N terminus by 0.4 kcal (2.1 × in HX rate).

due to an unfavorable interaction of the Barcelona histidine residue side-chain. It should be appreciated that the energy measured for the His146β salt link keys to the difference between the effective His146β pKa and the pH studied (pH 7.4).

The cross-subunit ion pair: desHis and NES Hb

The desHis146β modification removes the terminal histidine residue and both of its salt links, and thus breaks both the internal salt link and a cross-subunit link from His HC3(146)β-COO^- to Lys C5 (40)α-NH_3^+ (Figure 1(b)). The sensitive NH groups at the β-chain C terminus then exchange eight times faster (Figure 4(b)) corresponding to a destabilization free energy of 1.1 kcal, twice the free energy found for the Cowtown and Bunbury mutants. These results estimate 0.55 kcal/mol of free energy for each of the two salt links at the β-chain C terminus (0°C).

When the bulky N-ethyl succinimide reagent (NES) is bound to the exposed sulfhydril at Cys93β (Figure 1), it sterically interferes with the β-chain C terminus in deoxy Hb (Perutz, 1970; Kilmartin *et al.*, 1975) and produces an eightfold acceleration at that position, just as for desHis Hb. Does NES break both the internal salt link and the cross-subunit salt link as in desHis Hb, or do these modifications impose different effects which coincidentally appear equivalent? An identity test was performed using the doubly modified NES-desHis protein. If these different modifications impose different effects, then the two together should be more destabilizing than either one alone. The double modification has the same effect as either one separately (Figure 4(b)). Evidently these modifications, alone or together, remove both the internal and cross subunit linkages.

Surprisingly, these modifications also destabilize the distant α-chain N terminus, across the subunit interface with Hb still in the T-state (contrary to the concerted (MWC) model). Figure 4(a) shows that HX at the α-chain N terminus is made faster by a factor of 2.1, a destabilization of 0.4 kcal. The summed destabilization of the T-state measured at the chain termini for these modifications is 3.0 kcal/tetramer.

The global change in allosteric free energy due to the NES and desHis modifications was studied under similar conditions (5°C, same solvent) by their coupling to subunit dissociation (G. J. Turner & G. K. Ackers) and oxygen binding (J. A. Westrick & S. J. Gill). These approaches measure an overall $\Delta\Delta G$ for the T to R transition close to 3 kcal, in agreement with the HX results measured at the local positions.

The cross-subunit ion pair: Hb Kariya

The Hb Kariya mutation (Lys40αGlu; Harano *et al.*, 1983; Imai *et al.*, 1989) eliminates the cross-subunit salt link joining the terminal carboxylate group of His146β to the side-chain of Lys C5 (40)α. In deoxy Hb Kariya, the sensitive NH groups at

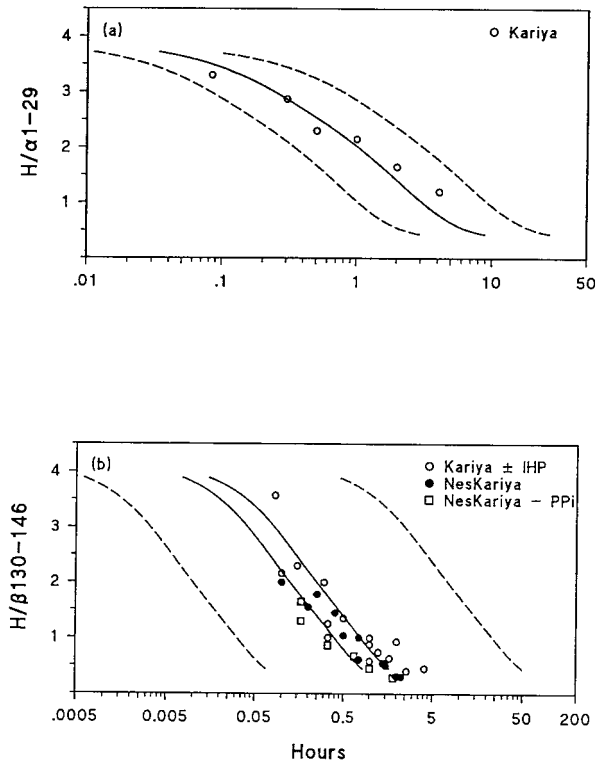

Figure 5. Hb Kariya (Lys C5(40)α to Glu). Data are for Hb Kariya with and without 1 mM IHP (but with PPi), Hb NES Kariya, and Hb NES Kariya with neither PPi or IHP present. The Kariya mutation destabilizes the β C terminus by 1.85 kcal (30 × in HX). It breaks both His146β salt links (insensitive to further destabilization by NES) and nullifies about half the stabilization normally due to PPi. Like NES and desHis, which also break both His146β links, it destabilizes the α N terminus by ~0.6 kcal (~3 × in HX).

the β C terminus then exchange faster by 30-fold (Figure 5(b)), indicating a local destabilization free energy of 1.85 kcal/site. The Kariya mutation also destabilizes the remote α-chain N terminus (Figure 5(a)) by threefold, or 0.6 kcal, similar to the effect of the NES and desHis modifications at the same positions.

Does the Kariya substitution also break the internal β-subunit salt link? An identity test was performed by imposing the NES modification which does break both salt links (previous section). Data for the doubly modified Hb have some scatter (Figure 5(b)) but show that any additional HX acceleration is less than twofold, much less than the eightfold acceleration seen for NES alone, indicating that the Kariya modification alone severs both the internal and the cross-subunit salt links. These two salt links account for 1.1 kcal (see above) of the 1.85 kcal/site destabilization measured for Hb Kariya at the β C-terminal segment.

Part of the additional destabilization at the β-chain termini in Hb Kariya may be explained by an inhibition of the DPG interaction. Figure 5(b) includes data for Hb Kariya in the absence of pyrophosphate effector (stripped Hb). The removal of PPi makes HX at the β-chain termini faster by twofold (0.4 kcal/site) or less, compared to a fourfold effect (0.75 kcal/site) normally found in unmodified deoxy Hb (see below). Thus the Kariya mutation also acts to nullify half of the stabilization provided by the phosphate effector, perhaps due to a local charge effect.

We considered the possibility that Hb Kariya might spend some small fraction of time in the R-state. A tiny equilibrium population of R-state Hb could account for a disproportionate HX acceleration since the exchange rate of the β C-terminal segment in the R-state is faster than in T-state Hb by 750-fold (Louie *et al.*, 1988a,b). A test for this effect (McKinnie *et al.*, 1991) used IHP (1 mM) which strongly biases the equilibrium toward the T-state (in addition to the PPi normally present). No effect was found (Figure 5(b)), indicating that no significant contribution to the HX acceleration was made by some fractional R-state population.

In summary, the Kariya modification breaks the two His146β salt links (1.1 kcal/site) and also negates half the phosphate effect (~0.4 kcal/site), accounting for 1.5 of the measured 1.85 kcal/site. In addition, the Kariya modification destabilizes the remote α-chain N terminus, as do other modifications that remove the β-chain C-terminal cross links. The total destabilization energy measured for the Kariya mutation at the two chain termini is 5 kcal/tetramer, in reasonable agreement with the overall $\Delta\Delta G$ measured by global methods under similar conditions (5°C), of ~6 kcal/tetramer, by subunit dissociation (G. J. Turner & G. K. Ackers) and oxygen binding (J. A. Westrick & S. J. Gill).

Allosteric effectors: phosphate and chloride

In vivo, the deoxy T-state is stabilized by salt links mediated by chloride anions at the α N terminus (Chiancone *et al.*, 1975; Arnone & Williams, 1977; Perutz *et al.*, 1994) and diphosphoglycerate (DPG) at the β C terminus (Benesch *et al.*, 1969; Arnone, 1972; Russu *et al.*, 1990). Our usual experimental solutions include pyrophosphate (PPi; 0.02 M) which serves as an analog of DPG. Figure 6 shows HX data for deoxy Hb with and without chloride and with and without PPi.

The removal of chloride speeds HX at the α N terminus by 3.6-fold compared to solutions containing NaCl up to 0.25 M (Figure 6(a); with or without PPi), indicating ~0.7 kcal in local stabilization energy for the chloride-mediated link. The removal of PPi speeds HX at the β-chain C terminus by fourfold (Figure 6(b); with or without Cl^-) corresponding to 0.75 kcal in stabilization free energy.

HX at the β C terminus is slowed equally by PPi, DPG, and IHP (Louie *et al.*, 1988b). These effectors bind T-state Hb with different affinities and accordingly stabilize the T conformation by different amounts because the T to R transition ejects the

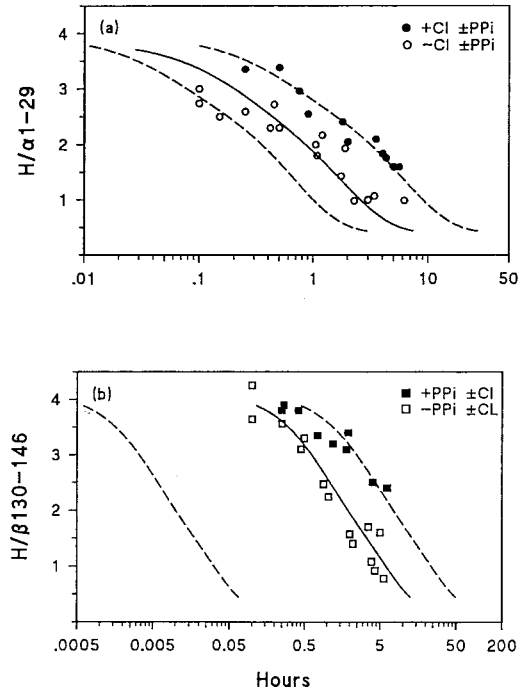

Figure 6. Allosteric effectors (PPi and chloride). Filled circles in (a) are for unmodified HbA in 0.25 M NaCl. Removal of chloride destabilizes the α N terminus by ~0.6 kcal (~3 × in HX), equal to desArg (the curve drawn is from Figure 7; ±PPi has no effect). PPi removal destabilizes the β C terminus by 0.75 kcal (4 × in HX) as for DPG and IHP (±Cl^- has no effect).

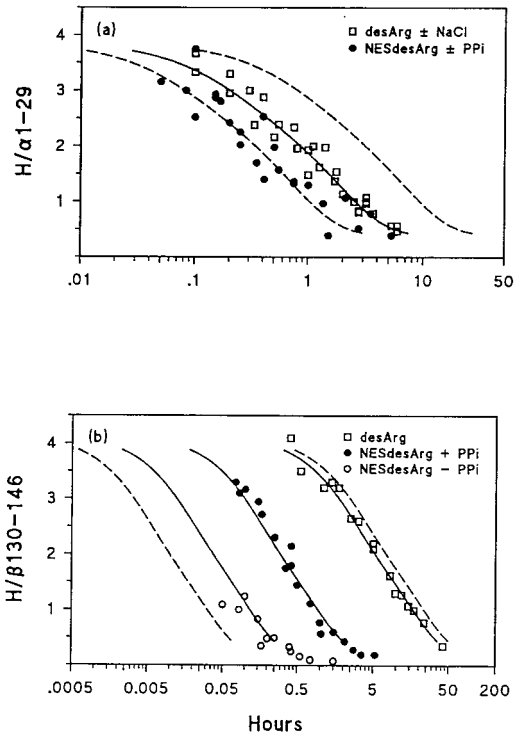

Figure 7. The terminal Arg HC3(141)α interactions. The desArg141α modification breaks both of the α to α C-terminal to N-terminal bonds (Figure 1(a)) and destabilizes the α N terminus by ~0.7 kcal (~3.6 × in HX), about equal to Cl^- removal alone (Figure 6). Removal of Cl^- in desArg Hb has no additional effect. The remote NES effect is still seen, indicating that the remote effect does not operate by breaking the C to N-terminal link. The desArg modification has minimal effect at the remote β C terminus (0.2 kcal; 1.4 × in HX), but NES and PPi removal have larger than usual effects, pointing to some population of the R-state and therefore a much larger underlying global effect for desArg than is measured at the two chain termini. All of these destabilizing modifications together, in deoxy Hb, cause HX rates to reach (a) or approach (b) the fully liganded R-state rates.

entire effector molecule and therefore feels the entire differential binding energy (Benesch *et al.*, 1969; Imai, 1982). Evidently the transient unfolding that allows HX at the β C-terminal segment does not dissociate these effectors but only breaks perhaps one bond, presumably at His143β (Figure 1(b)). The HX change registers only the energy due to the bond(s) broken and not the entire phosphate-binding energy.

Alpha chain linkages: desArg HC3(141)α

The two α-chains in deoxy Hb have a reciprocal N-terminal to C-terminal and also a C-terminal to C-terminal bonding pattern that help to stabilize the T-state (Figure 1(a); Perutz, 1970; Arnone & Williams, 1977; Kavanaugh *et al.*, 1995). Proteolytic removal of the C-terminal Arg141α to form desArg Hb accelerates HX of the allosterically sensitive NH groups near the α-chain N terminus by 3.6-fold (Figure 7(a)), indicating a destabilization of 0.7 kcal (equation (1)) at that position. NH groups at the remote β-chain C terminus are only slightly affected (1.4-fold, 0.2 kcal).

The $\alpha_1\alpha_2$ interaction is mediated by chloride ions apparently delocalized in the central Hb cavity (Arnone & Williams, 1977). Removal of chloride destabilizes the α-chain N terminus by ~0.6 kcal (Figure 6), about equal to the desArg effect. An identity test with both chloride and Arg141α removed shows the same effect as for either one separately (Figure 7(a)), confirming that both Arg141α and chloride are necessary to form the stabilizing bond.

An identity test was done to determine whether the modifications at the β-chain C terminus that destabilize the remote α-chain N terminus do so by breaking the cross link at that position (Figure 1(a)). In desArg Hb, the NES modification further destabilizes the α N terminus by 0.5 kcal (Figure 7(a)), about equal to its effect in unmodified deoxy Hb. Since the C-terminal α_1 to N-terminal α_2 linkage is already severed in desArg Hb, it appears that the long-range effect does not operate by severing this bond but must use some alternative pathway, perhaps involving Lys127(H10)α (see Figure 1(a)).

In the doubly modified NES desArgHb, the β C terminus shows an enhanced NES effect

(18-fold compared to the eightfold seen before). This suggests the rise of some significant R-state population (~1%). When NES desArg Hb is further destabilized by removal of PPi effector (a test for R-state occupation, a further larger than normal effect (eightfold) is seen at the sensitive β C terminus (Figure 7(b)). These results suggest that the initial desArg modification causes a global destabilization larger by several kcal than is measured at the two chain termini studied here, as was found in subunit dissociation studies (Turner *et al*., 1992).

In NES desArg deoxy Hb with PPi removed, the chain termini reach (α-chain) or approach (β-chain) the rate characteristic for the liganded R-state (Figure 7) even though the Hb is unliganded (Figure 7(b)). This result provides the important demonstration that the degree of HX slowing seen in the T-state depends on the condition of the protein structure and its stabilizing bonds and not on liganding *per se.*

Discussion

One now commonly attempts to ''explain'' protein function in pictorial terms based on crystallographic or NMR structures. Such explanations are unavoidably qualitative in nature. It is true that theoretical models of allosteric phenomena have emphasized change in structure (Wyman, 1948; Monod *et al*., 1965; Koshland *et al*., 1966; Wyman & Gill, 1990), but also fundamental in linked function theory is change in structural free energy. The measurement of structurally resolved energy relationships will be necessary to test structure-based inferences, to expose connections that may be less than obvious to structural studies, to develop insightful structure-function models of allosteric behavior, and to obtain quantitative tests of these models.

Proof of concept

The present work further develops and tests a quantitative energy-based approach to structure-function relationships. The HX methods used here apply several approaches in tandem: (1) function-based HX labeling using an exchange-in and exchange-out protocol to selectively place exchangeable tritium label just on sites that change their HX rates in the allosteric T to R transition; (2) analysis to identify the labeled positions and determine their HX rate behavior, using proteolytic fragmentation, fragment separation by fast HPLC, and liquid scintillation counting; and (3) interpretation of the HX results in terms of the free energy of structure change using the local unfolding model for HX behavior. These approaches have been summarized before (Englander & Englander, 1994; Bai *et al*., 1995).

The results obtained provide a broad-ranging test for the validity of these approaches. The functional labeling method successfully places HX label specifically on segments of the protein that experience change in the T to R transition. The fragment-separation method successfully isolates two protein segments, at the α-chain N terminus and the β-chain C terminus, that carry sites importantly involved in the allosteric function, and one found previously in the β-chain F-FG segment (Englander *et al*., 1983). As suggested by the local unfolding model, these segments demonstrate the concerted exchange of sets of amide NH groups (five, four, and seven amino acid residues) and each set changes as a group in the T to R transition to a new HX rate that is faster by a large factor (9, 750, and 30, respectively).

To study individual allosterically sensitive salt links that contribute to this behavior, we imposed modifications that interrupt known bonding interactions and measured the effects both locally and at a remote allosterically involved site. Measured changes in structural free energy show widespread internal consistency and agreement with external information. Removal of a given bond by different modifications produces the same destabilization free energy (desArg and removal of chloride; NES, desHis, and both together; Kariya and NES Kariya). Modifications that are structurally independent produce additive free energy values (NES and removal of DPG; Louie *et al*. (1988b)). Intersecting modifications show energetic overlap (Kariya and PPi removal). Good agreement was found with some independently measured values of cooperative free energy change (Table 1). These results demonstrate the ability of the HX approach to provide site-resolved structural and energetic information.

An important limitation should be noted. It now appears that many hydrogen atoms in typical stable proteins exchange by way of local fluctuations that may involve the motion of only one or two amino acid residues (Milne *et al*., 1998). Under those conditions it is not clear whether HX will simply correlate with the free energy of local structural stability.

Ion pair bonds

The role of ion pair bonds in structure stabilization has been the subject of considerable discussion (Hendsch & Tidor, 1994; Waldburger *et al*., 1995; Sindelar *et al*., 1998). The individual ion pairs measured here exhibit stabilization free energies between 0.4 and 0.75 kcal/mol (0°C, 0.35 M ionic strength). It can be noted that the free energy of bond formation and the free energy by which the bond stabilizes structure are different since $\Delta G_{bond} = -RT \ln K_{bond}$ while $\Delta G_{stab} = -RT \ln (K_{bond} + 1)$ (Sharp & Englander, 1994). Therefore the stabilization energies measured here correspond to bond free energies of only 0.0 to 0.6 kcal/mol and equilibrium constants for bond formation (K_{bond}) between 1 and 3.

As suggested by crystallographic results (Arnone & Williams, 1977; Perutz *et al*., 1994), the present results confirm that Cl^- mediates an α-chain to α-chain bonding interaction (Figure 1(a)), since the α N terminus is destabilized equally on removal of Cl^-, or the Arg141α residue (desArg Hb), or with both removed together. It seems remarkable that this should be so even though the chloride appears to be disordered in the central cavity of Hb rather than site bound (see Perutz *et al*., 1994). In this regard the fact that the bond formation equilibrium constant is only ~2.6 might be an issue.

The allosteric machinery

An earlier survey using low resolution HX measurements showed that only a quarter of Hb's peptide NH groups are affected by the T to R transition, indicating that only a limited part of the protein participates actively in the allosteric mechinery. This result in fact probably overstates the amount of the protein that is involved since change at a single bond can affect all of the hydrogen atoms that exchange through a sizeable unfolding reaction. All of the effects seen are in the direction of destabilization of the Hb structure upon liganding. When ligand is bound, binding energy is sacrificed to raise allosterically involved regions of the stable deoxy form to a higher energy level (Englander & Englander, 1994).

The present results indicate that about 70% of the overall allosteric free energy in Hb couples to the region near the β-chain C terminus. The important role of this region has been stressed before (Gelin *et al*., 1983). In many of the modified Hbs tested, the energetic change measured at the β C terminus plus the α N terminus is about equal to the total free energy change seen in the overall T to R transition (Table 1; Englander *et al*., 1992). Better understanding of why this should be so must await further elaboration of the allosteric machinery.

Other information makes it clear, however, that important changes must occur elsewhere as well. Previous HX work found a change of 4 kcal/tetramer in the T to R transition at the F-FG segment of the β-chain (Englander *et al*., 1983). The desArg modification has a large effect on allosteric free energy (Turner *et al*., 1992) that is not measured at the two termini studied and therefore must be ''stored'' at positions that do not fully couple to the chain termini measured here. The remote effects that connect the two termini require that intervening regions become involved. These and other observations (Ackers, 1998) ensure that additional regions of the allosteric machinery are utilized to store and transfer energy.

Cooperativity within the T-state

There has been much discussion concerning the assumption made in the two-state concerted allosteric model (Monod *et al*., 1965) that structure and energy changes do not cross subunit interfaces within the quaternary T-state (Edelstein, 1996; Ackers *et al*., 1997; Henry *et al*., 1997; Huang *et al*., 1997; Shibayama *et al*., 1997; Ackers, 1998; Shibayama *et al*., 1998). The present results uncover some additional counter examples. When the intersubunit interaction, His146β to Lys40α, is broken (NES, desHis, Kariya), stability changes are felt at other sites, both nearby and at the distant α-chain N terminus about 30 Å away from the site of the experimental modification. This occurs while Hb remains in the quaternary T-state. That these sites, which together account for most of the alkaline Bohr effect (Riggs, 1988), may functionally interact has been suggested before (Kwiatkowski & Noble, 1987). Results presented in the accompanying paper and elsewhere (Holt & Ackers, 1995; Paoli *et al*., 1996) show that heme liganding itself also produces cross-subunit effects in the T-state.

These observations and the other results presented here argue that efforts to understand allostery must move past the domain of simplified models and focus on defining the detailed intramolecular pathways that carry allosteric information within protein molecules. The present results illustrate the contribution that energy-based approaches, including the HX methods used here and their further generalization, can add to this effort.

Materials and Methods

Experimental conditions

Experimental solutions usually contained 0.1 M NaCl, 0.1 M sodium phosphate buffer (pH 7.4). For exchange-out experiments, deoxy conditions were maintained using 20 mM sodium pyrophosphate together with 5 mM ferrous ion (from $Fe(NH_4)_2(SO_4)_2$). Pyrophosphate in addition mimics DPG, the naturally occurring phosphate effector. In phosphate removal experiments, Hepes buffer was used for pH control, and glucose oxidase (20 ug/ml), glucose (0.3%, w/v), and catalase (2 ug/ml) were used for deoxygenation (Englander *et al*., 1987). In chloride-removal experiments, pH buffers were prepared by weighing predetermined ratios of mono and dibasic phosphate. All procedures were performed at 0°C.

Mutants and modifications

Human Hb was isolated from freshly drawn blood by standard methods. DPG was removed and buffer conditions adjusted by rapid microdialysis (Benesch *et al*., 1969) overnight.

NES Hb (N-ethyl succinimidyl Cys93β; Kilmartin & Hewitt, 1971) was prepared by treating hemoglobin with freshly dissolved N-ethyl maleimide (NEM; 2-3 mM Hb, 6 mM NEM, 1.5 hours, 0°C, 0.1 M $NaPO_4$, 0.1 M NaCl (pH 7.4)). Excess NEM was removed by dialysis or gel filtration. The degree of reaction was checked using [^{14}C]iodoacetamide. DesArg Hb was prepared with carboxypeptidase B (Sigma; 3 mM Hb, 4 uM enzyme, five hours, 22°C, 0.1 M Tris (pH 8)). Release of the C-terminal residue was followed by amino acid analysis as arginine

plus ornithine, and the purity was checked by slab gel electrophoresis. NES desArg Hb was prepared by treating desArg Hb with NEM.

Gifts of modified Hbs were generously supplied by Drs Gary Ackers and George Turner (Kariya, Bunbury and Barcelona), Dr Daniel Shih (Cowtown), and Dr Chien Ho (desHis146β).

Functional labeling

Allosterically sensitive amide NH groups in Hb exchange much more rapidly in the R-state than in the T-state. The HX functional labeling method exploits these rate differences to selectively label and study individual allosterically involved regions (Englander & Englander, 1994).

Hb (0.5 ml, ~1 mM in heme) was initially labeled by exchanging-in in tritiated water (pH 7.4, 0°C; 5-15 μl of 1 Ci/ml) in the fast-exchanging oxy R-state for a limited time period (35 minutes), then switched to the slowly exchanging deoxy T-state (~2 mg dithionite). Immediate passage through a G25 Sephadex column (equilibrated with pH 7.4 buffer deoxygenated by ferrous pyrophosphate or the glucose oxidase-catalase system (Englander *et al.*, 1987)) removed dithionite (exposure less than one minute at 0°C) and initiated the exchange-out of tritium (Englander *et al.*, 1987). Tritium on allosterically insensitive NH sites that become labeled in the exchange-in time period exchanges-out equally rapidly in deoxy Hb and is soon lost. However, the tritium label on allosterically sensitive sites is locked into the slower-exchanging deoxy Hb. T-state Hb, under argon, was allowed to exchange-out for predetermined time periods and then was analyzed for label still remaining on particular segments by a fragment-separation method (Englander *et al.*, 1985). Timed HX points generate an HX curve that is highly enriched in the targeted allosterically sensitive NH groups.

The background curve

The HX behavior of the sensitive NH groups is partly obscured by the fact that some allosterically insensitive NH groups contribute to the early part of the measured HX curve. A background curve that portrays the HX of mainly these insensitive NH groups was produced by reversing the selection procedure (deoxy exchange-in, oxy exchange-out). The protein is exchanged-in for the same time as before but now in the slowly exchanging deoxy T-state. The very same insensitive NH groups as before become labeled, but the sensitive NH groups, now in their slow form, tend not to become labeled. The protein is then switched to the fast-exchanging, liganded form (addition of CO-containing buffer) and exchanged-out. The few sensitive NH groups that became labeled in the deoxy exchange-in are rapidly lost, but the insensitive NH groups exchange-out just as before so that mainly these are measured, generating the background curve. Subtraction of the background curve from the experimental curve yields a curve for sensitive NH groups only.

When the oxy and deoxy exchange rates of the allosterically sensitive NH groups are not very different, as for the Hb α-chain N terminus where oxy and deoxy rates differ by a factor of 9, even the background curve is contaminated by sensitive NH groups. To measure these sensitive NH groups, we exchanged-in deoxy Hb for the usual 35 minutes, then exchanged-out initially in the deoxy form for 20 minutes to remove the large number of fast exchanging allosterically insensitive NH groups while retaining the contaminating sensitive NH groups. The Hb was then liganded by mixing with CO-saturated buffer. The contaminating sensitive NH groups switch to the fast form. Their resulting accelerated exchange was measured. For the α N-terminal segment the result shows one NH group exchanging with a half time of four minutes. The corrected background curve contains two insensitive NH groups with half time ~15 minutes.

The ability of the functional labeling method to portray the HX behavior of functionally sensitive NH groups decreases with a decrease in the ratio of oxy to deoxy HX rates. The experimental and background curves draw together and the difference becomes small. In compensation, as this ratio becomes smaller, the contribution of the measured site to the allosteric energy ($\Delta\Delta G$ in equation (4)) becomes less important and approaches zero.

Fragment separation analysis

The measurement of tritium label on allosterically sensitive protein segments was accomplished by fragmentation of the labeled protein with acid protease and separation of the fragments by HPLC (Rosa & Richards, 1979; Englander *et al.*, 1982; Rosa & Richards, 1982; Mallikarachchi *et al.*, 1989). After specified exchange-out time periods, labeled hemoglobin samples (~0.3 ml) were changed to ~pH 2.7 by gel filtration (1 cm × 4 cm column of G25 fine Sephadex, 0.1 M sodium phosphate, ~30 seconds). Under these conditions Hb is unfolded and the freely exposed NH groups exchange with half time ~one hour (Bai *et al.*, 1993; Connelly *et al.*, 1993). The eluant protein with label in place was collected (~one minute), fragmented with pepsin (60-120 ug/ml, five minutes), and the fragments were separated by reverse phase HPLC (12-20 minutes). HPLC columns immersed in ice water were run at 1 ml/minute with a linear gradient of 20% to 28% solvent B in 20 minutes to recover both the α1-29 and β130-146 fragments or with a narrower gradient to recover just one of the fragments more rapidly (solvent A, 0.05 M sodium phosphate; solvent B, 10% solvent A and 90% acetonitrile (pH 2.7)). To correct for tritium loss during the separation, counts measured on the sensitive sites of the α1-29 and the β130-146 fragments were multiplied by 1.23 and 1.5, respectively, as calibrated in previous work (Ray & Englander, 1986; Louie *et al.*, 1988b).

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