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Allosteric Sensitivity in Hemoglobin at the α-Subunit N-Terminus Studied by Hydrogen Exchange†

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ABSTRACT: Allosteric structure change in human hemoglobin was studied by hydrogen–tritium-exchange methods. The functional labeling method used takes advantage of the change in H-exchange rate at allosterically involved sites to selectively label, with tritium, H-exchange sites that are fast in one protein state and slow in another. The position of the labeled sites can then be located by the medium-resolution fragmentation–separation method. These methods reveal 5 allosterically sensitive, H-bonded, peptide NH's within the first 12 residues of the α chain. All five exchange with solvent protons at similar rates in deoxyhemoglobin (T form), and all shift to a new rate, about 30-fold faster, in the liganded protein (R) form. This indicates a decrease in structural stability at the α-chain N-terminus in going from the T to the R form, consistent with the loss of stabilizing interactions in that segment. The results indicate a loss of perhaps 2 kcal/mol in stabilization free energy and thus document a significant role for changes at the α-chain N-terminus in the allosteric transition.

Since the early work of Wyman and others on linked functions (Wyman, 1964, 1968; Monod et al., 1965; Koshland et al., 1966) and its widespread application to hemoglobin and other systems, the fundamental role of protein structure change in the regulation of protein function has become abundantly clear. Also fundamental in the linked function analysis is the issue of change in protein structural energy. In order to *understand* the regulation of function in any protein molecule, it will be necessary to detect the individual structure changes that contribute to the overall allosteric transition and to measure the contribution of each component change in terms of free energy.

At present, the molecular structures of about 200 protein molecules are known to atomic detail, and a large number of regulatory proteins that utilize the principles of structure change have been identified. Yet only in hemoglobin can it be said that the probable component changes are known in

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some detail (Perutz, 1970; Baldwin & Chothia, 1979; Ho & Russu, 1981; Friedman, 1985b), and even here, it has not been possible to verify their importance by measuring the energy contribution that each change makes to the overall allosteric transition, though some work has begun to address this basic issue (Englander, 1975; Englander et al., 1983; Smith & Ackers, 1985; Friedman, 1985a).

Hydrogen-exchange measurements have promise in this regard. The exchangeable hydrogens of proteins provide a multitude of probe points distributed throughout every protein molecule. Local changes in protein structure seem to be quite generally reflected in the exchange rates of protons on affected segments. Thus, H-exchange measurements have the capability of defining, in solution, those segments of a protein that do and do not experience changes in any protein functional transition. Further, unlike most other measurements one can make, the size of the change in H-exchange rate may key to the change in structural free energy of each affected segment (Englander, 1975; Englander & Kallenbach, 1984). Thus, H-exchange measurements may additionally indicate the importance of individual changes in terms of the energy invested in each one, and so help to trace out the way in which allosteric energy change is conducted through a protein.

Our previous hydrogen-exchange work with hemoglobin has been directed at developing these capabilities by finding the various structure changes that occur and understanding the relationships connecting structure energetics with H-exchange rate [see Malin & Englander (1980) and prior papers]. The present experiments continue this effort. The functional labeling method (Englander & Englander, 1983) was used to induce the self-labeling of some allosterically sensitive H-exchange sites that exchange in a particular time region of the overall hemoglobin H-exchange curve. Sites grouped in a segment of the α chain became labeled. The position of this allosterically active segment, at the α-chain N-terminus, was determined by using the fragment separation method (Rosa & Richards, 1979, 1981, 1982; Englander & Englander, 1985). This result is fully consistent with the conclusions of Perutz (1979) concerning crystallographic changes at that position, and the qualitatively large effect on H-exchange rate, which is thought to correlate with structural energy change, tends to establish the importance of this change in the equilibrium allosteric transition.

Materials and Methods

Hemoglobin was prepared from fresh human red cells washed with 1% NaCl, lysed by hypotonic shock, centrifuged to remove stroma, stored in liquid nitrogen, and dialyzed into 0.1 M sodium phosphate and 0.5% NaCl at pH 7.4 for use.

Three acid proteases were used in this work. Pepsin, obtained from Worthington, was dissolved in and dialyzed against 0.05 M sodium acetate buffer (pH 4.5, 0 °C) to remove small fragments and then stored frozen. Surecurd, an impure acid protease preparation from *Endothia parasitica*, obtained from Pfizer in aqueous solution (rennet), was dialyzed against 0.05 M sodium acetate and chromatographed through a C18 semipreparative high-performance liquid chromatography (HPLC)[1] column. The gradient was 0–50% C18B solution for 30 min at 0 °C. The protein peak was dialyzed against cold water to remove organic solvent, lyophilized, dissolved in 0.05 M sodium acetate, and kept frozen in small aliquots until use. Acid protease from *Aspergillus saitoi*, obtained from Sigma, was dissolved in 0.05 M sodium acetate, filtered (5-μm Millipore filter), and then gel filtered through a TSK-SW G2000 column equilibrated in the same buffer. The protease peak was lyophilized, redissolved in and dialyzed against 0.01 M sodium acetate buffer at pH 4.1, purified by anion-exchange chromatography (Mono Q column from Pharmacia; gradient 0–0.15 M NaCl in 0.01 M sodium acetate buffer at pH 4.1; protease emerges at ~0.1 M salt), and stored frozen. Homogeneity was demonstrated by SDS gel electrophoresis.

Proteolytic fragments of hemoglobin were separated by HPLC methods. A two-pump gradient system and HPLC columns were from Waters. Columns used were C18 7.8 × 300 mm semipreparative columns for separation of α and β chains (Congote et al., 1979), C18 3.9 × 300 mm columns for amino acid analysis, and a 3.9 × 300 mm phenyl column for separation of proteolytic fragments. Solutions used to generate chromatographic gradients were as follows. For the separation of peptide fragments, the solutions used were the following: phenyl A—0.05 M sodium phosphate (monobasic) adjusted to pH 2.7; phenyl B—60% acetonitrile, 30% dioxane, and 10% phenyl A. For Surecurd purification, solutions were as follows: C18A—0.05 M sodium acetate, pH 4.5; C18B—60% acetonitrile, 30% dioxane, and 10% C18A. For amino acid analysis, the stock solution was 11.5 g of $NaH_2PO_4 \cdot H_2O$ and 30.7 g of Na_2HPO_4 in 1 L of water, pH adjusted to 6.5; solution A was stock solution diluted 10-fold; solution B was 100 mL of stock solution, 400 mL of water, and 500 mL of acetonitrile. Some further details are given in the text.

Amino acid analysis was done to establish the identity of peptide fragments and to measure the concentration of eluant peptide peaks. Peptides eluted from HPLC columns were lyophilized and hydrolyzed in 6 N HCl in sealed tubes under vacuum (110 °C, 20 h). The HCl was then evaporated, and amino acid analysis was done by reverse-phase C18 HPLC using precolumn OPA derivatization (Hill et al., 1982; Fernstrom & Fernstrom, 1981). The gradient used was 30–85% of solution C18B for 30 min at room temperature with 2 mL/min flow rate. An added internal standard of fluorophenylalanine (FPhe) was used for quantitation. This was added at a known concentration to a known volume of the original peptide sample so that it carried through the whole procedure just described. A mix of standard amino acids (Pierce Chemical Co.) plus FPhe, all at 0.5 nM, was run through the OPA analysis, eluant peaks were measured, and the ratio R_1 was calculated for each residue. The peptide sample, with FPhe added at concentration A_0, was hydrolyzed and run through the OPA analysis, and the ratio R_2 was calculated for each residue. Each amino acid was identified by its eluant position and quantified according to eq 1. This

$$A_x = (R_2/R_1)A_0 \quad (1)$$

also leads to the extinction coefficient for each peptide peak, subsequently used to calculate eluant peptide concentrations. In eq 1, A_x = the concentration of the amino acid residue in the peptide sample, A_0 = the concentration of FPhe added to the peptide sample, R_2 = amino acid peak height/FPhe peak height in the peptide sample, and R_1 = amino acid peak height/FPhe peak height in the standard run. General tritium-exchange techniques have been described before (Englander & Englander, 1972, 1978).

Deoxygenation of hemoglobin samples and Sephadex column buffers was accomplished in one of several ways, using either dry dithionite, a ferrous pyrophosphate reduction system, or a glucose oxidase–glucose–catalase system (Liem et al., 1980). Samples were kept deoxygenated by continual flushing

[1] Abbreviations: HPLC, high-performance liquid chromatography; THO, tritiated water; SDS, sodium dodecyl sulfate; OPA, *o*-phthaldialdehyde; FPhe, fluorophenylalanine.

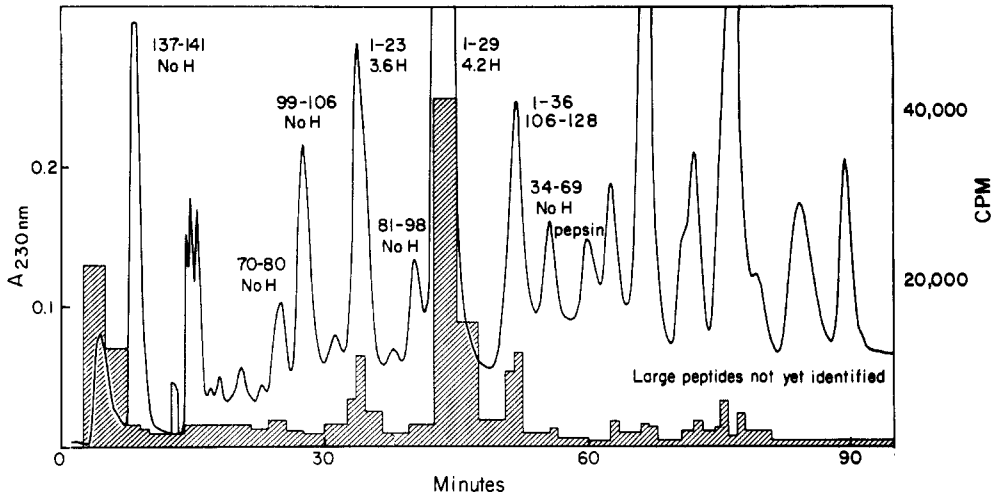

FIGURE 1: α-Chain peptide fragments from hemoglobin selectively labeled at the allosterically sensitive sites in the intermediate II and slow I classes. Oxyhemoglobin was labeled by exchange-in (3.5 h, 0 °C, pH 7.4), made deoxy, exchanged-out for 3 h, passed through a Sephadex column (oxygenated) to remove free THO, and then quenched to slow-exchange conditions (pH 2.7, 0 °C). The α and β chains were separated, then the α chain was proteolyzed with pepsin, and the fragments were separated as shown. Eluant fragments were measured by the area of the 230-nm absorbance profile. Tritium carried on each eluant sample (shaded histogram) was measured by liquid scintillation counting. These data were computed in terms of net peptide NH labeled per segment. Tritium label is found on allosterically sensitive sites near the α-chain N-terminus.

with treated argon gas (Meites & Meites, 1965).

RESULTS

Earlier H-exchange results, obtained by the functional labeling method, have shown that a sizable number of peptide group protons in hemoglobin, about 70 per $\alpha\beta$ dimer, representing one-fourth of hemoglobin's exchanging peptide NH, change their rate of exchange with solvent protons in response to the allosteric form of hemoglobin [see Malin & Englander (1980) and earlier papers]. The sensitive sites can be selectively labeled (functional labeling) by taking advantage of the fact that they exchange at quite different rates in deoxy and liganded hemoglobin, while the insensitive sites exchange at the same rate in the two protein forms.

In the *functional labeling* approach, hemoglobin is allowed to exchange-in for some defined time period in tritiated water while in the fast-exchanging oxy form and then is switched to the slower, deoxy form and exchanged-out. Allosterically *insensitive* sites exchange at the same rate in both protein forms, so that tritium that has exchanged into allosterically insensitive sites during the short oxy form exchange-in period is lost at a similarly rapid rate in the deoxy form exchange-out. In contrast, tritium on allosterically *sensitive* sites is locked in during the slow form exchange-out. Thus, sensitive sites can be selectively labeled and studied. The overall H-exchange curve for hemoglobin (and other proteins generally) is spread out over many orders of magnitude on the time axis. By adjusting the exchange-in and exchange-out time periods and the solution conditions used in the functional labeling steps, one can window in any part of the overall H-exchange curve and selectively label functionally sensitive sites that exchange in that time region.

The functionally involved sites, labeled in this way, can be located by use of a medium-resolution fragmentation–separation method (Rosa & Richards, 1979; Englander et al., 1985). In this method, the selectively labeled protein sample is quenched to slow exchange conditions (pH ~3, temperature 0 °C) and fragmented by use of an acid protease. The fragments are then separated as quickly as possible, by HPLC, and analyzed for carried tritium.

Allosteric Sensitivity in the α Subunit. Figure 1 shows the results of such an experiment. Oxyhemoglobin was initially labeled by exchange-in in tritiated water (3.5 h, pH 7.4, 0 °C). This exposure is expected to label a number of previously studied allosterically sensitive sets of exchangeable peptide group protons. [These have, for record keeping purposes, been named the jump class (5 H per $\alpha\beta$ dimer; Liem et al., 1980), the fast class (7 H; Ghose & Englander, 1974), the intermediate I (4 H) and II (12 H) classes, and the slow I (14 of 18 H labeled) class (Englander & Rolfe, 1973).] The hemoglobin sample was then switched to the deoxy form and exchanged-out for 3 h. According to previous work, the 3-h deoxy exchange-out period should remove, in addition to a lot of tritium on insensitive sites, the label on the fast class and most of the label on intermediate I and should retain label on the jump, intermediate II, and slow I sites. The jump class label is lost during the subsequent Sephadex column run (oxygenated) used to remove the free THO in preparation for the HPLC runs.

Samples labeled in this way (intermediate II and slow I classes labeled) were subjected to the fragmentation–separation analysis. Figure 1 shows the result. The identified fragments isolated as in Figure 1 by HPLC methods from the α subunit of hemoglobin account for most of the α chain, and the rest is presumably represented in the large trailing peaks. Tritium recovered with each fragment was corrected for losses expected during the fragment separation procedures (Englander & Englander, 1985) and calculated in terms of the number of (peptide group NH) sites labeled per fragment. Counts at the very beginning of the elution profile (Figure 1) represent unbound tritium lost during the proteolysis period. Significant label is found only on the fragment α1–29 and its subfragment α1–23. The label on α1–29 must represent either intermediate II or slow I class sites. Experiments were done to test the accuracy of the analysis, to measure the H-exchange rates of these sites in both protein forms, and to better define the location of these sites within the α1–29 segment.

Rapid Isolation of α1–29. To obtain the result indicated in Figure 1, the fragment separation analysis was used. In these experiments, a sample of hemoglobin selectively labeled as just described was initially separated by HPLC into α and β subunits, the α chain was dialyzed and proteolyzed, and the fragments were separated in a second HPLC column. The overall procedure required about 1 h during which time a

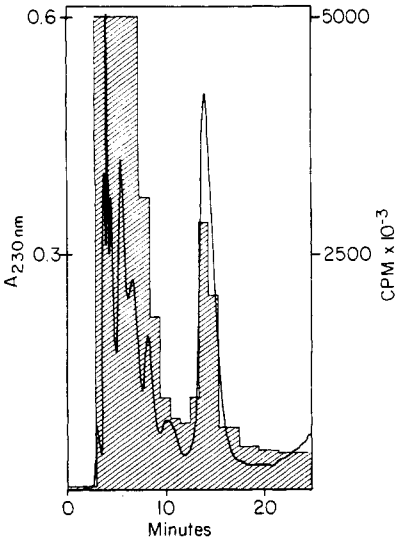

FIGURE 2: Rapid separation of the α1–29 fragment. Hemoglobin was labeled by exchange in tritiated water, then adjusted directly to pH ~2.7 (the slow-exchange condition) by addition of a small predetermined volume of 5 M phosphoric acid, passed through a Sephadex column held under these conditions to remove the free tritium, and treated with pepsin (7 min; no prior α–β separation). The HPLC gradient was designed to obtain α1–29 directly as shown.

significant amount of the label to be measured was lost (~50% overall loss). In further work, it proved possible to obtain the α1–29 fragment directly from whole hemoglobin, without the intervening α–β separation. Figure 2 shows a typical result. The separation directly from hemoglobin produces the α1–29 fragment within 25 min after initially quenching the tritium-labeled protein into the slow-exchange condition. The faster separation results in significantly less loss of carried tritium during the separation procedures (26% loss), therefore smaller correction factors and improved accuracy.

Tests for Accuracy. The accuracy of the fragment separation analysis was tested in experiments like that shown in Figure 2. Samples of hemoglobin were essentially fully labeled by rigorous exchange in tritiated water (pH 9, 37 °C, 48 h). One can then expect to find 27 labeled peptide NH on the α1–29 fragment (less than 29 due to one proline and the N-terminal amino NH). Samples of oxyhemoglobin labeled in this way were analyzed as in Figure 2. In 11 such samples measured over a 2-year period in various contexts, the value found varied between 24.6 and 28.0 H per fragment, with an arithmetic average of 27.0 H. This compares with the expected value of 27 H.

Similar experiments were done to measure the number of H-bonded peptide NH on this fragment, which we define experimentally in terms of a relatively slow exchange rate. Samples of fully labeled hemoglobin were adjusted to pH 5.0 with a predetermined volume of 0.5 M phosphoric acid and then passed into a Sephadex column (pH 5, 0 °C) to remove most of the free tritium and initiate exchange-out. At these conditions, freely exposed peptide NH's exchange with a half-time of 100 s or less (Englander & Poulsen, 1969; Molday et al., 1972); H-bonded NH's are much slower. After 10 min of exchange-out in the column, the sample was eluted, quenched to pH 2.7, and subjected to the fragmentation analysis. In four such samples, values ranging from 25.2 H to 25.8 H were found with an average of 25.4 H. This is somewhat higher than expected. Ladner et al. (1977) infer 20 H-bonded peptide NH on this segment in horse hemoglobin from X-ray diffraction results. The excess NH may represent a species difference, more H bonding than inferred from the crystallographic results including possible H bonding to bound

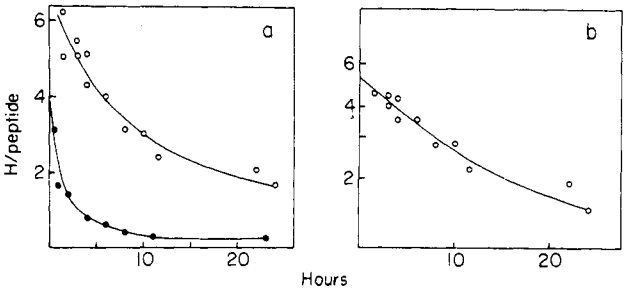

FIGURE 3: Exchange of functionally labeled peptide NH in the α1–29 segment. Oxyhemoglobin was exchanged-in for 3.5 h (pH 7.4, 0 °C), then made deoxy, and exchanged-out in the deoxy form. Samples taken as a function of exchange-out time were put through the fragmentation analysis to measure NH on α1–29 not yet exchanged. The upper curve in panel a shows these results. The background curve (lower curve in panel a), obtained as described in the text (exchange-in deoxy, exchange-out oxy/CO), portrays the contribution made to the upper curve by allosterically insensitive NH. The difference curve, in panel b, then shows the sensitive NH only.

water, some unexpected slowing on non-H-bonded NH, or experimental error.

In all these experiments, the procedures described by Englander et al. (1985) were followed. The HPLC separations were performed at 0 °C and low pH to minimize tritium loss, and corrections for the losses experienced were applied by using a predicted exchange-out curve for the random-chain α1–29 sequence under these conditions, calculated according to Molday et al. (1972), which leads one to expect a loss of 26% of the carried tritium during the overall analysis period.

Number of Allosterically Sensitive Sites. Results described to this point show that some allosterically sensitive sites reside near the α-chain N-terminus, that the N-terminal segment α1–29 can be rapidly isolated from selectively labeled hemoglobin with minimal loss of label, and that the label carried can be measured with good accuracy. Experiments were done to measure the H-exchange rates of allosterically sensitive sites on the α1–29 segment.

Oxyhemoglobin was selectively labeled by exchange-in in tritiated water at 0 °C and pH 7.4 for 3.5 h, then made deoxy by addition of dry dithionite, and passed immediately through a Sephadex column (pH 7.4, 0 °C, deoxygenated with a ferrous pyrophosphate system) to remove the free tritium and initiate exchange-out. Samples were collected under argon and held under deoxy conditions while exchange-out proceeded for increasing periods of time. At various times, aliquots were removed and subjected to the fragmentation analysis to isolate the α1–29 segment and to measure its remaining tritium label (peptide NH not yet exchanged). Results are shown in the upper curve of Figure 3a. This curve represents sites—both allosterically sensitive and insensitive—that become labeled during the 3.5 h (oxy form) exchange-in period and have not yet lost their tritium at the experimental (deoxy form) exchange-out time.

The contribution made to this curve by residual allosterically insensitive NH can be portrayed by carrying through similar experiments with the *exchange-in* done in the *deoxy form*. Here the allosterically *insensitive* sites become labeled just as before, since these exchange at the same rate in oxy and deoxy forms. However, the *sensitive* sites, now in their slow (deoxy) form, tend not to become labeled. Exchange-out was done in the oxy form. The exchange-out kinetics of the sites thus selected were followed by the fragmentation analysis. These experiments produce the bottom, *background* curve of Figure 3a, which represents, as just indicated, the contribution made by allosterically insensitive sites to the upper curve. Subtraction of the smoothed background curve (insensitive H)

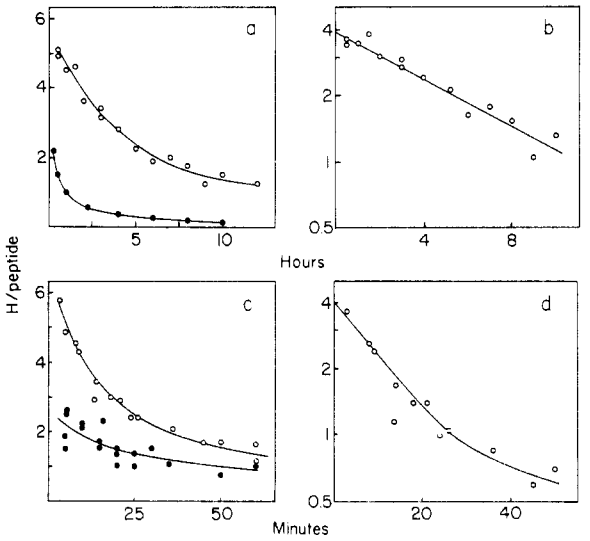

FIGURE 4: Exchange of the allosterically sensitive NH in α1–29 in both hemoglobin forms. Oxyhemoglobin was exchanged-in for 35 min (pH 7.4, 0 °C) and then exchanged-out in either the deoxy (a) or the oxy (c) form. The background curve (lower curves in panels a and c) was obtained by exchanging-in the deoxy form and then exchanging-out oxy. Subtraction yields the difference curves which display the exchange of allosterically sensitive sites on α1–29 in deoxy- (b) and oxyhemoglobin (d).

from the points in the upper curve (insensitive + sensitive H) displays specifically the allosterically sensitive NH, shown in the semilog difference plot in Figure 3b.

The resulting difference plot (Figure 3b) portrays the exchange of five allosterically sensitive peptide NH's (5.2 measured) in the segment α1–29 as they behave in deoxyhemoglobin in solution at pH 7.4 and 0 °C. The semilog plot shows some spread in rates with an average half-time about 10 h. In previous H-exchange work on whole hemoglobin (no subfractionation experiments were done), an "intermediate II" class of allosterically sensitive sites was recognized, consisting of 12 protons with a half-time under these conditions of 9 h. Apparently, the five sites found here represent part of the intermediate II set.

Exchange Rates of Allosterically Sensitive Sites. The fast form, oxyhemoglobin half-time previously determined for the intermediate II class of protons is 15 min (Englander & Rolfe, 1973). Thus, it should be possible to label these sites initially in oxyhemoglobin with a much shorter exchange-in period than the 3.5 h used here, with concomitantly lesser labeling of background-insensitive sites.

Experiments were done in which oxyhemoglobin was initially labeled for only 35 min (~15 min exchange half-time expected). This shorter exchange-in time is convenient but does sacrifice full labeling of the sensitive sites so that one can only expect to find an amount of label corresponding to about four sensitive NH's in this set. Samples labeled in this way were subjected to the fragmentation analysis just described to obtain exchange-out curves for the sensitive protons in α1–29 in both deoxy- (Figure 4a) and oxyhemoglobin (Figure 4c). Conjugate background curves were generated with exchange-in in the slow deoxy form and exchange-out in the fast oxy form (lower curves in Figure 4a,c). Subtraction of the background produces difference curves which portray the exchange behavior of the α1–29 allosterically sensitive NH in deoxy- (Figure 4b) and oxyhemoglobin (Figure 4d).

The difference curves display about four allosterically sensitive NH's per α1–29 segment exchanging with approximate half-times of 6 h in deoxyhemoglobin and 13 min in oxyhemoglobin. The true number of NH's must be a little larger since exchange-in was incomplete. For the same reason, the initial incomplete labeling causes the measured exchange-out curve (Figure 4b) to appear somewhat faster, and more purely single exponential, than before (Figure 3b). Thus, these results seem fully consistent with the estimate of five total NH's in this set and a half-time of ~10 h (deoxy form) obtained by using a much longer initial labeling period (3.5 h, Figure 3b). Also, both deoxy and oxy rates of these sensitive NH's place them within the previously characterized intermediate II class (9-h deoxy and 15-min oxy half-times; Englander & Rolfe, 1973).

Location of the Allosterically Sensitive Sites. The effort was made to define more precisely the location of the five allosterically sensitive NH's within α1-29 by further subfractionation.

Two different sets of subfractionation experiments were carried out. One set of experiments used the Surecurd enzyme, a commercially obtained acid protease derived from *Endothia parasitica*, and another set used an acid protease from *Aspergillus saitoi* (see Materials and Methods). Hemoglobin was labeled by H exchange in tritiated water, and the α1–29 peptide was obtained directly by HPLC, as in Figure 2. Most often the peptide was then kept frozen and stored for brief periods to await analysis. For the subfragmentation analysis, aliquots were defrosted by swirling the test tube in 0 °C water, dialyzed to remove organic solvent (10 min), and then treated with the acid protease (10 min). The subfragments were separated by HPLC and analyzed for carried tritium.

Figure 5 shows HPLC elution diagrams of the α1–29 subfragments. To test the accuracy of the method, experiments were done as before with fully labeled hemoglobin so that the number of labeled sites to be expected is known. Figure 5a shows one of several such experiments. Results obtained are listed in Table I. Good tritium recovery was obtained for the fragments 1–12, 13–19, and the mixed peak 16/19–29. Fortunately, these cover the length of α1–29. (The peak labeled 16/19–29 is a poorly resolved doublet; amino acid analysis for both shoulders indicates one residue of the α19–29 amino acids and ca. half a residue for amino acids α16–18). Poor results for the recovery of expected H were found for the trailing peaks 13–29 and 11–29 and for the early peak 1–9, which was most often only poorly resolved. Therefore, we focus mainly on the three fragments that yield the most credible results.

To localize the allosterically responsive (intermediate II class) sites within α1–29, hemoglobin was selectively labeled as before (exchange-in pH 7.4, 0 °C, 35 min, oxy form) and then exchanged-out in the deoxy form for 2 h to reduce the level of background label on the insensitive sites while retaining most of the label at the sensitive sites. Figure 4 shows that this should label the five sensitive, intermediate II class sites on α1–29 to an extent equivalent to approximately three H's. Hemoglobin labeled in this way was fragmented to obtain α1–29, and this peptide was subfragmented as just described. Three replicate experiments were done. Figure 5b shows some results. Conjugate background experiments were done (exchange-in deoxy, exchange-out oxy) to display the contribution of allosterically insensitive sites to the label on these subfragments (Figure 5c).

Results of the Surecurd subfragmentation experiments are summarized in Table I. Label measured on the initial isolate of α1–29 was in good agreement with the number of sensitive NH's expected (2.3, 2.8, and 3.2 H after 2-h deoxy exchange-out, average of 2.8). In two of the three subfragmentation runs, some residual uncleaved α1–29 peptide was

Table I: Surecurd Fragments[a]

peptide	full labeling			functional labeling		
	measured	av	expected	measured	background	sensitive
1–29 (initial)	25–28	27	27	2.7–3.7	0.36–0.46	2.8
1–9	3.8–4.4	4	7			
1–12	9–11	9.6	10	1.8–2.2	0.04–0.06	2
13–19	5.5–6.8	6.2	6	0–0.1	0.04–0.01	~0
16/19–29	8–11	9.0	11.5	0.15–0.27	0.13–0.2	~0
13–29	7.7–9.7	8.4	16	0.4–0.6	0.2–0.3	0.3
11–29	9.6–12	11	18	0.3–0.5	0.2–0.2	0.2
1–29 (residual)				2.5–3.3	0.3–0.4	2.5

[a] Measured label on the initial α1–29 fragment and on subfragments obtained by proteolysis with the Surecurd enzyme is listed in terms of NH per fragment. For fragments obtained from fully labeled hemoglobin samples, the table lists the range of values found (two to four replicate samples), the average, and the value expected (equal to the total NH present on the fragment). For fragments obtained from hemoglobin that was initially selectively labeled (functional labeling, 35-min oxy exchange-in, 2-h deoxy exchange-out), the table lists the range of values measured (two to four replicate samples), the range found for background-labeled samples, and the difference between these which estimates the allosterically sensitive sites. In interpretation of the subfragmentation results, emphasis is placed on the fragments that test well in the full labeling experiments (1–12, 13–19, and 16/19–29).

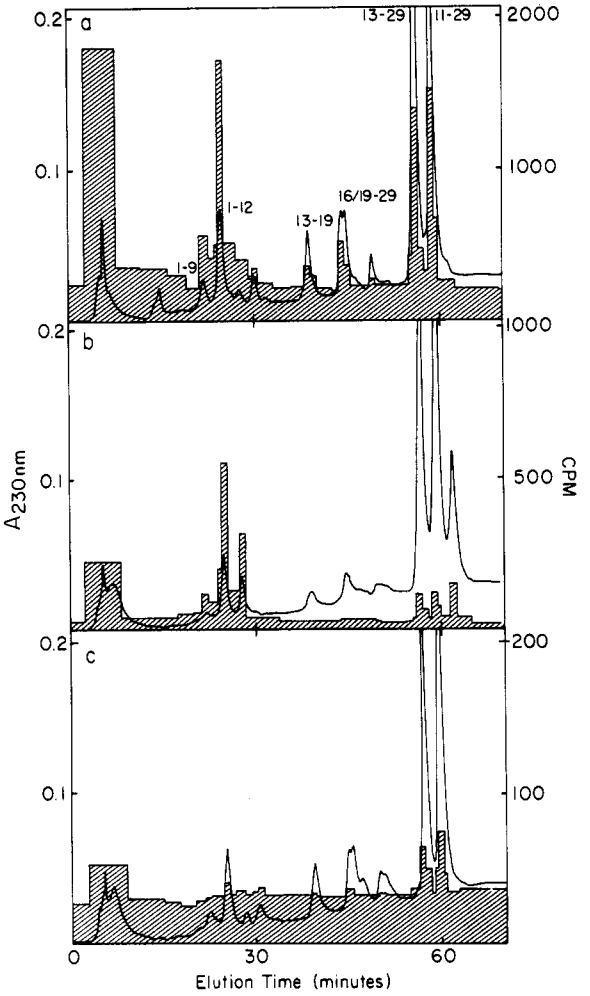

FIGURE 5: Elution diagrams from α1–29 subfractionation experiments using the Surecurd enzyme. Panel a is from hemoglobin initially fully labeled. Fragments 1–12, 13–19, and 16/19–29 yield accurate results (Table I). Panel b shows results from functionally labeled hemoglobin (exchange-in 35-min oxy, exchange-out 2-h deoxy). Panel c shows results from a background experiment (exchange-in deoxy, exchange-out oxy). Results are in Table I.

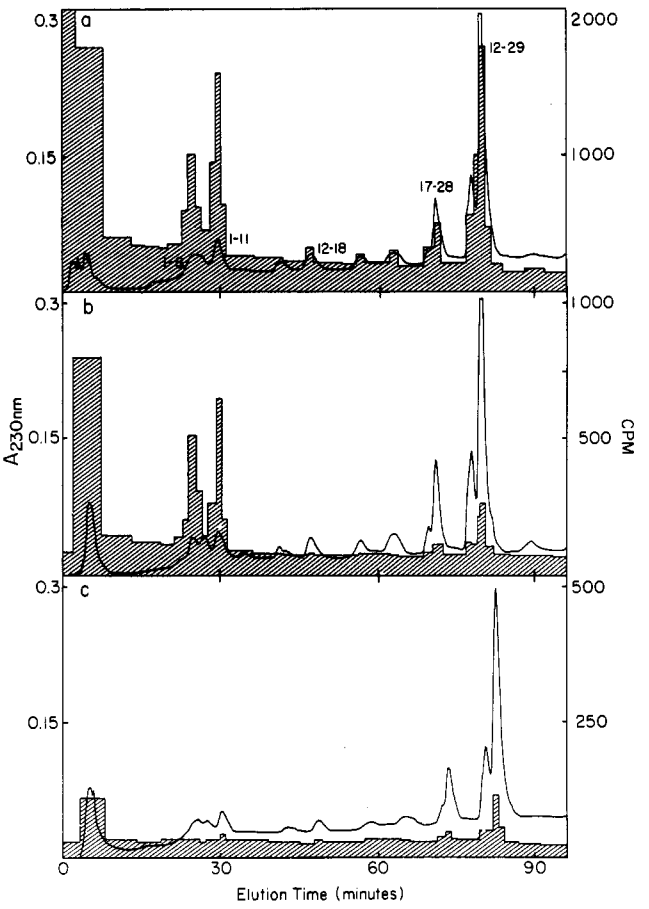

FIGURE 6: Elution diagrams from α1–29 subfractionation experiments using the acid protease from *Aspergillus saitoi*. The experimental design is identical with that for Figure 5; panels a, b, and c show results from experiments involving full labeling, functional labeling as described, and background labeling, respectively. Results are in Table II.

recovered, and ~90% of the originally measured NH label (2.2 and 2.9 H) was calculated thereon. Among the α1–29 subfragments, 70–80% of the labeled NH (2 H average) was recovered on α1–12. The other subfragments that tested well on the prior total labeling tests (α13–19 and α16/19–29) had zero label (less than 0.05 H). (The questionable peaks, α11–29 and α13–29, had 10% of the indicated H.) These results indicate that all the allosterically sensitive sites measured by the differential labeling protocol used reside on the α1–12 segment.

In a parallel attempt to define the location of these allosterically responsive sites, similar subfragmentation experiments were done by using an acid protease from *Aspergillus saitoi*. The α1–29 subfragmentation pattern obtained (Figure 6) is similar to that for Surecurd, although here the early peaks are quite small and not very well resolved. For this reason, part of each sample from the subfragmentation run was used to measure peptide concentration by quantitative amino acid

Table II: *Aspergillus* Fragments[a]

peptide	full labeling			functional labeling		
	measured	av	expected	measured	background	sensitive
1–29	25–28	27	27	3.0–3.3	0.5	2.8
1–8	6.8	6.8	6	1.2–2.1	0.02	1.6
1–11	7–10	8.6	9	1.5–2.3	0.04	2.0
12–18	4.6–6.1	5.6	6	0.2	0.06	0.1
17–28	5.2–6.4	6.0	11	~0.3	0.2	0.1
12–29	11.9–12.5	12	17	~0.5	0.2	0.3

[a] Label recovered on subfragments obtained by proteolysis with the *Apsergillus saito* enzyme is listed in terms of calculated NH per fragment. Other comments are the same as for Table I.

analysis rather than depending upon absorbance measurements (area) as before. Otherwise, the experimental design was as described above.

A typical HPLC result is shown in Figure 6, and Table II summarizes the results obtained. In full labeling tests, good results were obtained for the fragments α1–8, α1–11, and α12–18, but again here recovery was low for the large trailing peaks α17–28 and α12–29. In the selective labeling experiments (35-min exchange-in oxy, 2-h exchange-out deoxy), 2.8 sensitive sites were found for the α1–29 peptide as before. Again, the results indicate that these allosterically sensitive sites are clustered near the N-terminus. Less than 10% of the responsive sites appear to be on the C-terminal fragments.

When serial fragment separation steps are utilized, recovery of tritium is something less than quantitative even after correcting for expected losses due to continuing H exchange during the separation steps. Nevertheless, the results obtained indicate that the five allosterically sensitive peptide NH's in the hemoglobin α chain, which have H-exchange rates characteristic of the intermediate II class (Figures 3 and 4), reside on the α1–29 segment and evidently are clustered close to the N-terminus, within the first 12 residues of the α chain (Tables I and II).

Discussion

Location of Functionally Involved Segments. Experience with protein hydrogen exchange has shown that when a protein engages in any functional interaction or structural change, some of its hydrogens change their exchange rates (Hvidt & Nielsen, 1966; Woodward & Hilton, 1976; Woodward et al., 1982). For hemoglobin's allosteric transition, it has been shown that only a fraction of the exchanging hydrogens (approximately one-fourth) are affected, in most cases by a factor of about 30 in rate; the majority of the exchanging hydrogens are not affected at all (Malin & Englander, 1980). It therefore seems very probable that the affected hydrogens identify regions of the protein structure that are directly affected by the allosteric changes.

The functional labeling method was designed to label sites on proteins that change their H-exchange rates in any functional interaction. In the experiments described here, the functional labeling protocol used selected five peptide NH protons with exchange rates sensitive to the allosteric state of hemoglobin. All of these protons exchange with solvent at approximately the same rate in deoxyhemoglobin (half-time about 10 h at pH 7.4 and 0 °C), and all move to a new rate faster by about 30-fold when hemoglobin is liganded. These protons were found to represent amino acid residues clustered within the first 12 residues of the α chain. (The H-exchange rates observed also indicate that these protons are H bonded in both protein forms.)

An analogous result was obtained by use of similar methods in previous work (Englander et al., 1983), which uncovered a change in exchange rate at the F-FG segment of the β chain. There, seven to eight amide NH's, located sequentially through several turns of the F helix, were all found to exchange at about the same rate in deoxyhemoglobin and about 30 times faster in the liganded protein.

Both of these protein regions are believed to experience allosterically important structure changes. Crystallographic results indicate that a salt-linked network involving the α-chain N-terminus occurs in deoxyhemoglobin but is lost in the liganded form (Perutz, 1970, 1979). Changes in the β-chain F helix, which carries the proximal histidine, have been extensively documented [e.g., see Baldwin & Chothia (1979) and Perutz (1979)]. A comprehensive study of allosteric effects in a large number of mutant and modified hemoglobins (Pettigrew et al., 1982) indicates that the allosterically sensitive region of hemoglobin covers the $\alpha_1\beta_2$ interface, including the α-chain N-terminus and the β-chain F helix. The H-exchange results are fully consistent with these conclusions and thus provide further support for the importance of these regions in allosteric function. Alternatively, given the prior conclusions of others, the present results can be taken to support the correlation between H-exchange changes in particular protein segments and their significant involvement in function.

Determination of Change in Local Structural Energy. Previous papers from this laboratory have discussed the possibility that changes in H-exchange rate may additionally measure the change in structural free energy that the segment experiences. This possibility arises from the unfolding model for protein H-exchange, which pictures that hydrogens H bonded in protein structure can exchange with solvent only during the fraction of time when the H bonds are transiently cleaved and re-formed to solvent, as in eq 2 [see also Linderstrom-Lang (1958) and Eigen (1964)]. A quantity of

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\leftrightarrow}} \text{open} \xrightarrow{k_{ch}} \text{exchange} \qquad (2)$$

available H-exchange data, when interpreted in these terms, shows that exchange rate in almost all cases obeys eq 3, where

$$k_{ex} = K_{op}k_{ch} \qquad (3)$$

$K_{op} = k_{op}/k_{cl}$. When exchange rate is proportional to an opening equilibrium constant (K_{op}), it necessarily keys to the free energy of the opening reaction, as in eq 4. Here k_{ch} is

$$\Delta G = -RT \ln K_{op} = -RT \ln (k_{ex}/k_{ch}) \qquad (4)$$

the exchange rate characteristic of the freely exposed NH, so that k_{ex}/k_{ch} is the slowing factor imposed by involvement in structure. *Change* in structural stabilization may then connect with change in H-exchange rate according to

$$\delta\Delta G = -RT\delta \ln (k_{ex}/k_{ch}) \qquad (5)$$

For the small N-terminal segment of the α chain studied here, exchange rate is increased by about 30-fold upon

switching from the deoxy to the fully liganded protein. The increase in rate in the liganded form is expected, since the loss of a stabilizing salt link (Perutz, 1970) would promote transient unfolding of this segment. Similarly, studies of hemoglobin subunit dissociation (Pettigrew et al., 1982) document a loss of stabilizing interactions in the $\alpha_1\beta_2$ interface region. According to eq 5, the 30-fold rate increase translates into a 2 kcal destabilization of structure near the N-terminus. This is generally consistent with the energy expected for the breakage of a stabilizing salt link.

Interpretation of these results in quantitative energy terms, however, requires caution. Considerable evidence does now point to the role of local unfolding reactions in determining protein H-exchange behavior [reviewed in Englander & Kallenbach (1984); see also Wand et al. (1986)], so that a *qualitative* connection between change in H-exchange rate and change in structural energy seems likely. However, in order to place this kind of interpretation on a quantitative basis, considerable further information will be necessary concerning, for example, the conformation and standard H-exchange rates characteristic of open forms and the extent of overlap of effects felt by allosterically involved segments.

Unfolding Units. The size and conformation of local unfolding units are of great interest. The previously characterized intermediate II class of allosterically sensitive protons involves 12 protons all exchanging at a similar rate in oxyhemoglobin and much slower in the deoxy form. The results obtained here characterize five sensitive protons, near the α-chain N-terminus, with the exchange rate of the intermediate II class in both oxy- and deoxyhemoglobin. The present results suggest but do not prove that these five intermediate II protons are located in a tight cluster, perhaps on sequential amino acid residues. The results do show that all 12 of the intermediate II class of protons are not located side by side on one protein segment but must exist on distributed segments, 5 on α1–12 and the others on other segments. Whether these different segments all "breathe" open together (as in eq 1) or just happen to display very similar exchange rates by chance, both in oxyhemoglobin and in deoxyhemoglobin, remains to be seen.

Conclusions. In summary, the H-exchange results described here, obtained by use of the functional labeling method and fragment separation analysis, indicate a significant energetic destabilization at the N-terminus of the hemoglobin α chain in the allosteric transition from the deoxy to the liganded form (T to R transition). The location of this change is fully consistent with prior crystallographic studies that point to the breakage of a salt link at this position and with studies of modified hemoglobins that localize the allosteric function to the $\alpha_1\beta_2$ interface. The large effect, about 30-fold, on H-exchange rate tends to establish an important role in the overall transition for this change. The change in H-exchange rate suggests the play of about 2 kcal in allosteric free energy at the α-chain N-terminus.

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