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Identification of an Allosterically Sensitive Unfolding Unit in Hemoglobin

JOAN J. ENGLANDER, JOSE R. ROGERO† AND S. WALTER ENGLANDER

Department of Biochemistry and Biophysics
University of Pennsylvania School of Medicine
Philadelphia, PA 19104, U.S.A.

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Hydrogen-exchange studies locate a set of seven allosterically sensitive amide NH protons side by side around two turns of the F–FG helical segment in the hemoglobin beta chain. Some of these protons are on the aqueous protein surface and some deeply inside, yet they all exchange with solvent protons at similar rates. Further, they move in unison to a new common rate when hemoglobin changes its allosteric form. These observations and analogous results for other proteins appear to be inconsistent with penetration-dependent models which relate H-exchange rate to solvent accessibility in the native state. Rather, these results point to sizeable fluctuational distortions that make small sets of protons more or less equally accessible in some transient H-exchange transition state, as visualized in the local unfolding model.

The set of allosterically sensitive protons studied here exchanges 30-fold faster in liganded hemoglobin than in the deoxy form. In terms of the unfolding model, this means that the F–FG structure is relatively destabilized in oxyhemoglobin, so that the allosterically linked change in structural free energy at F–FG favors the deoxy state. The 30-fold change in H-exchange rate suggests a contribution to the allosteric free energy by this segment of 2 kcal (1 cal = 4·184 J).

These experiments utilized a labeling technique, described earlier, that selectively places tritium on sites whose H-exchange rates are sensitive to the protein functional state, and used a method introduced by Rosa & Richards (1979,1981) to locate this label in the protein. The latter method, which rapidly separates protein fragments under conditions that can preserve exchangeable label, was here brought to a more quantitative level. Taken together, these techniques provide a "functional labeling" method capable of selectively labeling and identifying protein segments that participate in functional interactions.

1. Introduction

It has long been appreciated that protein hydrogen-exchange behavior is determined by thermally driven fluctuations in protein structure (Linderstrom-Lang & Schellman, 1959), but a variety of viewpoints on the workings of this process are still being considered. Structural unfolding models center on H-bond breaking (Hvidt & Nielsen, 1966) and the transient unfolding (Englander, 1975;

† Present address: Istituto De Pesquisas Energeticas E Nucleares-IPEN; Caixa Postal 11049; Pinheiros-01000; Sao Paulo, Brazil.

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Schrier & Baldwin, 1976,1977; Kossiakoff, 1982) of small regions of structure, or more global distortions (Wagner & Wuthrich, 1979). Penetration models (Woodward *et al.*, 1982) visualize the entry of H-exchange catalysts into the essentially native protein, either directly through the protein matrix by liquid-like diffusion (Ellis *et al.*, 1975), or by way of transiently opening channels formed by the thermal expansion of pre-existing voids (Richards, 1979), or mediated by the mobile translation through the protein of defective H-bonding (Nakanishi *et al.*, 1973) and/or free volume (Lumry & Rosenberg, 1975). The penetration process is pictured to be supported by small, rapid, dynamical fluctuations of the kind that has been extensively studied in recent years by calculational, spectroscopic and diffraction methods, and other approaches (Gurd & Rothgeb, 1979; Karplus & McCammon, 1982). The underlying fluctuational mechanism of protein H-exchange can best be studied by observing the exchange of defined protons in defined protein structures. Here we report a study of this kind.

A small fraction of the hemoglobin amide NH groups display allosterically sensitive H-exchange rates; these probably mark segments of the protein that are involved in structure changes. In previous work we described H-exchange methods that make it possible to label selectively and study the allosterically sensitive protons. In this work, additional methods, introduced by Rosa & Richards (1979,1981), were applied to locate some of these protons in the molecule.

This work focuses on the so-called Slow I class, a set of 18 allosterically sensitive hydrogen atoms characterized earlier (Englander & Rolfe, 1973). A subset of these, involving about seven protons, can now be placed on the F-FG helical region of the beta chain. Earlier phases of this work have been described (Englander *et al.*, 1980; Englander, 1980; Rogero *et al.*, 1981).

2. Materials and Methods

(a) *Materials*

Hemoglobin obtained from fresh human red cells (washed, lysed by osmotic shock, centrifuged to remove stroma), was stored frozen in liquid nitrogen (70 mg/ml), and dialyzed into 0·1 M-sodium phosphate (pH 7·4), 0·1 M-NaCl for use. Tritiated water (1 Ci/ml) was from New England Nuclear. Exchange-in utilized tritiated water from 10 mCi/ml (fully labeled hemoglobin) to 100 mCi/ml (Slow I class). Sephadex G-25 (fine grade; Pharmacia) was used for tritium separations as described and liquid scintillation counting utilized a cocktail (dioxane, toluene, naphthalene, PPO) described elsewhere (Englander & Englander, 1978).

Samples and solvents were deoxygenated using either dithionite, a ferrous ammonium sulfate/pyrophosphate system, or an enzymatic method involving glucose oxidase (Boehringer-Mannheim), catalase (Sigma) and glucose (Liem *et al.*, 1980). Experimental deoxyhemoglobin samples were flushed with argon washed through a vanadyl sulfate/sulfuric acid/zinc scavenging system (Meites & Meites, 1965).

HPLC† grade acetonitrile and dioxane were from Burdick and Jackson, and methanol was from Waters; sequenal grade trifluoroacetic acid was from Pierce. The separation of alpha and beta chains used a semipreparative μbondapak C18 column; proteolytic fragments were separated in a fatty acid analysis (phenyl) column (3·9 mm × 30 cm). A 2-pump gradient system was used (all HPLC equipment was from Waters).

†Abbreviation used: HPLC, high pressure liquid chromatography.

Pepsin (Worthington) was dissolved at 12 mg/ml in 0·05 M-sodium acetate (pH 4·5), dialyzed for several hours to remove fragments (Englander & Crowe, 1968), and stored frozen: about 0·1 mg was used with 2 mg of beta chains for 10 min of proteolysis. Except for the total labeling of hemoglobin at 37°C, all operations, including the HPLC separations, were performed at 0°C. H-exchange and related methods were as described (Englander & Englander, 1978; and references therein).

(b) *Tritium labeling and exchange-out*

For experiments with fully labeled fragments, hemoglobin was initially incubated at 37°C, pH 9, for 24 to 48 h in tritiated water (10 mCi/ml; no buffer; pH maintained by the protein itself) in order to obtain full labeling. Samples were then adjusted directly to pH 2·7 (slow exchange condition) with a small predetermined volume of 5 M-phosphoric acid, run through a Sephadex column G-25 at pH 2·7 to remove free tritium, and separated into alpha and beta chains by HPLC. The beta chains were then fragmented with pepsin, and the fragments were separated rapidly by HPLC and analyzed for carried tritium. The label measured was calculated in terms of amide NH per fragment.

To measure the number of slowly exchanging, presumably H-bonded, amide NH groups on each fragment, fully tritiated samples were initially adjusted to pH 5 with a tiny volume of 0·5 M-phosphoric acid and exchanged-out briefly at pH 5 (Sephadex column method) to remove label on non-H-bonded amide groups before the alpha/beta separation, fragmentation and analysis steps.

To measure the Slow I class of allosterically sensitive NH, hemoglobin was initially labeled at 0°C for 3·5 h in the standard pH 7·4 buffer. For the deoxyhemoglobin exchange-out experiments, samples were deoxygenated with dithionite, passed through a deoxygenated Sephadex column (equilibrated with ferrous pyrophosphate) to remove free tritium and initiate exchange-out, and kept under argon during the subsequent exchange-out. Samples taken at various times were bubbled with CO for 1 min (antifoam added) to remove the contaminating Jump class of protons (halftime 20 s in liganded hemoglobin, 125 h dexoyhemoglobin: Liem *et al.*, 1980), then passed through a Sephadex column at pH 2·7, and subjected to alpha/beta separation, fragmentation and analysis for carried tritium. To follow the accelerated exchange-out of the Slow I class in the liganded form (drop-out curves), hemoglobin prepared as described was first exchanged-out in the deoxy form for 3 h to remove most of the background label, then liganded by bubbling with CO, and allowed to exchange-out in the liganded form. Samples taken at various times were separated into chains, fragmented and analyzed for carried tritium.

To generate background curves, samples were exchanged-in for 3·5 h as before but in the deoxy form, then exchanged-out in the oxy form and analyzed as usual.

(c) *HPLC separation of alpha and beta subunits*

All HPLC separations were performed at 0°C by immersing the injector and the analytical and guard columns in ice. For alpha/beta chain separations, the solvents used, designated C18 A and C18 B were: C18A, 40% (v/v) acetonitrile, 57% (v/v) 0·05 M-monosodium phosphate, 3% (v/v) trifluoroacetic acid, adjusted to pH 3·3 with 10 M-NaOH; C18 B, 50% (v/v) acetonitrile, 50% (v/v) 0·05 M-monosodium phosphate, adjusted to pH 3·3 using 1 M-HCl. The chains were separated isocratically using approximately 40% (v/v) C18 A, 60% (v/v) C18 B (46% (v/v) acetonitrile), but the exact proportions are critical and may drift from day to day. The flow-rate was 1 ml/min. This approach adapts the method of Congote *et al.* (1979) to 0°C (note the reversed elution order).

Hemoglobin collected from a Sephadex column run at pH 2·7 was diluted with the C18 column solvent before injection into the C18 column: 0·4 ml of the solvent was added to 0·6 ml of the pH 2·7 eluant. This relieves an artifact at the HPLC elution front, perhaps by fully solubilizing the heme: all the protein then appears in the alpha and beta peaks (see Fig. 1). Protein elution was followed by measuring absorbance at 280 nm using an 80-μl

flow-cell (Hellma) held at 0°C. Several fractions were collected through the alpha and beta peaks for determination of bound tritium label at this point, and for further analysis of subfragments. Samples saved for later processing were stored frozen (−80°C) in this mixed solvent.

Calculations for amide NH not yet exchanged (Englander & Englander, 1978) require readings of protein concentration and tritium label carried. Concentration of initial pH 2·7 hemoglobin samples, diluted into a Drabkin's solution (pH 6·5) with 1% (w/v) sodium dodecyl sulfate, was determined by measuring absorbance at 402 nm: the millimolar extinction coefficient per heme is 86 cm^{-1}. The concentration of alpha and beta chains was found by measuring absorbance at 280 nm and 400 nm. The contribution at 280 nm due to contaminant heme, equal to 0·22 × absorbance at 400 nm, was subtracted from the 280 nm reading. The millimolar extinction coefficients are 10·5 cm^{-1} for alpha chains and 16·4 cm^{-1} for beta chains. Tritium carried by the chains was measured by liquid scintillation counting and calculated in terms of amide NH as yet unexchanged in the initial hemoglobin preparation. The sum of the alpha and beta determinations should match the H still carried by hemoglobin samples taken after the pH 2·7 Sephadex column run.

(d) *Beta chain fragmentation*

For proteolysis, the beta chain sample was taken directly from the alpha/beta chain separation, or thawed in ice-water if previously stored frozen. Organic solvent was removed by rapid dialysis (10 min: Englander & Crowe, 1968). Pepsin was added (10 min). The sample, usually 2 ml, was then injected into the phenyl column, which was initially in phenyl A solvent. HPLC solvents used for fragment separation, designated phenyl A and B, were: A, 0·05 M-monosodium phosphate (pH 2·7): B, 60% (v/v) acetonitrile, 30% (v/v) dioxane, 10% (v/v) phenyl A, readjusted to pH 2·7 using 1 M-HCl, then 0·1 M-HCl, with care. The running program (1 ml/min) was: 5 min in phenyl A, then 15% (v/v) phenyl B for 5 min, followed by a linear gradient from 15% to 45% (v/v) phenyl B over 100 min (see Figs 2 and 3). Fragment elution was monitored by measuring absorbance at 230 nm, and carried tritium was measured by counting 0·5 ml samples.

(e) *H-exchange analysis of peptide fragments*

Samples were collected manually through the elution sequence, with emphasis on obtaining peak fractions and representative background samples (see Figs 2 and 3). The concentration of samples was calculated from the recorder peak area collected, sample volume, and the extinction coefficient for each known peptide. The identity of each peak peptide and their molar extinction coefficients were determined by quantitative amino acid analysis, and checked by calculation from calibrated values for absorbance of the peptide bond, tryptophan and tyrosine (aromatic residues make a dominant contribution when present).

The values obtained for H/peptide in this way were corrected for losses sustained by exchange-out of bound tritium during the separation procedures. This was done by calculating an exchange-out curve for the oligopeptide segment in question according to Molday *et al.* (1972) and Englander & Englander (1978), taking into account newly done calibrations of the slowing effect of the solvents used here on H-exchange rate. The effective exchange-out time for each isolated peptide was measured from the time of first switching the native hemoglobin sample to the low pH analysis condition to the time of obtaining the HPLC eluant peptide isolated in a test-tube. This period, when matched to the computed loss curve, indicates the fraction of H lost during the analysis procedure, and provides the multiplying factor needed to correct for this loss. In this work, loss factors were close to 2 ×.

Further discussion on these and related issues, including control of pepsin cleavage to restrict products obtained, calibration of tritium loss during the separation procedures,

testing for the analytic accuracy of these methods, etc., will be given in greater detail elsewhere.

3. Results

In developing the capability for locating allosterically sensitive protons in the hemoglobin molecule, we have studied a set of protons designated the Slow I class. In earlier work (Englander & Rolfe, 1973), this proton set was estimated to contain 18 H atoms per alpha-beta dimer with an exchange halftime of 100 minutes in carboxyhemoglobin and 50 hours in deoxyhemoglobin (pH 7·4, 0°C).

(a) *Difference hydrogen exchange*

These sites were selectively labeled and studied by a differential labeling approach. Hemoglobin was labeled in tritiated water for 3·5 hours (2 H-exchange halftimes for the Slow I class), then deoxygenated, and exchange-out was initiated by passage through an oxygen-free Sephadex column (1 min) to remove free tritium. Exchange-out in the deoxy form was continued for long times. During the long exchange-out period in the deoxy form, label on allosterically insensitive sites, which exchange at the same rate in both allosteric forms, is lost early on. The responsive sites, which are labeled relatively rapidly in the fast exchanging oxy form, retain their label for much longer times in the slow deoxy form. The exchange-out behavior of the responsive sites, selectively trap-labeled in this way, could then be followed both in the deoxy and the religanded forms.

To locate remaining label as exchange-out time progressed, hemoglobin samples were fragmented, the fragments were separated, and label on each peptide fragment was measured and calculated in terms of the number of amide NH not yet exchanged-out. Fragmentation and separation were performed at slow exchange conditions, namely pH 2·7 and 0°C, where exchange halftime for freely exposed amide NH is over one hour (and longer in mixed organic solvent), so that the loss of tritium label during the separation procedure was minimized.

To facilitate the resolution of the many peptic fragments of hemoglobin, we found it useful to separate first the alpha and beta chains. A sample of the selectively labeled hemoglobin preparation, after exchanging-out under the normal experimental conditions (0°C and pH 7·4) for a given time, was passed through a short Sephadex column to adjust solvent to the slow exchange condition, then run on an HPLC column to separate the alpha and beta chains (Fig. 1). The eluant sample to be subjected to proteolysis was freed of organic solvent by rapid dialysis, treated with pepsin, and the resulting fragments were separated on a second HPLC column (Figs 2 and 3: see Materials and Methods for details).

(b) *Accuracy of proton recovery*

Experiments were done to test the ability of these HPLC methods to measure the true number of labeled NH sites present on each hemoglobin segment in the native protein. In these test experiments, hemoglobin was initially subjected to

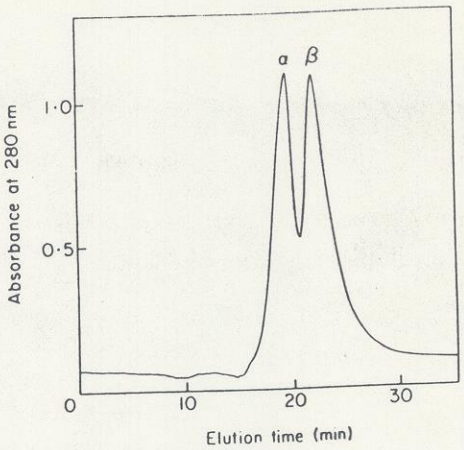

FIG. 1. Separation of alpha and beta chains at pH 3·3, 0°C. The 25 min of run time in the solvent used corresponds to 7 min of exchange-out time in aqueous (pH 2·7) solution. Samples taken from the peak regions were dialyzed, fragmented with pepsin, and resolved as shown in Fig. 2.

exhaustive exchange in tritiated water so that near-equilibrium labeling was achieved (Ghose & Englander, 1974). Samples, while still in the high-level tritiated water, were adjusted directly to pH 2·7 to inhibit all exchange-out, passed through a short Sephadex column (pH 2·7) to remove free tritium, then fragmented and resolved as just described (Fig. 2). The tritium label recovered

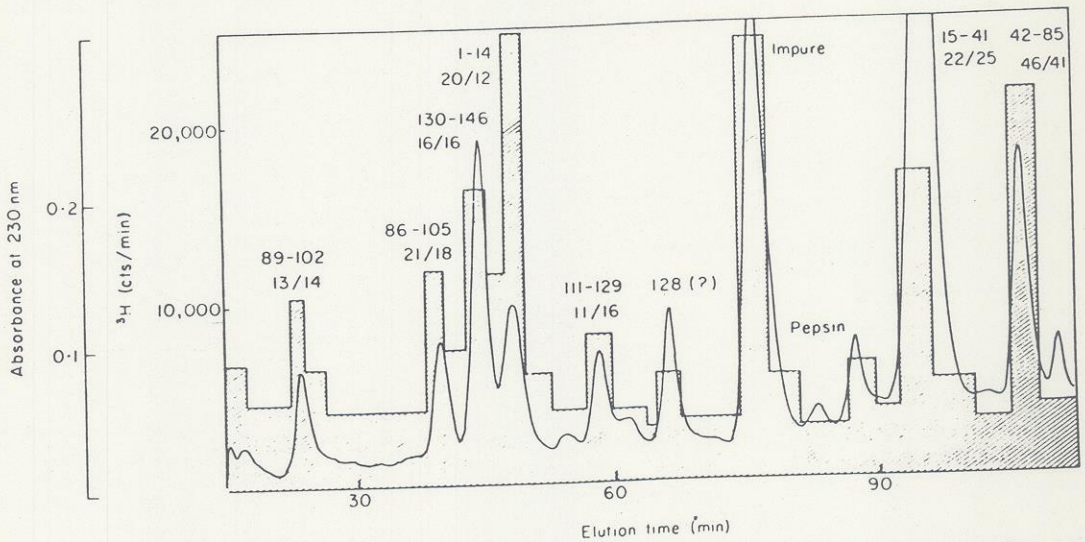

FIG. 2. Pepsin fragments of beta chains from fully labeled hemoglobin. The fragments, separated at pH 2·7 and 0°C, were measured by absorbance at 230 nm (peaks), and carried tritium (histogram) was measured by liquid scintillation counting. Fragments are identified by their residue numbers (e.g. 89–102). The label recovered was corrected for expected losses during the separation procedures (approximately 2×) and calculated in terms of amide NH indicated on each fragment: this is compared to amide NH actually present on each fragment (shown as NH measured/NH present).

was computed in terms of the number of sites initially labeled on each fragment. For these fully labeled samples, the correct value is of course the number of peptide group protons in each fragment.

Figure 2 shows an elution profile of beta chain peptic fragments and their carried tritium label. The numbers given above each peak in Figure 2 identify the fragment in terms of residue number. The present work focuses upon the first two fragments identified in the elution diagram. The peptide fragment 86–105 was obtained in pure form but peptide 89–102 was fractionally contaminated (approx. 25%) with the fragment 82–102. All calculations (extinction coefficient, H present, etc.) were treated accordingly.

The number of amide NH measured for each fragment is also shown in Figure 2 (and see Table 1). For peak 86–105, 18·3 H atoms were recovered compared to the true value of 18 (values measured in replicate experiments were 15·7, 21 and 18·2). For peak 89–102(+82–102), 13·9 H atoms were measured compared to the correct value of 13·8 (values measured were 11·7, 13, 15·5 and 15·5). These calculations take into account and correct for the expected loss of label during the separation procedures, which can be as high as 50%. Loss rates assumed were computed from previous rate calibrations (Englander & Poulsen, 1969; Molday *et al.*, 1972) and more recent calibrations of exchange in pertinent mixed solvents. Accuracy of the loss-rate calculation was checked in control experiments set up to recover one fragment or another with only a brief loss period. All these procedures will be described more extensively elsewhere.

In summary, trial experiments with fully labeled samples, for which the correct number of labeled NH groups in each fragment is known, show that the methods used can provide an accurate measurement of labeled amide NH in definable segments of the hemoglobin molecules.

TABLE 1

Peptide NH in the F–FG region

Fragment	Number of† peptide NH	Slow† (H-bonded)‡	Allosterically responsive† Liganded	Deoxy
89–102 (+82–102)(25%)	14(14)	9(8)	4·5(5)§	6·3(7)§
86–105	18(18)	11(10)	6·4(6)	9·6(8)

† The numbers of peptide NH protons in fragments 86–105 and 89–102 were measured under differing conditions to obtain: the total number of peptide NH protons, the number of slowly exchanging (H-bonded) NH protons, and the number of allosterically sensitive NH protons in liganded and deoxyhemoglobin. These are compared to (in parentheses) the total number expected on the basis of amino acid composition, the number of H-bonded peptide NH protons inferred from crystallographic data, and the number of responsive NH protons if these represent the H-bonded residues in the F–FG helix acting as a co-operative unfolding unit.

‡ H-bonded NH protons were computed by M. Liebman & S. Amato (personal communication) using the results of Baldwin & Chothia (1979) for human carboxyhemoglobin (energy refined) and Fermi (1975) for human deoxyhemoglobin (see Fig. 6).

§ Comparison of the H-exchange results with the H-bonding present indicates that the allosterically sensitive protons run from residues 89 to 95. The proteolytic cleavage at residue 89 loses this proton: when added back to the values shown for peptide 89–102, this changes the entries (for peptide 89–102) to 5·5(6) for the liganded form and 7·3(8) for the deoxy form.

(c) *H-bonded amide NH groups*

Similar experiments were done to measure the apparent number of H-bonded amide NH groups in these hemoglobin segments. Previous work with myoglobin (Englander & Staley, 1969) and collagen (Yee *et al.*, 1974) showed that the non-H-bonded amide NH groups in those proteins exchange relatively rapidly, at just the rates calibrated for fully exposed amides (Molday *et al.*, 1972). Intramolecularly H-bonded amide NH groups are far slower, and we expect these to account for almost all of the slowly exchanging allosterically sensitive protons of hemoglobin (for one exception, see Lamar *et al.*, 1981). Thus, these are the protons that provide the reporter groups detected in the difference H-exchange experiments.

To measure the number of slowly exchanging (H-bonded) amides on each fragment, samples fully labeled as just described were exchanged-out for five and for ten minutes at pH 5 and 0°C; label on free amides is lost with a halftime of about one minute, while label at H-bonded sites is fully retained. Samples were then quenched to pH 2·7 and carried through the procedure for determining the number of still-labeled NH groups on the various fragments. The number of slowly exchanging H atoms measured on peptides 86–105 and 89–102 are shown in Table 1 and are compared to the number of H-bonds on each segment (shown in parentheses). Values actually measured were 10·6 and 10·8 H for 86–105, compared to the 10 H-bonded protons present, and 7·4 and 9·9 H for 89–102 compared to the 7·8 present. (The fractional number for H present reflects the fractional contamination (25%) of peptide 82–102 in the 89–102 fragment peak.)

Figure 6 illustrates the H-bonding in this region of the protein (M. Liebman & S. Amato, personal communication) inferred by computer analysis of crystallographic results for human hemoglobin on file at the Brookhaven Data Bank. This computation, using available crystallographic information, analytically places protons on the amide N, then evaluates the distance and orientation to all potential H-bond acceptors (see also Ladner *et al.*, 1977).

The measured number of slowly exchanging protons, summarized in Table 1, are in good agreement with the number of H-bonded amide NH groups derived from crystallographic data. This lends credence to the derived H-bonding scheme (Fig. 6) and, reciprocally, to the conclusion previously reached in experiments with other proteins that the slowly exchanging NH groups represent H-bonded amides. The slow protons studied here exchange over 10^4 times more slowly than the rate characteristic of non-H-bonded amides (i.e. those H-bonded to solvent). We assume in the following that the slow protons must be assigned from among the intramolecularly H-bonded amides shown in Figure 6.

(d) *Allosterically responsive sites*

The methods tested as just described were used to study some of the allosterically sensitive amide NH groups of hemoglobin. Figure 3 shows a peptide elution profile taken from an experiment in which hemoglobin was initially labeled for 3·5 hours in the fast-exchanging oxy form, then switched to deoxy to lock in tritium on responsive sites, and exchanged-out in the deoxy form for

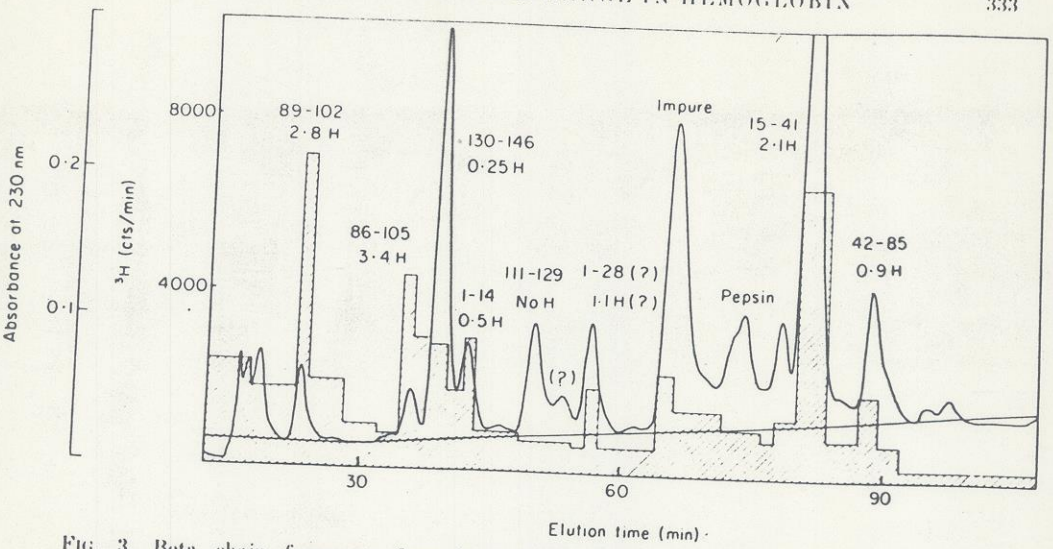

FIG. 3. Beta chain fragments from hemoglobin selectively labeled at the Slow I class. Oxyhemoglobin was labeled in tritiated water for 3·5 h, then switched to the deoxy form and exchanged-out for 45 h (all at pH 7·4, 0°C). The HPLC analysis was then performed. Tritium still carried (histogram) was computed in terms of amide NH/fragment not yet exchanged at the 45 h sampling time, and is indicated for each fragment. These data provide 1 time-point (45 h) on the exchange-out curves in Fig. 4.

45 hours. Analysis by the HPLC method shows (Fig. 3) that, even after this lengthy exchange-out period, a significant number of unexchanged protons are still retained on the overlapping fragments 89–102 and 86–105 and also on peptide 15–41. Evidently, there are present on these segments some allosterically responsive amide NH groups that exchange on the three hour time-scale in oxyhemoglobin and far more slowly in the deoxy form. This is characteristic of the Slow I class studied by Englander & Rolfe (1973).

Some other classes of responsive sites, previously calibrated, will also become labeled during the 3·5 hour exchange-in used here. Among these, label in the fast class (Ghose & Englander, 1974) is lost within 40 minutes of exchange-out in the deoxy form, and from the Intermediate I class (Englander & Rolfe, 1973) within four hours (4 halftimes for each). Also, the Jump class (Liem *et al.*, 1980) is removed in the one minute exposure to CO before the alpha/beta subunit separation. The Intermediate II class, which exchanges out with a halftime of nine hours in deoxyhemoglobin (Englander & Rolfe, 1973), however, should be fractionally retained over part of the exchange-out period used here, and should appear in the earlier-time HPLC elution profiles.

For each peptide fragment, results like those in Figure 3 supply one time-point on an exchange-out curve. A series of analogous experiments provide the results shown in Figures 4 and 5. Figure 4 shows data for the Slow I class of protons in peptide 89–102(+82–102) and in peptide 86–105 as they exchange in deoxyhemoglobin (upper panels), and also conjugate background data (low-lying curves). The deoxy form difference curves, shown on semilogarithmic plots in the

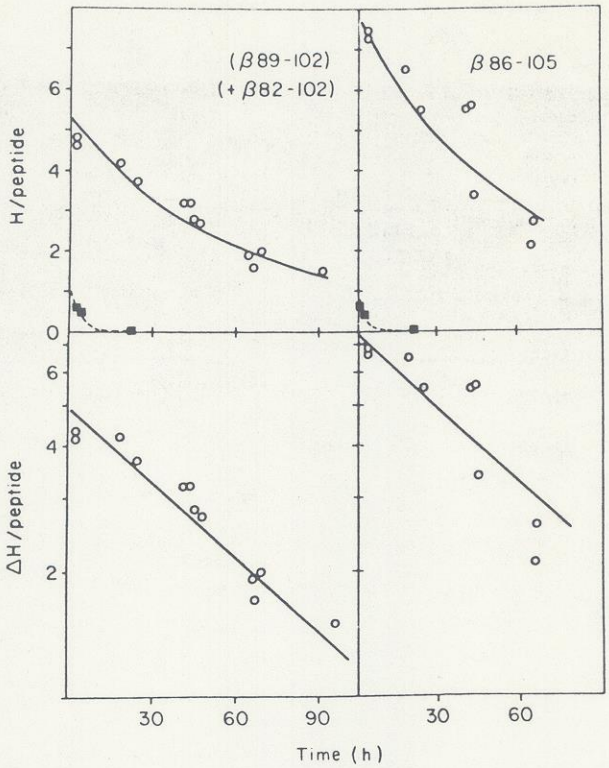

FIG. 4. Exchange-out behavior of allosterically sensitive protons in the beta chain F-FG region of deoxyhemoglobin. The upper panels show exchange-out data obtained from HPLC analyses of beta chain fragments (as in Fig. 3): the minor low-lying curves indicate residual background label on allosterically insensitive sites. The lower panels show semilog plots of the H-exchange behavior of the allosterically sensitive NH in each fragment, obtained as the difference between each upper curve and its background. A correction to the number of NH measured is required owing to the incomplete (77%) initial labeling.

lower panels of Figure 4, were obtained by subtracting the background from the deoxy data. A background curve indicates the fractional labeling on allosterically insensitive sites that has survived the differential kinetic filtering steps applied to retain selectively the allosterically sensitive label. (The curve was generated by carrying out the differential labeling the other way round, labeling 3·5 h in the slow form and exchanging-out in the fast, so that allosterically sensitive sites are selected against, while the background-insensitive sites appear just as before, since their exchange is, by definition, indifferent to the allosteric form).

The same methods can portray the behavior of the very same responsive sites as they exchange in liganded hemoglobin. Here, deoxyhemoglobin was selectively labeled as before (exchanged-in 3·5 h oxy, deoxygenated, exchanged-out deoxy for 3 h to lose most of the label on insensitive sites while retaining the Slow I class), then was religanded by bubbling with CO, and the accelerated exchange behavior in the religanded protein was monitored by the HPLC method as described. The results are shown in Figure 5. Semilogarithmic difference curves are shown in the lower panels.

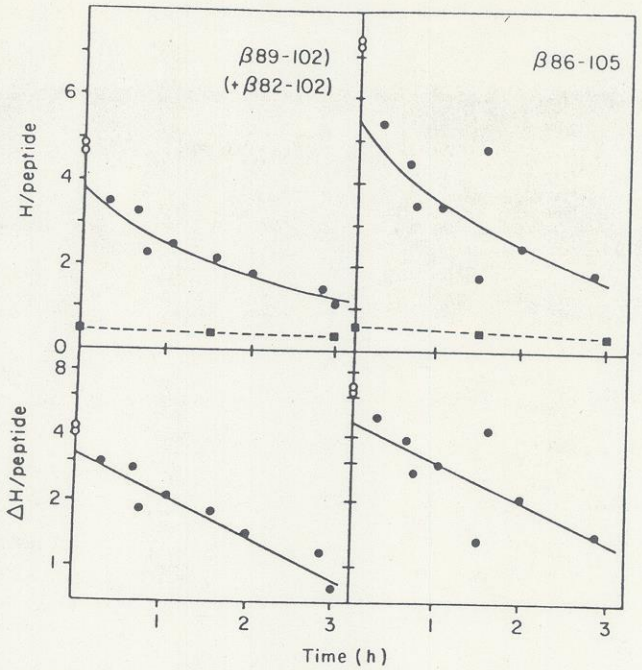

FIG. 5. Exchange-out behavior of allosterically sensitive protons in the beta chain F–FG region of carboxyhemoglobin. Samples labeled to tag the Slow I class, as in Fig. 4, were exchanged out in the deoxy form for 3 h (open circles) to cleanse label from non-Slow I class sites, then liganded with CO. The accelerated exchange in the liganded form was then followed by the HPLC method. The lower panels show semilog difference plots (exchange-out minus background data).

The results in Figures 4 and 5 display a set of allosterically sensitive NH groups, in the F–FG region of the beta subunit, exchanging with apparent halftimes of 100 minutes in the liganded protein and 50 hours in the deoxy form. The same halftimes (100 min liganded, 50 h deoxy) were measured previously for the entire set of 18 Slow I class protons (Englander & Rolfe, 1973).

The number of sensitive H atoms in this set measured in Figures 4 and 5 is an underestimate, since the initial exchange-in was incomplete: the 3·5 hour exchange-in (liganded) corresponds to only 2·1 halftimes (77% labeling). Also, in the liganded exchange-out experiments (Fig. 5), some label (4%) was lost during the preliminary three hour exchange-out in the deoxy form. When the appropriate corrections are applied, the values given in Table I are obtained. The standard error of estimating these values (data in Figs 4 and 5) is 0·65 H for peptide 89–102, and considerably worse, 2 H, for 86–105.

(e) *The F–FG region*

Figure 6 compares the number of responsive H atoms found in each fragment to the H-bonding scheme in the F–FG region, computed by M. Liebman & S. Amato (personal communication: see also Table I).

The measurements on the two fortuitously overlapping fragments from the F–FG

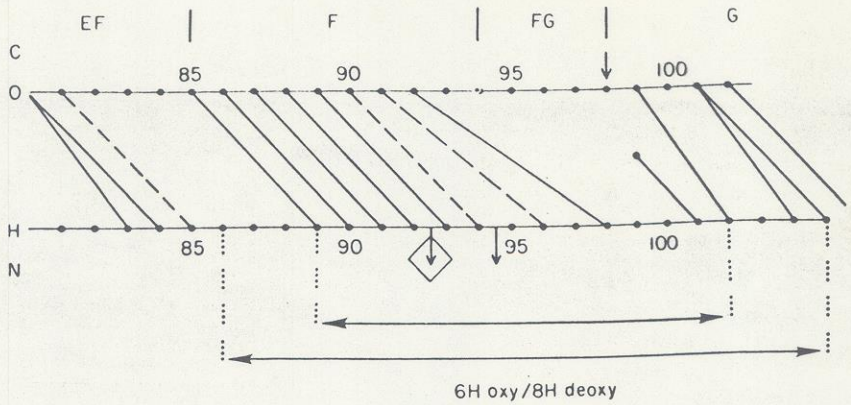

FIG. 6. H-bonding and allosterically sensitive NH in the F–FG region of the beta chain. Diagonal lines represent H bonds from amide NH (bottom line, residue number indicated) to peptide carbonyl groups (top line) or to a side-chain (Asp99). Bonding is taken to be present for NH to OC orientation linear and H to O distance less than or equal to 2·35 Å. Broken lines indicate bonds present by these criteria in deoxy but not in carboxyhemoglobin. H-bonding computations are due to M. Liebman & S. Amato (personal communication: see Table I and footnotes). The arrows indicate interactions suggested by Perutz (1970) to be important in the allosteric transition. The location of the 2 peptic fragments obtained in this region are shown, along with the number of allosterically sensitive NH groups measured on the fragments in experiments with the liganded and deoxy protein (see also Table I).

region provide independent estimates of the number of allosterically sensitive NH groups in this region of the beta chain. Fragment 89–102 shows values lower than those found for 86–105. This is probably due, in part, to the peptide cleavage at residue 89, which causes the immediate loss of exchangeable tritium at that position. When this proton is added back, these results indicate six responsive H atoms (5·5 H) in the carboxyhemoglobin experiments, in good agreement with the results for 86–105 (6·4 H). In the deoxy case, 89–102 shows (with one proton added back) seven responsive protons (7·3 H): 86–105 is a little higher (9·6 H).

Comparison with the H-bonding scheme in the F–FG region (Fig. 6; Table I) indicates that the measured Slow I class protons correspond to the H-bonded amide NH from residues 89 to 98. The only alternative possibility allowed by the results would include some protons from the G helix together with a fraction of the F helix. This grouping cannot be ruled out by the available H-exchange data, but seems quite unlikely on structural grounds, since all the responsive protons exchange with similar rates and display similar sensitivity to the allosteric transformation, while the candidate protons in the G helix are spatially quite far removed from those in F–FG. (Note that this alternative assignment would not relieve the paradox posed here: one would still have deeply buried and surface protons exchanging at the same rate.)

Interestingly, the computations by M. Liebman & S. Amato (personal communication) suggest that two H bonds in this segment (residues 94 and 96) are lost upon liganding (broken lines in Fig. 6). The measured difference between our results for the oxy and deoxy forms (Table I) is consistent with this possibility. Independently, this kind of liganded deoxy difference is suggested by

the rapid drop-out of one to two H atoms seen in Figure 5 when the exchanging deoxy sample (indicated by open symbols in the graphs at zero time) is religanded: the deoxy points in Figure 5 are higher than the best-fit line to the liganded data by 2 to 1·5 standard deviations.

In summary, the results shown in Figures 4 and 5 provide four independent sets of data on a cluster of protons in the hemoglobin beta chain. The rates observed for these are identical with the rates previously found for the entire set of 18 H atoms in the Slow I class. The number and positioning of the allosterically sensitive protons measured in the fragmentation experiments correspond well with the H-bonded amide NH protons in the beta chain F–FG segment. Secondarily, this identification is also consistent with the small difference in number of H-bonded protons observed between the two overlapping fragments studied, and with H-bonding differences between the liganded and deoxy protein in this segment. We conclude that this identification provides the probable interpretation of our data. Figure 7 represents the hemoglobin tetramer and displays the backbone folding of the oligopeptide segments studied. The smallest concerted motion necessary to unfold this segment and sever the H-bonds shown in Figure 6 would

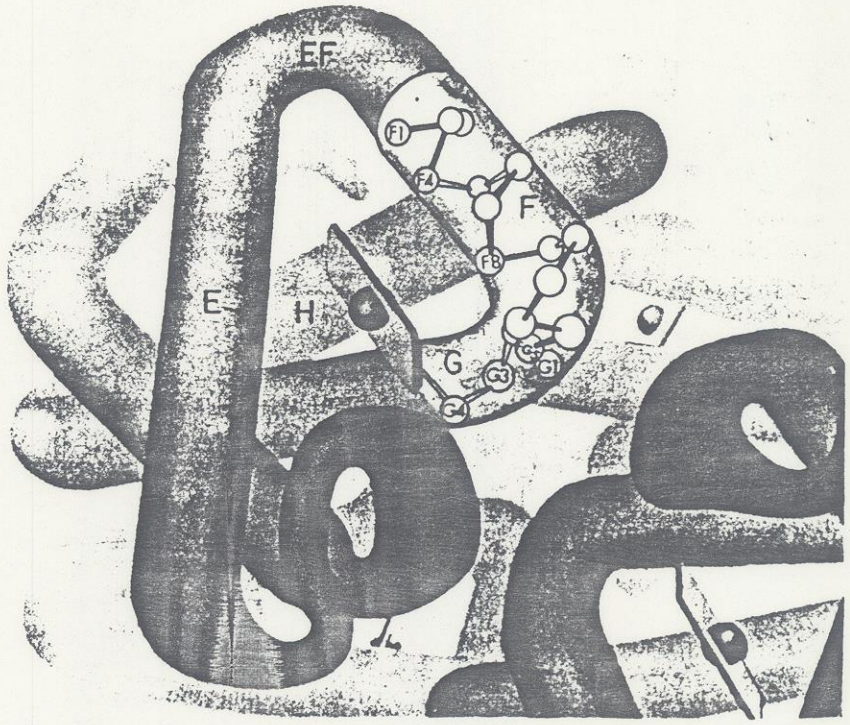

FIG. 7. Position in the hemoglobin tetramer of the oligopeptide fragments studied. Beta subunit residues in the F–FG region are represented as alpha carbons with stick connectors: side-chains, not shown, fill the empty spaces (adapted from Dickerson & Geis, 1969). The environment of the H-bonded peptide NH from 89F5 to 98FG5 (see Fig. 5) ranges from aqueous surface to hydrophobic core. These NH protons all exchange with solvent protons more slowly than the free peptide rate by 6 decades in deoxyhemoglobin and are accelerated by 30-fold upon liganding.

involve one turn of helix, the length of backbone chain between the alpha carbons of residues F4 to F9 (88 to 93 in Fig. 6, see also Fig. 7).

(f) *Other allosterically responsive regions*

The Slow I class, previously characterized by difference H-exchange, includes approximately 18 H atoms (Englander & Rolfe, 1973). The seven H atoms found on the segment characterized here evidently represent a subset of the overall Slow I class, which now appears to occur in three small groupings in the hemoglobin molecule. The location of some of the other Slow I class protons is suggested in Figure 3; the fragment beta 15 to 41 carries four to five NH groups with exchange rates similar to the Slow I class. An alpha chain fragment, 1 to 23, carries another four or five similar H atoms. We will report on these in further communications.

4. Discussion

This work assigns a set of allosterically responsive protons to the F–FG region of the hemoglobin beta chain. About seven amide protons, side by side along contiguous turns of a helical segment, are dissimilarly located: some are along the aqueous surface of the protein and some are deeply buried, within the apolar core about the heme group (see Fig. 7). Nevertheless, all these amide protons exchange with solvent protons at similar rates. Further, the allosteric structure change, which variably affects the immediate environment of only a few of these protons (Perutz, 1970; Baldwin & Chothia, 1979), changes the exchange rate of all by a factor of 30. These results seem significant for present views of internal protein dynamics and H-exchange mechanism, and also for ongoing efforts to localize the important allosteric structure changes in hemoglobin and to assess the contribution of each in energy terms.

(a) *Protein dynamics and H-exchange mechanism*

The H-exchange behavior of the various protons in a protein is affected by structure, structure change, and structural dynamics all through the protein. In order to extract that information from measured data, it is necessary to understand these relationships. It has long been supposed that the exchange with solvent of a protein's slowly exchanging hydrogen atoms depends on some kind of internal protein motion (Linderstrom-Lang, 1955; Linderstrom-Lang & Schellman, 1959). Over the years, two different kinds of protein motions have been invoked, and have been codified in penetration models and in unfolding models.

We find it extremely difficult to construct a feasible penetration-based mechanism that could account for the present results. Several protons with grossly differing accessibility to solvent are seen to exchange at about the same rate, and a structure change that hardly alters their solvent accessibility changes their common exchange rate by 30-fold. These observations seem inconsistent with the basic assumption of penetration models, that exchange rate relates to solvent accessibility in the native structure. Since the contiguous protons studied

here are so dissimilarly placed in the native protein, yet experience such similar H-exchange rates, they must be made similar in the H-exchange transition state. It follows that the distorted H-exchange transition state must be very different in conformation from the native form and involve a significant degree of conformational unfolding, so that all the protons, those initially on the aqueous surface of the helix and those on the buried surface, become more or less equally available for exchange in the transient open state.

Unfolding models assume that slowly exchanging hydrogen atoms of proteins are slow because they are H-bonded, and that the exchange process requires a transient separation of the H-bond (as suggested initially by Linderstrom-Lang, 1955; see also Hvidt & Nielsen, 1966) and its reformation to water. The slowing effect of H-bonding in small molecules has been amply substantiated by Eigen (1964) and others (Haslam & Eyring, 1967; Rose & Stuehr, 1968). Work with hemoglobin has shown that the slowly exchanging protons in this highly alpha-helical protein tend to exchange in coherent kinetic sets, suggesting that transient H-bond breakage in alpha helices, and perhaps in other regular secondary structures, tends to occur in co-operative unfolding reactions that concertedly unwrap a segment of structure and expose the protons thereon to attack by solvent catalysts (Englander, 1975; Malin & Englander, 1980). A completely analogous result with the ribonuclease-S peptide system was described by Schrier & Baldwin (1976,1977). These investigators labeled the isolated S-peptide, reconstituted the S-protein complex, and found that the amide NH groups in the re-formed S-peptide helix all exchange at about the same rate.

Linderstrom-Lang (1955) alluded to the possibility of co-operative H-bond breakage based upon the early work of Schellman on co-operativity in the helix–coil transition. The results described here for neighboring protons in hemoglobin, and in the accompanying paper for similar protons in ribonuclease (Kuwajima & Baldwin, 1983), now provide striking examples of this kind of behavior.

(b) *Other evidence on H-exchange mechanism*

A great deal of information is now available concerning the dependence of protein H-exchange rate on solution and ambient variables (Woodward *et al.*, 1983; Barksdale & Rosenberg, 1983), but this has not resolved the uncertainties concerning H-exchange mechanism. More insight can be expected from the study of defined protons in defined protein structures and, in addition to the present work, some information of this kind is becoming available from nuclear magnetic resonance and neutron diffraction studies with several different proteins.

The accompanying paper (Kuwajima & Baldwin, 1983) describes a similar observation: six protons disposed around a helical segment in ribonuclease are all found to be protected from exchange with solvent protons. Even though some are on the aqueous surface and some "inside", they all experience a similar degree of protection and exchange about 1000 times more slowly than free peptide group NH protons. Interestingly, neighboring protons as the N-terminal end of the

protected helix taper off into faster exchange, as in typical helix fraying behavior (e.g. see Kallenbach *et al.*, 1976).

Considerable work on the most slowly exchanging protons in pancreatic trypsin inhibitor (Wagner & Wuthrich, 1979; Hilton & Woodward, 1979) has led to the conclusion that their exchange depends on a co-operative structural opening reaction, but whether the opening involves reversible denaturation of the whole protein or some more limited unfolding is still at issue (Wuthrich *et al.*, 1980; Hilton *et al.*, 1981). Work on other protons in BPTI (Wagner & Wuthrich, 1982) does not suffer from this ambiguity. Six contiguous protons along the C-terminal alpha-helix of pancreatic trypsin inhibitor exchange at roughly similar rates, about 10^4-fold more slowly than the characteristic free amide rate. When the intrinsic chemical exchange rates of the different residues are taken into account (Molday *et al.*, 1972), it can be seen that five of the six protons exchange with very similar slowing factors, within a range of 3-fold; one (Cys55) is significantly slower, by tenfold. As in the ribonuclease S-peptide helix (Kuwajima & Baldwin, 1983), the protons on both sides of these well-protected NH groups, i.e. at the ends of the helix, taper off to fast rates.

The same kind of behavior has been found in the small but stable protein, apamin, by Wemmer & Kallenbach (unpublished data). Here, five contiguous alpha-helical protons are all slowed by about 100-fold in rate, in light of the Molday factors within a 3-fold range. As in the trypsin inhibitor and ribonuclease, the protons on both ends of the helix taper off to much faster rates.

Thus, in ribonuclease, basic pancreatic trypsin inhibitor and apamin, as in hemoglobin, contiguous protons distributed around the length of a helical segment are found to exchange at remarkably similar rates, even though some are on the aqueous face of the helix and some on the inner side facing the protein core. These observations seem very difficult to rationalize with a penetration-based mechanism that would connect exchange rate with degree of accessibility in the native protein form. Rather, they point to a localized concerted structural unfolding as a requisite intermediate in the H-exchange pathway.

The detailed behavior uncovered in the ribonuclease, the trypsin inhibitor and apamin investigations display some interesting features that may begin to illuminate the conformation of the transiently opened state. It can be expected that in the rather fluid, locally denatured open form, some amide NH groups that find themselves hemmed in by an apolar face of the remaining protein structure and by surrounding groups of the unfolded segment may well exhibit slower exchange than their neighbors, perhaps because they tend to reform weak H-bonding to an available donor. The slowing factors seen for the individual most slowed proton in the trypsin inhibitor can be explained by this kind of transient H-bond being formed 80% to 90% of the time in the unfolded state. This corresponds to an equilibrium constant of less than 10 for formation of the partially protected H-bond, i.e. a formation free energy of about 1 kcal (=4·184 J). Similar H-exchange slowing factors have been found for individual protons in a number of tenuously structured small molecules, and have been interpreted in terms of this kind of weak H-bond formation (e.g. see Krishna *et al.*, 1980; Krauss & Cowburn, 1981; Oberholzer *et al.*, 1981). Again, with respect

to the gradient toward faster exchange seen for protons at the boundaries of the helical regions in ribonuclease and the trypsin inhibitor, this points to classical helix end fraying behavior.

Some protons selected by virtue of their very slow H-exchange rates have been identified in neutron diffraction studies of trypsin (Kossiakoff, 1982) and ribonuclease (Wlodawer & Sjolin, 1982). These investigators conclude that their results favor a local unfolding model for the H-exchange process. With respect to the details of the unfolding process, it seems possible that some of the individually observed slowest protons may outline a pattern of "left-over" protons, like that in the trypsin inhibitor suggested to reform a protecting H-bond in the transient unfolded state.

It can be noted that the hemoglobin work described here used a mode of H-exchange measurement in which the summed exchange behavior of multiple protons is recorded. This tends to mask moderate differences in exchange rate of individual protons. Thus, it is not possible to say whether some rate differences of the kind seen in the cases just discussed may also occur among the beta chain F–FG protons in hemoglobin. If any of these are greatly slowed in exchange rate, they may have escaped labeling and detection in these experiments. If so, then an equivalent number of NH groups from the G helix must be considered for inclusion in the Slow I set. The results at hand (Fig. 6) do allow several NH groups at the beginning of the G helix to be so considered, but this seems unlikely on structural grounds.

(c) *On hemoglobin allostery*

Previous hydrogen-exchange experiments with hemoglobin have shown that only a small fraction of its exchanging amide NH groups, 0·25 of the total, are affected in the allosteric transition (Malin & Englander, 1980). This is true even though it appears that a minimal structure change can affect the exchange rate of a sizeable set of protons (one or more co-operative unfolding units). Apparently, only a small fraction of the protein is actively involved in the allosteric function (see also Gelin & Karplus, 1977; Baldwin & Chothia, 1979; Pettigrew *et al.*, 1982).

This work points to the F–FG segment of the beta chain as a part of the allosterically involved region. Certainly that conclusion is not surprising; the beta chain F–FG segment contains crosslinks that are severed in the T to R transition (Perutz, 1970; Baldwin & Chothia, 1979) and it is the site of the proximal histidine that co-ordinates the heme iron. The 30-fold increase of exchange rate in the R state indicates, according to the local unfolding model, that the F–FG segment experiences a net destabilization in the T to R transition, measuring 2 kcal in free energy.

Development of the general significance of these findings will require more complete information. This work locates only a fraction of the 18 protons that account for the previously characterized Slow I class of allosterically sensitive protons. In addition, a number of other segments that display sensitivity to the allosteric form of hemoglobin (Malin & Englander, 1980) have yet to be analyzed. The relations among these will be dealt with in future reports.

(d) *Functional labeling*

The methods and concepts used here may provide a widely applicable "functional labeling" approach to protein structure–function problems (Englander & Englander, 1983). The reporter groups in hydrogen-exchange studies are the amide NH protons, which are of course distributed throughout all proteins. It has been very generally observed that when any protein engages in any interaction, some of its protons display a change in their exchange rates (Woodward & Hilton, 1979). The functionally sensitive protons are, by definition, faster in one functional form and slower in the other. Therefore, functionally involved segments may be selectively labeled by the differential labeling methods used here and identified.

Measurement of the change in exchange rate may provide another rather special kind of information. The unfolding model makes H-exchange rate (k_{ex}) proportional to an equilibrium constant (K_{op}) for a local structural denaturation reaction ($k_{ex} = K_{op}k_{ch}$: Hvidt & Nielsen, 1966): in turn, the unfolding equilibrium constant relates in the usual way to the structural free energy stabilizing against the unfolding of that particular segment ($-RT\ln K_{op} = \Delta G_{op}$). Accordingly, a change in H-exchange rate of the kind observed in this work may indicate the change in stabilization free energy experienced by that particular segment in the oxy/deoxy transition (Englander & Rolfe, 1973: Englander, 1975). The linked function formalism (Wyman, 1964: Monod *et al.*, 1965) relates this kind of change in structural energy to the change in liganding energy. For example, a strain energy of 2 kcal developed by the beta F–FG segment in the hemoglobin R state should favor transition to the T state, and dissociation of the ligand, by 2 kcal in free energy.

This chain of reasoning suggests that it may be possible to label selectively and identify those segments of any protein that participate in any functional interaction, and perhaps also to read out the contribution of each segment to the functional process in quantitative energy terms (see also Gelin & Karplus, 1977: Warshel, 1977: Ackers, 1980).

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