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Protein Hydrogen Exchange Studied by the Fragment Separation Method

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The potential of hydrogen-exchange studies for providing detailed information on protein structure and structural dynamics has not yet been realized, largely because of the continuing inability to correlate measured exchange behavior with the parts of a protein that generate that behavior. J. Rosa and F. M. Richards (1979, *J. Mol. Biol.* **133,** 399–416) pioneered a promising approach to this problem in which tritium label at exchangeable proton sites can be located by fragmenting the protein, separating the fragments, and measuring the label carried by each fragment. However, severe losses of tritium label during the fragment separation steps have so far rendered the results ambiguous. This paper describes methods that minimize losses of tritium label during the fragment separation steps and correct for losses that do occur so that the label can be unambiguously located and even quantified. Steps that promote adequate fragment isolation are also described.

KEY WORDS: HPLC, peptides; hydrogen exchange; isotope analysis; protein structure.

The measurement of protein hydrogen exchange (HX)[1] can, in principle, provide information on structure, structure change, and structural dynamics at positions throughout a protein molecule (1–3). HX approaches can also selectively tag and thereby help to identify intermediates in protein-folding pathways (4,5). To realize these objectives, it is necessary to correlate measured H-exchange behavior with identifiable regions of the protein molecule. This has so far been possible only in a limited way. The recent development of two-dimensional NMR techniques (6,7) makes possible the assignment of individual proton resonances in very small protein molecules and the observation of their exchange with solvent protons. Neutron diffraction methods can distinguish ^1H and ^2H at individual proton sites, and thus can portray, in the crystal state, the detailed ^1H–^2H exchange picture at what is essentially one time point on the overall H-exchange curve (8–11).

A similar capability, using ^1H–^3H exchange, may be applicable to all proteins in solution, or in any other state, and to all points along the protein H-exchange curve. It is now possible to label selectively with exchangeable tritium just those protein segments of interest for any function being studied (4,12). A protein fragmentation method pioneered by Rosa and Richards (13–15) can make it possible to specify the location of exchangeable sites labeled in this way. We have been working for some time to make the latter method reasonably quantitative so that it might adequately serve this purpose (16–18). In applying this method, one can selectively label a protein with exchangeable tritium, using either "functional labeling" (12,18) or "kinetic labeling" (19,20), or other trapping approaches (4,5,21). One then wants to determine the position of the label. In the method dealt with here, this is accomplished by quenching the ^3H-labeled protein into slow exchange conditions, cleaving it into fairly small fragments, and then separating the fragments and analyzing them for carried tritium.

It may seem that such an effort is doomed to failure since the exchangeable tritium label must be lost before any useful separation of

[1] Abbreviation used: HX, hydrogen exchange.

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the fragments can be obtained. In the early development of this method (13–15), major tritium losses were in fact experienced. Nevertheless it is possible to perform this kind of analysis under conditions that retain most of the label. Furthermore, losses that do occur can be accurately estimated and accounted for so that a fairly precise measurement can be obtained for the amount of label originally present on each fragment as it existed in the intact protein. Here we illustrate the means for accomplishing this, using hemoglobin as a test protein.

Another difficulty with this approach is the requirement for obtaining defined protein fragments in good yield at low pH, using acid proteases which are notoriously nonspecific. Progress on this problem is also discussed.

METHODS

Hemoglobin was obtained from human blood by standard methods. Pepsin, from Worthington, was dialyzed (0.05 M acetate, pH 4.5) for 2 h at 0°C (31) to remove small-molecule contaminants and kept frozen in small aliquots. Initial labeling of hemoglobin samples was done with tritiated water at levels between 2 and 100 mCi/ml. These levels are fully adequate for the experiments described here, so that protein labeling can be done under common solution conditions. Special freeze-drying methods designed to utilize and recover much higher level solutions [1 Ci/ml (13–15)] are unnecessary.

For full equilibrium labeling of even the most slowly exchanging sites in hemoglobin, relatively rigorous conditions were used (e.g., pH 9 to 10 at 40°C for 2 days). For selective labeling experiments (18), labeling can be done under any functional solution conditions. Separation of tritiated protein from the tritiated solvent used columns of Sephadex G-25 with bed size up to 2×8 cm for unusually large sample volumes (2 ml), but a 1×6-cm column was usually employed. Much larger columns described by others are unnecessary.

To prepare selectively labeled protein for the HPLC analysis, samples were put into the slow exchange condition, usually pH 2.7 at 0°C, by passage through a cold Sephadex column buffered at this pH with 0.1 M sodium phosphate or by adding a predetermined volume of phosphoric acid to the sample. The sample was fragmented with an acid protease (pepsin at 0.1 to 0.2 mg/ml for 10 min), and the fragments were separated by HPLC. For general fragment separation, we use phenyl (fatty acid analysis) μBondapak columns (Waters Associates) with linear solvent gradients running from about 15 to 45% of solvent B, where solvent A is 0.05 M sodium phosphate at pH 2.7 and solvent B is 10% A, 30% dioxane, 60% acetonitrile, readjusted to pH 2.7. To minimize losses of exchangeable tritium all these steps were performed at 0°C.

For more complete descriptions of ^1H–^3H exchange methodology, see Refs. (19,27,28).

RESULTS

A Test of the Method

Some results obtained with the method described here are shown in Fig. 1A and B. In these experiments hemoglobin was initially labeled to nearly full exchange equilibrium in tritiated water. This provides a stringent test sample; if the subsequent analysis is correct, it should indicate an amount of label corresponding to the known number of peptide NH on each fragment.

For this analysis the fully labeled protein was placed into the slow exchange condition, pH 2.7 at 0°C, via a Sephadex column (2 min), α and β chains were separated by reverse-phase HPLC at pH 3.3 in 45% acetonitrile, 1.2% trifluoroacetic acid (25 min), the chains were collected and dialyzed (31) to remove the organic solvent (10 min) and then fragmented with pepsin (10 min), and the fragments were separated by HPLC (all at 0°C). Figures 1a & b show elution profiles of α- and β-chain fragments and the tritium counts carried by each fragment. The identity

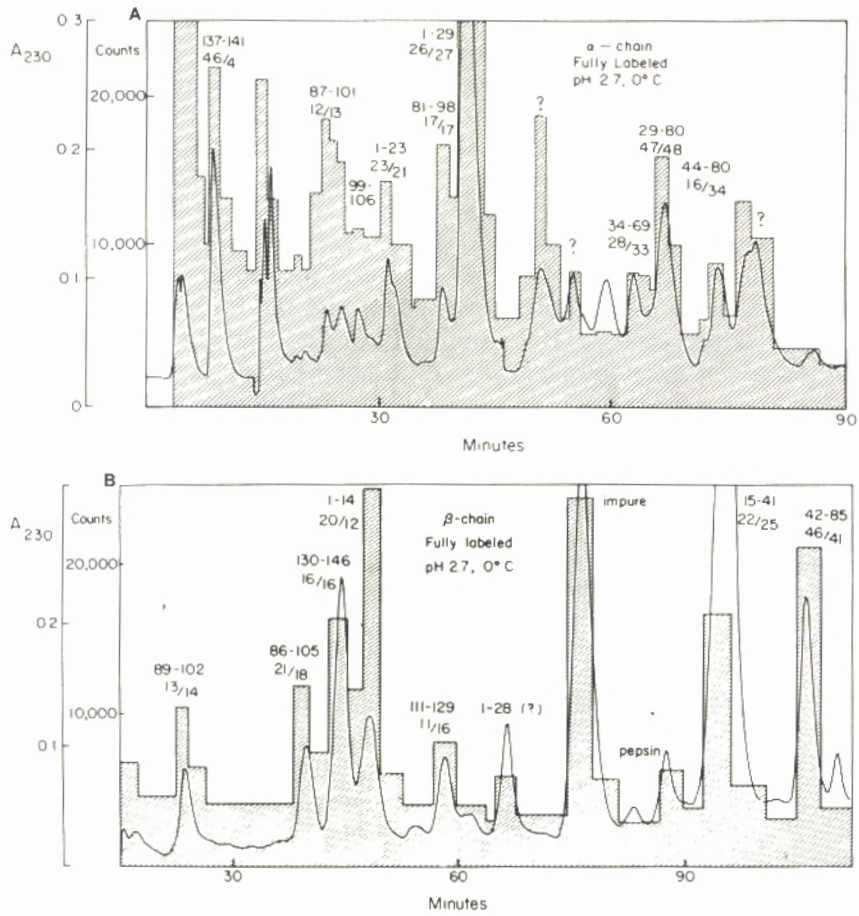

FIG. 1. Analysis of hemoglobin by the fragment separation method. Hemoglobin was fully tritiated at exchangeable proton sites, placed into slow exchange conditions, separated into subunits, fragmented with pepsin, and resolved by HPLC. The solid line (absorbance at 230 nm) traces elution profiles for the α- and β-chain fragments. The histogram indicates tritium count level in each fraction taken. Numbers above each peak give the fragment identity and number of protons measured/number of protons present. The value for number of protons present counts 1 for each peptide NH and zero for prolines and the N-terminal residue. Side chain Asn and Gln NH have a lifetime less than 1 min under these conditions (24) and are not expected to be recovered.

of each fairly pure fragment is indicated by its sequence position in the native chain. The tritium label recovered on each fragment was computed in terms of the number of peptide NH and compared to the true number of peptide NH, indicated above each fragment in Fig. 1 as NH measured/NH present. Good quantitative accuracy was found for many of the hemoglobin fragments.

In these experiments the major challenge is to minimize the loss of exchangeable tritium from the peptide fragments as the separation proceeds, and to correct for the unavoidable losses that do occur. The low pH and low temperature condition used here produce a halftime over 1 h for the exchange of fully exposed peptide NH, and the organic solvents used in the HPLC procedures slow exchange still further. Table 1 lists the real time required for each step in this procedure and the equivalent exchange time, in terms of time at the standard slow exchange con-

TABLE 1

CALCULATION OF EQUIVALENT LOSS TIME

Operation	Real time (min)	Equivalent time (min)
Remove THO (gel filtration)	2	2
$\alpha\beta$ Separation	25	5
Dialysis	10	10
Pepsin cleavage	10	10
HPLC pregradient	12	12
Time in gradient		
β130–146	33	12
β15–41	83	31
Total time	92 and 142	51 and 70
Loss factor		1.8 and 1.9

Note. The entries show, for each step in the fragment separation procedure, the real time used and the equivalent exchange time, calculated as real time/SF. SF is the H-exchange slowing factor due to the presence of organic solvent in the HPLC separations, calculated according to Eq. [4]. Given the total equivalent loss time, one can calculate the tritium loss (correction) factor as in Fig. 5. These are 1.8 for β130–146 after 51 min of equivalent time and 1.9 for β15–41 after 70 min equivalent time. When β130–146 was isolated more rapidly (see text), real time was 27 min and equivalent time was 19 min, leading to a loss factor of 1.3.

dition (pH 2.7, 0°C) in fully aqueous medium. To compute the numbers of exchangeable NH shown in Fig. 1, the tritium label actually measured for each fragment was multiplied by the overall loss factor expected for that fragment during the procedures in Table 1, as described below.

A further experimental check of the validity of these corrections can be obtained by carrying out the separation under conditions that lead to a much faster recovery of particular fragments. The experiment depicted in Fig. 2 was designed to recover rapidly the fragment β130–146. This was accomplished within 27 min (19 min equivalent time) of quenching the initial fully labeled hemoglobin sample into the slow exchange condition (pH 2.7, 0°C). Here several of the steps listed in Table 1 were eliminated, so that the overall correction factor for tritium loss was reduced from 1.8 to 1.3 (real tritium recovery increased from 55 to 77%). These experiments measured 16 NH compared to the true value of 16 NH actually on the β130–146 segment.

Tritium Loss

In these experiments it is of paramount importance to limit the loss of exchangeable label during the fragment separation steps to perhaps one halftime or so. If this is accomplished, then knowledge of tritium loss rates at the various steps can allow an accurate correction for the losses sustained. Steps for minimizing losses, and information for calibrating loss rates and correcting for the losses that do occur, are dealt with here.

Temperature and pH. The peptide group H-exchange reaction has a relatively high temperature coefficient, with activation energy approximately 17 kcal, leading to a rate decrease of threefold for a 10°C drop in temperature (22,23). Thus as a first step in minimizing tritium loss, one uses low temperature. All the handling procedures discussed here were carried out at 0°C, including

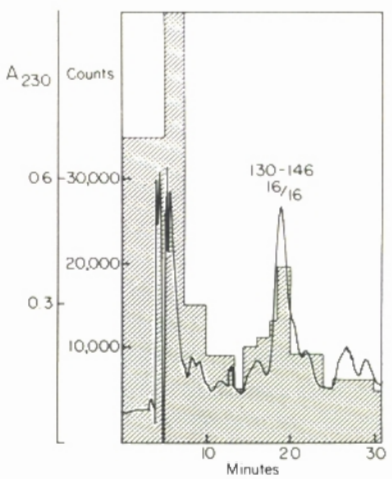

FIG. 2. Accelerated analysis of the fragment β130–146 by the fragment separation method. Fully tritiated hemoglobin, without prior separation into subunits, was fragmented with pepsin and run on an HPLC gradient designed to deliver fragment β130–146 directly. Profiles show the elution trace measured by absorbance at 230 nm and the carried tritium (histogram).

the HPLC separations for which the columns and injector valve were kept in ice water. Rosa and Richards (13–15) used 8°C, which accounts for a large part of the major tritium losses they experienced.

H-Exchange rate is also a sensitive function of pH. Figure 3A displays the rate–pH dependence of the peptide NH in random chain poly-DL-alanine at 0°C. If we read from the alkaline limb of the curve, the rate decreases with pH by a factor of 10 per pH unit, reflecting catalysis by OH^- ion, passes through a minimum at pH 3, and then increases by 10-fold for each further pH unit owing to H^+-ion catalysis. When the amino acid side chain is more polar than alanine, the exchange rate of neighboring peptide NH are increased in the alkaline region and reduced below pH 3. The shadowed region in Fig. 3A indicates the range of rates calibrated by Molday *et al.* (24) for the naturally occurring amino acids. In a heteropolypeptide these same effects also tend to lower the overall minimum in the catalysis curve (pH_{min}) to approximately pH 2.7.

Figure 3A shows that at pH_{min} and 0°C, the exchange halftime for freely available peptide NH is over 1 h. This fact, together with the ability of HPLC to produce oligopeptide separations on this same time scale, makes the method described here possible. The parameters in Fig. 3A can be used directly to predict and correct for the losses in exchangeable tritium sustained during the steps that occur in aqueous solvent (Table 1). To correct for losses during the HPLC separations, which use organic-aqueous mixtures, further information is required. Here exchange is even slower than indicated in Fig. 3A.

Organic solvents. Figure 3B displays the effect on the amide HX rate of a number of organic solvents that find use in reverse-phase HPLC. Added organic solvents have little effect on the H^+-catalyzed exchange rate, evidently because H^+ activity is empirically reset in each solvent mixture by the direct pH meter reading. The apparent OH^- rate, however, can be greatly slowed largely due to a decrease in K_w and thus in OH^- activity (25,26). This effect is helpful in reducing tritium losses during the HPLC separations. Note that as the cosolvent is increased, the pH_{min} shifts progressively to higher pH. This effect also can be turned to some advantage (see below).

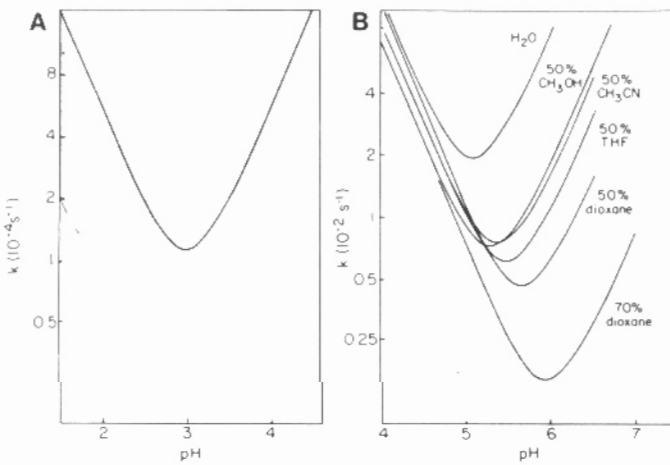

FIG. 3. Dependence of amide NH-exchange rates on pH and organic solvents. The solid line in panel A shows exchange rates of random-chain poly-DL-alanine in water at 0°C (22). The shadowed region indicates the range of rates experienced by peptide groups with various polar side chains (24). The shift in HX rate due to various mixed HPLC solvents is shown in panel B, measured using acetamide at 25°C.

The results shown in Fig. 3B were obtained using acetamide as a model amide, which allows this kind of information to be efficiently collected by spectrometric procedures (23). An acetamide solution in D_2O was diluted 10-fold into an H_2O–organic solvent mixture buffered at the pH to be studied, and the rate of the ^1H–^2H exchange reaction was followed by recording the absorbance change in time at 230 nm (23). Since the effects seen here reflect solvent phenomena alone, the relative slowing factors for the OH^- and H^+ reactions and the measured shifts in pH_{min} are directly applicable to peptide group behavior or to any similar pH-dependent reaction. From these data, loss rates for real peptide NH can be directly computed.

Alternatively these methods can provide a more direct calibration of HX slowing factors under the actual HPLC conditions to be used. We separate oligopeptide fragments in a gradient of two HPLC solvents (A and B, see Methods) at 0°C. Figure 4 shows the slowing factors for varying mixtures of solvents A and B. These calibrations were obtained as in Fig. 3B, but using poly-DL-alanine as a test molecule. Since it was difficult to perform the spectrometric measurements at 0°C directly, these measurements were done at 23 and 13.5°C. The relative slowing in these solvents compared to water is essentially temperature independent; i.e., the apparent activation energy through this range is 17 kcal, equal to the activation energy found in water (22,23).

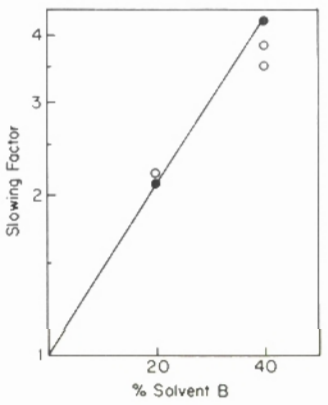

FIG. 4. HX slowing factors in HPLC solvents. Slowing factors are from direct HX measurements like those in Fig. 3b, using poly-DL-alanine in mixtures of solutions A and B (see text) preset to pH 2.7. Slowing factors were measured at 25°C (●) and 13.5°C (○).

In the HPLC experiments, solvents A and B are initially set at pH 2.7, the general minimum for peptide NH exchange. As more B is added, the position of pH_{min} shifts to higher pH (Fig. 3B). In our gradients the pH_{min} shifts by about 0.3 pH units. Fortunately, as B is added, the apparent pH increases in step with the HX rate shift so that the exchanging peptides are spontaneously kept close to their pH_{min} as the changing solvent gradient sweeps over them in the HPLC column. [The pH shift occurs due to the solvent dependence of phosphate pK_a, even though A and B are individually adjusted to read pH 2.7. This effect probably accounts for the apparent deviation from Molday *et al.* (24) behavior noted by Rosa and Richards (13).]

Correcting for tritium losses. If one knows the loss rate of exchangeable tritium during the fragmentation analysis, then the label measured at the end of the analysis can be corrected by multiplying by the expected loss factor. For any peptide fragment, the apparent number of peptide NH remaining unexchanged as a function of exchange-out time can be written as

$$H_t = \sum_i e^{-k_i t} \qquad [1]$$

where the summation is taken over each individual NH with exchange rate constant k_i. The ^1H–^3H exchange rate for the exposed peptide NH in poly-DL-alanine at 0°C (Fig. 3A) is given by (22)

$$k\,(s^{-1}) = 5.8 \times 10^{-5}(10^{pH-3} + 10^{3-pH}). \qquad [2]$$

To obtain the loss factor, $L(=H_0/H_t)$, required to correct the label measured at the end of the analysis to the value present in the native protein at the time of initiating

the analysis, a predicted HX loss curve should be generated and used for each fragment studied. As an example, the ^1H–^3H exchange curve expected for the hemoglobin peptide β130–146 at pH 2.7 and 0°C in water is shown in Fig. 5. This curve was calculated using the peptide NH-exchange rate given in Eq. [2] for these conditions, modified to take into account the effect on the HX rate of each peptide NH in β130–146 due to inductive effects exerted by its neighboring side chains, according to the rules of Molday and co-workers (23,24). The variety of peptide NH present, though they exhibit some range of exchange rates, produce a roughly exponential exchange curve with a halftime under these conditions of about 1 h (Fig. 5).

In computing L, one also wants to take into account the fact that tritium is lost more slowly than is indicated in Fig. 5—effectively time moves more slowly—while the fragment is in the partially organic HPLC solvent. For the bookkeeping involved in this correction, we define a slowing factor, SF, which relates the losses experienced at each step in the peptide separation procedure to the time in aqueous solvent that would have produced the same loss (see Table 1). Figure 4 shows that, through the limited solvent gradient useful for peptide separations, the H-exchange slowing factor, SF, increases essentially exponentially with the concentration of added HPLC solvent B, according to Eq. [3a], or with the time dependence indicated in Eq. [3b], since we use a linear solvent gradient.

$$SF = SF_0 e^{a[B]} \qquad [3a]$$

$$SF = SF_0 e^{ct}. \qquad [3b]$$

In separating β-chain fragments, we use a gradient that runs from 15 to 45% solution B in 100 min. From Fig. 4 then, SF_0, the slowing factor at the initial 15% B, corresponds to 1.75, and the constant c (Eq. [3b]) is $\ln(SF_t/SF_0)/t = 0.011$. The real time, t, spent in the organic solvent gradient corresponds to a shorter loss time (t_{cor}) in the standard aqueous solvent according to Eq. [2],

$$t_{cor} = \int_0^t [SF_0 e^{ct}]^{-1} dt$$

$$= (1 - e^{-ct})/(SF_0 c). \qquad [4]$$

The integration sums the effect of the changing gradient.

According to this analysis, in the $\alpha\beta$ separation performed isocratically in 45% acetonitrile, exchange losses occur at one-fifth the rate in aqueous solvent, so that the real 25-min separation period corresponds to a corrected loss time of only 5 min on the exchange time scale utilized in Fig. 5 (Table 1). For the subsequent isolation of fragment β130–146 (see Fig. 5), the 33 min spent in the column gradient corresponds to an equivalent time of 12 min (Table 1). When β130–146 emerges from the HPLC column and is collected, 92 min after the initial native hemoglobin sample was placed into the denaturing slow exchange condition, it has experienced a loss of exchangeable label corresponding to 51 min under the fully aqueous slow exchange condition. From Fig. 5, the loss factor L at 51 min is 1.8. The tritium label actually measured on the recovered fragment must be multiplied by L to correct for these losses (Table 1). The experiment shown in Fig. 2 was designed to obtain

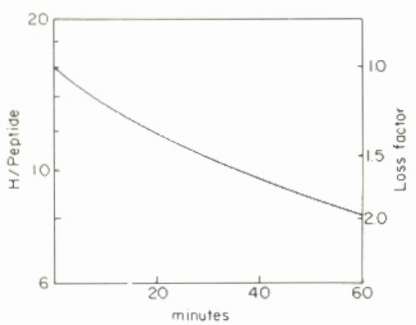

FIG. 5. Tritium loss curve for fragment β130–146 (semilog). The predicted HX curve was computed from Eq. [2] for poly-DL-alanine at pH 2.7 and 0°C taking into account the effects of Molday *et al.* (24). From this curve one can obtain the loss correction factor (L) for this fragment if the equivalent exchange-out time is known.

β130–146 more directly. Here the preliminary $\alpha\beta$ separation was not used; whole hemoglobin was treated with pepsin and the fragments were separated in a gradient beginning with 18% solvent B so that β130–146 elutes soon after the front. A similar accounting shows the real time of 27 min corresponds to a corrected loss time of 19 min. Figure 5 then indicates a loss-correction factor, L, for the rapidly isolated β130–146 peptide of 1.3.

The number of exchangeable peptide NH on the fragment β130–146 is 16. Both the longer analysis (Fig. 1B) and the accelerated analysis (Fig. 2) indicate close to 16 NH after correction for the losses during the fragment separation period, in good agreement with the true value.

The free NH assumption. How valid is the assumption that exchange proceeds as just indicated from the time of placing the hemoglobin sample into the slow exchange condition? An independent test is shown in Fig. 6 which compares tritium exchange-out data, measured (27,28) by ^1H–^3H exchange at pH 2.7 and 0°C, with the theoretical curve computed as just indicated for denatured hemoglobin (random chain assumed). Good agreement is found down to about 80% exchange, past which point the measured loss rate is a little slow, perhaps indicating some residual structure in the acid denatured protein at 0°C. Analogous experiments were also performed using pepsin-fragmented hemoglobin; here the exchange was followed by diluting fully deuterated hemoglobin fragments from D_2O into H_2O at these standard conditions, and observing the exchange spectrometrically (23). Again the measured exchange tracked well the predicted curve.

Thus it appears that for most segments in hemoglobin, and presumably also other similarly denatured proteins, tritium loss during the fragment separation procedures can be expected to proceed at the rate predicted for structure-free oligopeptides. Since these have been well calibrated (22–24), the tritium losses sustained can be estimated with good accuracy and corrected for. Still, in further use of these methods one should be alert for possible deviations from this simple behavior.

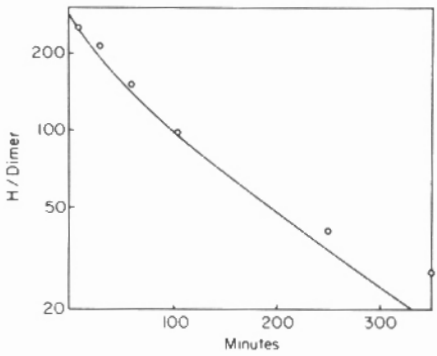

FIG. 6. A test of the HX rate prediction. The solid line is the ^1H–^3H exchange curve predicted from Eq. [2] modified by the Molday *et al.* (24) factors for (random coil) human hemoglobin at pH 2.7 and 0°C. The data points are from ^1H–^3H exchange measurements on hemoglobin at these conditions.

Data Calculation

The primary data obtained in these experiments are the optical density (OD) of the eluted fragment samples and their level of carried tritium counts (C). Optical density (OD) can be obtained most conveniently by integrating the absorbance trace (e.g., Fig. 1) of each collected sample. From these measured parameters, the number of labeled sites (H/peptide) on each fragment as it existed in the native protein at the beginning of the HPLC analysis is given by Eq. [5].

$$H/\text{peptide} = (111\epsilon_{230}/1.19C_0)(C/\text{OD})(L). \quad [5]$$

A similar equation has been described before (27,28,32). $C_0/111$ is the specific activity of the initial exchange-in solvent; i.e., 111 is the molar concentration of H in H_2O and C_0 is the tritium count level. C is the count level in the recovered fragment sample, so that $C \times 111/1.19\ C_0$ gives the molar concentration of initial H still carried on the fragment sample. The factor 1.19 accounts for the equilibrium isotope effect when exchangeable tritium is distributed between H_2O and peptide NH (1,22). The term OD/ϵ_{230}

(optical density/extinction coefficient) is the molar concentration of oligopeptide fragment in the final sample. OD is the integrated absorbance of the part of the elution profile relevant to the sample collected. L is the loss factor experienced during the entire HPLC analysis, as described above.

The value of ϵ varies greatly from one fragment to another and must be determined for each fragment analyzed. This can be done in two ways: (i) on a theoretical basis, by summing the absorbance contribution due to each chromophoric group in the fragment, namely the peptide groups themselves plus the number of tyrosine and tryptophan residues present; (ii) experimentally, by quantitative analysis of the isolated peptide fragments, requiring optical density measurements of purified fragment samples together with quantitative amino acid analysis to obtain the molar concentration of each fragment sample. We have used both approaches for many fragments of hemoglobin and found good agreement in most but not all cases. We use 500 M^{-1} cm^{-1} as an estimate of the average absorbance due to the peptide bond at 230 nm. (The true value varies as a function of solvent composition and neighboring side chains since 230 nm is on a sharply rising edge of the peptide absorbance bands.) When tyrosine or trytophan is present, these residues make a dominant contribution to fragment optical density. The molar extinction coefficients of the aromatic amino acids are somewhat dependent on solvent concentration, so that the ϵ value pertinent for the actual HPLC solvent concentration present in each fragment sample should be used. Figure 7 shows these values as a function of the percentage of solvent B in our HPLC system. Absorbance at 280 and 220 nm is relatively insensitive to solvent since these wavelengths represent approximate maxima in the spectrum.

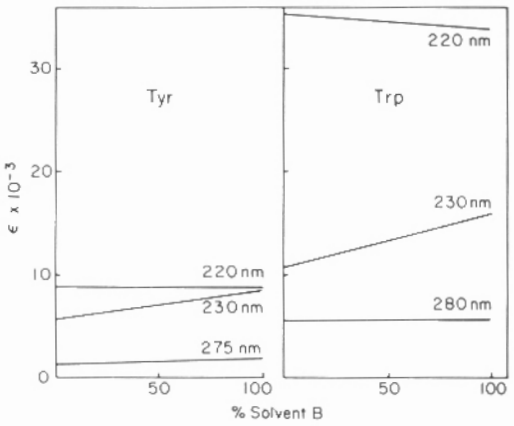

FIG. 7. Molar absorbance of aromatic amino acids. Extinction coefficients are shown for tyrosine and tryptophan in HPLC solvents at wavelengths pertinent for the HPLC analysis. These residues when present in a peptide fragment make a dominant contribution to measured absorbance in the HPLC elution profiles.

Production and Separation of Fragments

To implement the method described here, one wants to obtain a limited number of defined fragments in good yield. This is best achieved by use of proteases with high specificity. The special requirements of the present system, however, restrict the experimental pH to a small range about pH 3, dictating the use of acid proteases, which are unfortunately nonspecific. Konigsberg and his coworkers (29,30) described the peptic digest of hemoglobin; after exhaustive proteolysis (24 h at 25°C) about 60 fragments are obtained in variable yield. One can expect that a partial digest, for example one obtained in a limited time at 0°C, will be even more complex. This would make the fragmentation approach useless.

We have found, however, that pepsin displays a kind of kinetic specificity that makes it possible to obtain resolvable breakage products (see Fig. 1) from a partial digest. This phenomenon is exhibited in Fig. 8, which traces the kinetics of hemoglobin cleavage by pepsin in time. Approximately 12 breaks per chain are produced fairly rapidly, apparently representing a limited number of sensitive sequences. (Analysis indicates a relative specificity for the C-terminal side of leucine and phenylalanine.) The curve then enters a much slower phase of further

proteolysis that goes on for a long time, and ultimately involves about twice the number of cuts that occur in the more rapid phase. The population of fragments produced can be expected to pass through a region of minimum complexity around the break point in the curve of Fig. 8, where one can expect to find a dozen or so fragments in fair yield. HPLC runs of samples taken through this region support this concept (see Fig. 1) although yields of any individual peptide are still well below 100%. To aim for this region in the fragmentation step, we normally add 0.1–0.2 mg/ml of pepsin to hemoglobin or hemoglobin chains present at a concentration of approximately 5 mg/ml (pH 2.7, 0°C) and find that the peptide elution pattern (Fig. 1) is remarkably stable to moderate changes in these parameters.

When this is done with whole hemoglobin, the digest contains about two dozen fragments, which are dificult to separate cleanly. We simplify the patterns by first separating the α and β chains in a preliminary HPLC run, and then further fragment either chain. (The unused chain can be deep frozen without loss of tritium and processed later.) The initial $\alpha\beta$ separation requires 20 to 25 min of real time, which translates into only about 5 min of equivalent exchange-out loss time (Table 1). In addition, there is another loss of 10 min required to remove by rapid dialysis (31) the 45% acetonitrile/1.2% trifluoroacetic acid solvent used in the $\alpha\beta$ separation, which otherwise inhibits the subsequent pepsin proteolysis.

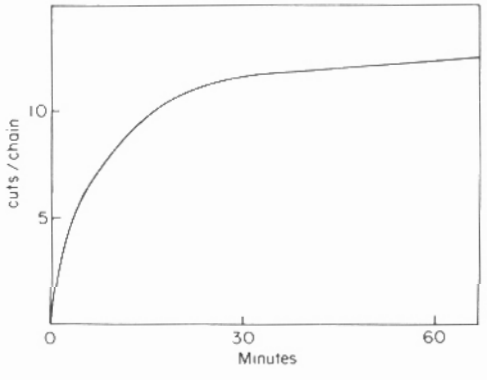

FIG. 8. Kinetic specificity in the proteolysis of hemoglobin by pepsin. About 12 sensitive sequences per chain are cleaved in a rapid kinetic phase that precedes the subsequent slower breakage at many sites.

Alternative strategies may be useful. One would depend upon the isolation of a few large fragments in a first HPLC separation, and their subsequent resolution into subfragments. We have not been successful at this with hemoglobin. Another possibility is suggested by the experiment shown in Fig. 2, in which the HPLC conditions were targeted to obtain directly one predetermined fragment. It may often be possible to design a high-resolution separation that will directly deliver any individual fragment for study. For further localization of tritium label on this fragment, it may be further subfragmented with another acid protease, or with more pepsin.

The difficulties of peptide separation can be relieved to some extent by realizing that it is by no means necessary in these experiments to obtain baseline resolution. When eluant peaks overlap, collection of the leading edge of the forward peak and the back edge of the trailing one can often yield samples sufficiently pure for the tritium analyses required.

DISCUSSION

From the early development of the hydrogen-exchange approach by Linderstrom-Lang and his co-workers (33), it has seemed clear that the measurement of protein H-exchange properties could provide a great deal of information on protein structure and behavior. The interpretation of HX data in these terms, however, requires good knowledge of the ways in which aspects of protein behavior translate into measurable H-exchange parameters. This is a matter of continuing debate (2). It now seems clear that the resolution of these issues and their subsequent use in structure and function studies will require the ability to measure the H-exchange behavior of defined protons in defined protein structures. Emerging NMR and neutron dif-

fraction methodologies and the protein fragmentation approach dealt with here show a great deal of promise along these lines. It is our view that the effective application of H-exchange approaches to sizable functioning proteins will be especially well served by the fragmentation approach when combined with selective functional labeling studies.

The methods described here provide improvements in certain aspects of the approach initially utilized by Rosa and Richards (13–15) and show how these measurements can be made essentially quantitative. One can look forward to further improvements. At present the total analysis of the HX behavior of a given protein by these methods is an immense task. In a large sense, the best strategies for undertaking such a task remain to be formulated. Also, these efforts would benefit from further technical improvements, for example in HPLC separation capability and perhaps especially in the development of additional acid proteases with properties adapted to the needs of these experiments.

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