INDIVIDUAL BREATHING REACTIONS MEASURED IN HEMOGLOBIN BY HYDROGEN EXCHANGE METHODS

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ABSTRACT   Protein hydrogen exchange is generally believed to register some aspect of internal protein dynamics, but the kind of motion at work is not clear. Experiments are being done to identify the determinants of protein hydrogen exchange and to distinguish between local unfolding and accessibility-penetration mechanisms.   Results with small molecules, polynucleotides, and proteins demonstrate that solvent accessibility is by no means sufficient for fast exchange.   H-exchange slowing is quite generally connected with intramolecular H-bonding, and the exchange process depends pivotally on transient H-bond cleavage.   At least in α-helical structures, the cooperative aspect of H-bond cleavage must be expressed in local unfolding reactions. Results obtained by use of a difference hydrogen exchange method appear to provide a direct measurement of transient, cooperative, local unfolding reactions in hemoglobin. The reality of these supposed coherent breathing units is being tested by using the difference H-exchange approach to tritium label the units one at a time and then attempting to locate the tritium by fragmenting the protein, separating the fragments, and testing them for label. Early results demonstrate the feasibility of this approach.

INTRODUCTION

"A protein cannot be said to have 'a' secondary structure but exists mainly as a group of structures not too different from one another in free energy... the molecule must be conceived as trying out every possible structure each in accordance with its Boltzmann factor." This modern-sounding statement was written 21 years ago by K. U. Linderstrom-Lang and John Schellman (1), working side by side on protein hydrogen exchange and on helix-coil transition theory. Lang put forward the proposition that the slowing of H-exchange in proteins may reflect internally H-bonded helical structures (2) and that the exchange process probably requires the transient cleavage of these bonds. For ideas about the mode of H-bond cleavage he drew on the pictures of polypeptide denaturation being dealt with by Schellman (3). Today, decades later, studies of protein dynamics have become most popular, but we are still undecided about the kinds of motion that determine protein H-exchange behavior.

With the benefit of technical advances made since that time, work in this laboratory and others has somewhat refined the so-called breathing hypothesis of H-exchange (Fig. 1 *a*) (hereafter called local unfolding), which holds that in the H-exchange pathway normally protected sites must be brought out into the solvent by a transient structural unfolding reaction (4, 5).   Others have taken an opposite view, that slow exchange is primarily a function of solvent inaccessibility, and that the H-exchange process requires the penetration of the native protein structure by H_2O and/or OH^--ion (6–8).   These exchange catalysts are thought to make their way into inaccessible protein regions via pathways opened by local fluctuations, then to bind the protected protons and carry them out to the solvent (Fig. 1 *b*). We will refer to this general viewpoint as the accessibility-penetration hypothesis.
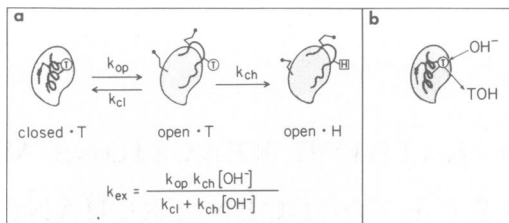
Figure 1   Representations of the competing local unfolding (*a*) and accessibility-penetration (*b*) models. Alternative H-exchange mechanisms are shown for an internal (labeled) proton, here H-bonded in an α-helix.   The kinetic expression relating exchange rate to opening and chemical parameters in the unfolding model is also shown.

In this paper, we summarize these models and the present state of knowledge bearing on the penetration--local unfolding question, and then describe results being obtained in this laboratory which favor a central role for local unfolding reactions in H-exchange processes. Also a new kind of experimental approach is illustrated which may be able to settle this question in a definitive way.

METHODS

Hydrogen-Tritium Exchange

In H-T exchange experiments, hemoglobin was incubated in solution at pH 7.4 and 0°C with a trace of tritiated water to label exchangeable H sites. Free tritiated water was then removed by fast passage through a Sephadex column to initiate exchange-out of bound T. After increasing exchange-out times, samples were taken, the newly freed T was removed, the amount of label still remaining bound was measured, and results were calculated and plotted in terms of original H per hemoglobin dimer not yet exchanged-out as a function of time (for further details see reference 9).

Difference Hydrogen Exchange

The difference H-exchange approach was designed to separate out from the overall exchange curve of hemoglobin just those hydrogens whose exchange rate is altered in the allosteric transition. The approach takes advantage of several factors: the slowly exchanging (H-bonded peptide) protons of hemoglobin are widely spread out over about eight decades on the H-exchange time axis, only a fraction of these (~1/3) respond to allosteric change, and the responsive H all take on faster exchange rates when deoxyhemoglobin is switched to the liganded form. The difference method applies three separate kinetic filters to isolate the allosterically responsive hydrogens. The initial exchange-in is limited in time so that relatively slowly exchanging sites are not labeled; behavior of the faster sites can then be studied without the usually occurring large background of slow tritium. To look most specifically at allosterically responsive sites, the hemoglobin sample is initially labeled for a limited time in the fast exchanging oxy form, then switched to the slow deoxy form for exchange-out measurements. Tritium on responsive sites, labeled rapidly in the oxy form, is then locked in and exchanges out much more slowly in the deoxy form. In contrast, allosterically indifferent sites that are fast enough to become labeled during the limited oxy form exchange-in experience equally fast exchange-out in the deoxy form, since their exchange is by definition insensitive to the allosteric form. Thus the tritium remaining after some initial exchange-out is largely on allosterically responsive sites. In general, however, there is still a residual contribution due to the labeling of a small fraction of the very slow allosterically indifferent sites. This contribution can be specifically portrayed in a "background curve" by reversing the above selective labeling approach. Thus hemoglobin can be initially selectively labeled for the same time period as before but now in the slow, deoxy form. This avoids labeling of (now slow) responsive sites but does label the very same indifferent sites as before. Exchange-out of this sample, especially in the fast oxy form, provides the desired background curve, and subtraction of the background from the sample curve portrays the exchange of responsive sites only. (For further description and examples, see references 5, 10–12).

Hydrogen-Deuterium Exchange

H-D exchange of nucleic acid components was studied by use of spectral absorbance differences that appear when exchangeable nucleotide protons are exchanged for deuterons. Polynucleotides in D_2O were diluted into H_2O buffers in a stopped-flow mode and exchange was monitored at 290 nm (13).

High Performance Liquid Chromatography

The location of exchangeable tritium placed on specific allosterically-responsive peptide groups of hemoglobin by the difference H-exchange technique was studied. Specifically labeled samples in the usual pH 7.4 buffer were dropped to pH 2.7 by fast passage through a short Sephadex column. Here H-exchange halftime of unstructured peptides is ~70 min. The hemoglobin was fragmented by added pepsin for 300 sec, injected into an HPLC column at the same pH, and immediately run through a low pH, dioxane-acetonitrile gradient. All these operations were at 0°C. Two Waters 6000A pumps (Waters Instruments, Inc., Rochester, Minn.) were used and effluent optical density was monitored at 230 nm in a flow cell-equipped Gilford spectrophotomer. Samples were collected and counted for tritium content. A similar approach has been previously described by Rosa and Richards (14). Effluent peaks were identified by amino acid analysis, end group Edman-dansyl determinations, and other approaches.

HYDROGEN EXCHANGE MODELS

It has long been appreciated that the H-exchange behavior of a protein can tell us about motions all along its backbone chain. However, to extract this information, it is necessary to understand the relationship between particular motional modes and the exchange behavior they generate. Two opposing models have been put forward. Fig. 1 *a* diagrams the local unfolding model and Fig. 1 *b* the penetration model. The slowly exchanging protein hydrogens under consideration here turn out with few exceptions to represent peptide group protons and their exchange, above pH 3, is catalyzed solely by OH^--ion (15).

The local unfolding model holds that the exchange of an H-bonded proton requires the prior cleavage of the H-bond and, in the presence of regular secondary structure, the separation also of neighboring H-bonds in a reversible, locally cooperative unfolding reaction. This allows the proton in question to be brought out into contact with water where normal OH^--catalyzed H-exchange chemistry can proceed. The kinetic equation for this reaction scheme (Fig. 1 *a*) shows that exchange rate should vary hyperbolically, in a Michaellian way, with concentration of catalyst. The exchange rate approaches a limiting value given by the structural opening rate (k_{op}) when OH^--ion catalyst is high or reclosing (k_{cl}) is slow (e.g., near a melting transition). At low OH^-, exchange rate is proportional to the catalyst concentration and here the normal exchange chemistry of the exchanging group is directly observable. According to this model, all the hydrogens exposed in a given breathing reaction share the same exchange behavior and the overall protein H-exchange curve represents the summation of a number of such sets. The model also suggests how protein H-exchange behavior should respond to the kind of localized structure changes known to occur allosteric transitions. For example the pre-breakage of the stabilizing (salt) linkage diagrammed in Fig. 1 *a*, as occurs in hemoglobin when 0_2 is bound (16), should promote the equilibrium opening of the segment pictured, and the exchange rate of all its protons by a factor corresponding to the free energy of the link broken (5).

In contrast to local unfolding, penetration models suppose that the catalyzing water species penetrate to the locus of individual inaccessible protons within the essentially native protein and there the important exchange event occurs. A number of suggestions concerning possible penetration modalities have appeared (6–8). Small atom-scale fluctuations rather than segmental unfolding is thought important for the penetration process. A role for H-bond

breakage may or may not be focused upon, but this is never viewed in the sense of a cooperative unfolding. Thus neighboring protons can generally be expected to have unrelated exchange rates, and small allosteric changes may alter neighboring protons differently and distant protons similarly. A quantity of recent non-H-exchange work does demonstrate the reality of small motional fluctuations within proteins (17–21) and suggests the deep penetration of proteins by small molecules such as dioxygen (22).

It is generally accepted that near a melting transition whole molecule opening can supersede the usual penetration and/or local unfolding pathways and determine exchange (15, 23). Here the same kinetic expression written for local unfolding is pertinent, and many if not all the protons can be expected to display essentially equivalent exchange rates. This case may be especially pertinent to recent studies on the H-exchange behavior of basic pancreatic trypsin inhibitor (24, 25).

RESULTS AND DISCUSSION

It appears to us that observations so far put forward as favoring accessibility-penetration models in fact do not do so, whereas considerable evidence argues against this viewpoint and favors local unfolding. In studies with a variety of proteins, the dependence of H-exchange rate on a wide range of solution parameters have been studied including pH, temperature, organic cosolvents, denaturants, and high pressure (23). Exchange rate generally shows a weak dependence on these factors though strong dependences have sometimes been found, especially under conditions approaching gross denaturation (15, 23). These results have been claimed to support the penetration hypothesis. The rationale for these assertions has seldom been clear to us and has more than once appeared self-contradictory, as when both high and low activations energy are in different cases claimed to support penetration. In fact the several available penetration models appear to make no specific predictions of the behavior to be expected under these various conditions, and the behavior found seems to us to be at least equally consistent with protein unfolding reactions of greater or lesser size. Here we organize a quantity of the pertinent evidence.

.The Importance of H-bonding and H-bond Breakage

Penetration models start with the assumption that H-exchange slowing stems from inaccessibility to solvent. In fact, the issue of contact with solvent appears to be more or less beside the point. In several well worked out cases, slow H-exchange can now be seen to result not from solvent inaccessibility but from intramolecular H-bonding and the requirements of the chemical exchange pathway. This occurs in small molecules, in double helical polynucleotides, and in some well-known proteins. Fig. 2 portrays examples of these. Let us consider them one at a time.

Small Molecules   Fig. 2 *a*, a salicylate, is one example of a large class of internally H-bonded molecules for which the H-bonded proton has been found to exchange far more slowly than the diffusion-limited rate (26). The maximum possible diffusion-limited rate, $\sim 10^{10}$ $M^{-1}s^{-1}$, is achieved when every collision with the exchange catalyst results in H-bond formation to the acceptor and successful transfer of the proton along the H-bond to the acceptor (26). The class of protons suggested in Fig. 2 *a* are hardly buried or inaccessible to solvent, yet in various known examples exchange is slower than the diffusion-limited rate by factors ranging up to 10^{5+}. Evidently the exchange process in these molecules is interrupted at the step of H-bond formation with the exchange catalyst (usually OH^--ion), which can occur only during the small period of time when the intramolecular H-bond is transiently severed
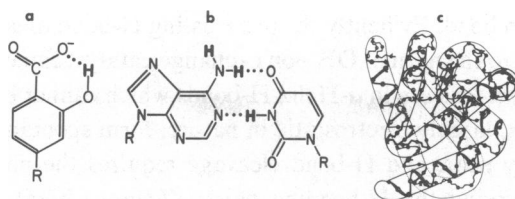
Figure 2 Several systems with fully accessible but nevertheless slowly exchanging protons. (*a*) Salicylate as one of many known small molecule examples. (*b*) A·U as one example of several Watson-Crick base pairs with known H-exchange character. (*c*) Myoglobin, with a large number of slowly exchanging protons on its aqueous surface.

(analogous to Fig. 1 *a*). Indeed a correlation between H-exchange slowing and pK in a series of such molecules has been demonstrated (27).

Polynucleotide duplexes   Fig. 2 *b* shows the standard Watson-Crick A·U base pair. The exchange behavior of these protons in the double helix is just the reverse of what would be expected from an accessibility point of view. It turns out that the ring NH proton of uridine, the most protected of the three exchangeable nucleotide protons, is the fastest to exchange. The two protons on the adenosine NH_2 group are slower and both of these, the external freely exposed A-NH_2 proton and the internally H-bonded one, exchange at identical rates. The same behavior occurs for the G·C base pair in which the central ring NH is even more impressively separated from contact with solvent. This behavior finds a straightforward explanation in the view that exchange can only occur from the transiently opened base pair. Here exchange rates are determined by the intrinsic exchange chemistry of the different groups rather than by their relative exposure in the native, structured form (13, 28). The observations that demonstrate these conclusions are summarized in a poster abstract by Mandal et al. in this volume.

For present purposes, we wish to focus mainly upon the perhaps obvious qualitative lesson that the too facile interpretation of H-exchange slowing simply in terms of relative accessibility is bound to be misleading. One must give due regard to exchange chemistry which very generally requires the breakage of pre-existing internal H-bonds. It appears that in many cases this will require a sizable structural opening reaction.

Myoglobin   Fig. 2 *c* is the familiar Dickerson-Geis representation of myoglobin. Perhaps half the peptide group protons of this molecule are at the protein surface, in contact with solvent water. Yet it is clear, simply from the numbers involved and results obtained in several laboratories, that many of the surface protons exchange more slowly than free, non-H-bonded peptide protons by orders of magnitude.

This result does not arise from any exotic physical or chemical situation on the surface of the protein, since it has been possible to show that the non-H-bonded peptide protons on the myoglobin surface exchange precisely according to expectations for free peptides, based on calibrations in small molecule models. This was demonstrated by Molday et al. (30) who used tritium exchange methods to study the fast exchanging peptide protons of myoglobin. Myoglobin was exposed to a brief exchange-in treatment, limited to a period calculated to label essentially all its nonbonded protons but very few slower ones. The subsequently measured exchange-out curve accurately matched a computed curve constructed by summing the expected exchange behavior of the known 33 non-H-bonded peptide protons of myoglobin. The same result has been obtained with collagen (31).

Thus, as with the cases previously discussed, the H-bonded protons on the freely accessible aqueous surface of proteins exchange slowly simply because they are involved in an

intramolecular hydrogen bond. Evidently the pre-existing H-bond must first be severed before H-bonding and transfer to the normal OH⁻-ion exchange catalyst can occur. In myoglobin the great majority of these protons are in α-Helix H-bonds which cannot be broken singly (except at helix ends). H-bonds, largely electrostatic in nature, form spontaneously when donor and acceptor are sufficiently near, and H-bond cleavage requires the physical moving apart of structural segments carrying the H-bonding pairs. Thus to break an α-helix bond, it is necessary to physically pull out, to unfold, at least one whole turn of helix and in so doing to break simultaneously all the H-bonds that either stem from or donate to that turn.

Let us look again at the myoglobin model (Fig. 2 c). The protein is not built like an onion with more and more deeply buried layers. In myoglobin no peptide proton is more remote from the surface than the thickness of one α-helix. Therefore the kind of structural unfolding we have just seen necessary to labilize even surface protons to exchange will perforce expose equally myoglobin's most deeply "buried" protons, those on the back surface of its helices. The issues of accessibility and penetration and internal water appear from this viewpoint totally irrelevant to the H-exchange process. It seems a reasonable conclusion that in myoglobin the exchange of all H-bonded protons proceeds from unfolded forms since unfolding is not only a necessary condition for any exchange but also a sufficient condition for all exchange to proceed.

This logic can be extended directly to most other proteins, though H-bond breakage in β-strands may require less severe structural dislocations than for α-helix. Some proteins do have stretches of main chain which are buried by one overlying layer of chain (32). This situation does not directly yield to the simple logic just applied, but we suspect that here too unfolding reactions, possibly of a more complex kind, must govern the H-exchange process.

Direct Measurement of Local Unfolding

Hydrogen exchange results of a special kind, obtained with hemoglobin, appear to measure individual local unfolding reactions. The difference H-exchange method, described in the Methods section, takes advantage of the allosteric nature of hemoglobin and the fact that some of its hydrogens exchange rapidly in the oxy form and much more slowly in the deoxy form. Thus allosterically responsive sites, exposed to tritium for a limited exchange-in period in the fast form, are labeled relatively rapidly, and this label can be locked in by deoxygenation while allosterically indifferent sites, labeled during the same brief exchange-in, rapidly lose their label. The small number of responsive protons in any given time region can then be studied in virtual isolation. The difference method mechanically maps out the exchange behavior of these protons. The results obtained suggest that their exchange is mediated by segmental unfolding reactions.

Examples of these results are given in Figs. 3 and 4. To obtain Fig. 3, oxyhemoglobin was labeled for 1 min only, then deoxygenated and exchanged out. A small number of sites, labeled in 1 minute in the oxy form, exchange out in deoxyhemoglobin over tens of hours, and when the allosteric effector, pyrophosphate, is present, this time is lengthened to hundreds of hours! Substraction of the minor background curve (see Methods) yields difference curves portraying only the allosterically responsive hydrogens (Fig. 3 b and 3 d). We find that three hydrogens per dimer share this extraordinary behavior and in deoxyhemoglobin these exchange-out with closely the same greatly retarded rate constant. Other experiments show that in oxyhemoglobin held at these conditions (pH 7.4, 0°C), the same three hydrogens exchange with a halftime of 20 s.

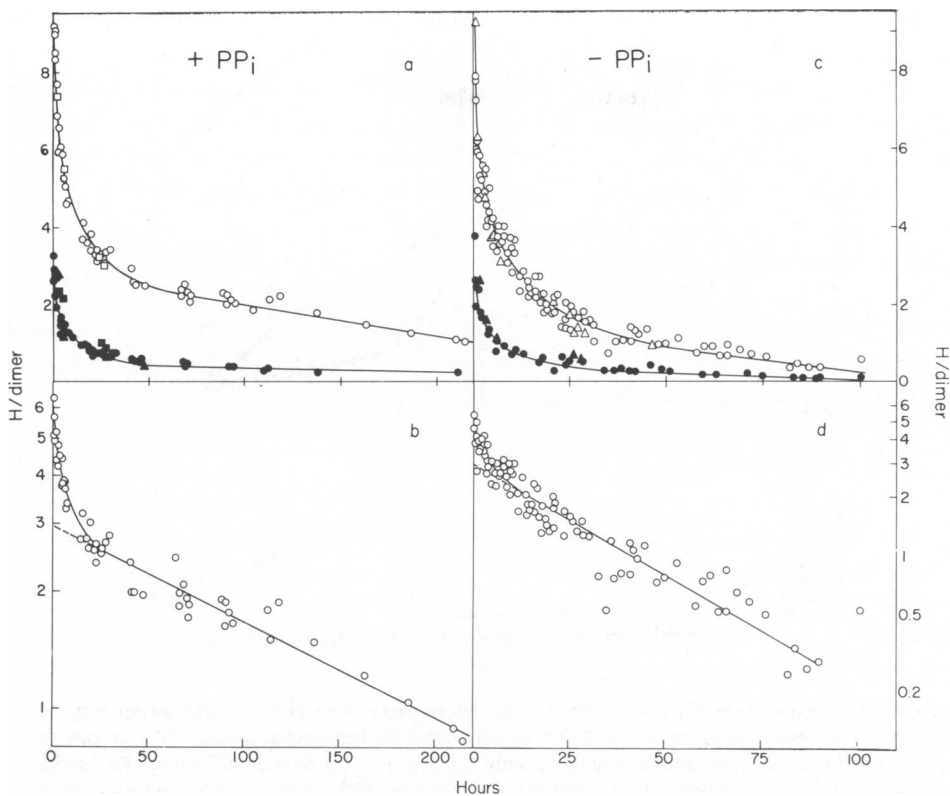We have now used this approach to sweep hemoglobin's entire exchange curve for

Figure 3 Exchange of the "jump class" in deoxyhemoglobin with and without the allosteric effector, pyrophosphate. Oxyhemoglobin was labeled for 1 min, deoxygenated, and exchanged out as deoxyhemoglobin (upper curves). The reverse sequence generated the background curves (filled symbols). The semilog plots give the difference curves (sample minus background). Conditions are pH 7.4 and 0°C. Exchange of the same protons in the liganded form was studied by adding CO back to exchanging deoxyhemoglobin and observing the resulting greatly accelerated exchange of the 3 remaining protons (not shown).

allosterically responsive hydrogens. Results obtained most recently in the slowest part of the exchange curve are shown in Fig. 4. Exchangeout data for fully labeled oxy and deoxyhemoglobin (Fig. 4 *a*) show that responsive hydrogens are present here. Fig. 4 *b* views these at higher resolution. Here exchange-in in the fast oxy form was continued for only 1 1/2 h to reduce the labeling of the slower background seen in Fig. 4 *a*. Both panels show drop-out curves obtained by adding ligand (CO) back to exchanging deoxyhemoglobin. The drop-out curve displays the behavior of oxyhemoglobin's responsive sites in this general time region, and the semilog plot in Fig. 4 *c*, obtained by subtracting the background from the drop-out data in Fig. 4 *b*, show that all these exchange at essentially the same rate. The very same hydrogens can be observed in the deoxy form simply by following the deoxy curve out to long times. Fig. 4 *d* shows such measurements together with the pertinent background, and Fig. 4 *e* gives the corresponding semilog difference plot. The results show that all the responsive hydrogens in this time region exchange with a halftime of 35 min in oxyhemoglobin and the very same set of hydrogens shifts as a unit to a new rate in deoxyhemoglobin slower by 50-fold. Other previously characterized responsive hydrogens exchanging in different time regions of the exchange curve display analogous behavior (10–12).

This extraordinary but characteristic behavior is just what one would expect if H-exchange
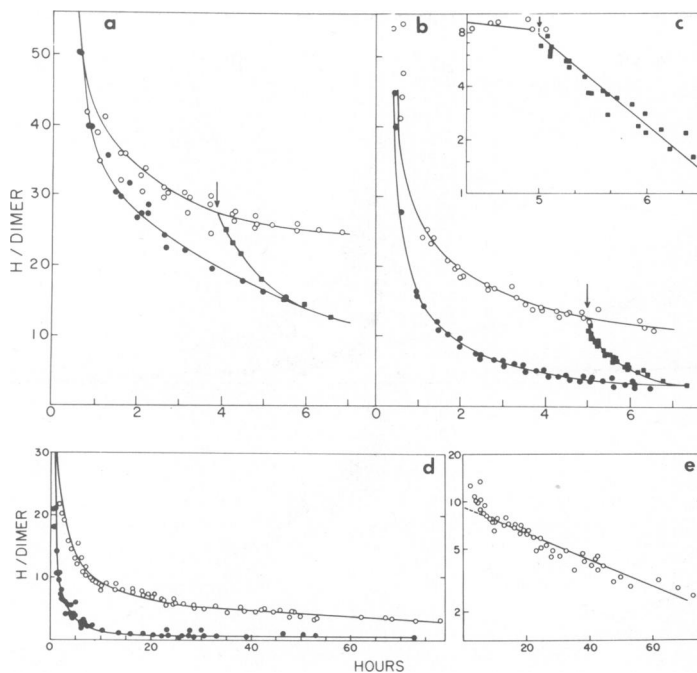
Figure 4 Application of the difference method to the slowest part of hemoglobin's exchange curve at pH 7.4, 37°C. (*a*) Deoxy, background, and CO drop-out curves for hemoglobin initially fully labeled to equilibrium. (*b*) As in *a*, but labeled initially for only 1 1/2 hours. (*c*) Semilog difference plot for the drop-out data in *b*. (*d*) Long time exchange-out data for deoxyhemoglobin initially labeled in the oxy form for 1 1/2 h, together with its background curve. (*e*) Semilog difference curve from data in *d*.

is mediated by local unfolding reactions. A set of hydrogens exposed by a given breathing reaction should all exchange at about the same rate, and when the unfolding reaction is perturbed by a structure change, all the segment's hydrogens should move as a unit to a new, common rate. A penetration model, together with the kinetic selection procedures employed here might also generate, within a selected time region, a set of protons which exchange at about the same rate in one form of the protein. However, it does seem rather unlikely that a kinetically coherent set, artificially generated in this way, would maintain its coherence and shift as a unit to a new rate as a result of allosteric change. It seems even more unlikely that all the allosterically responsive hydrogens in the protein would display this character.

A Search for the Allosterically Responsive Sites

A decisive test of the competing penetration and unfolding models might be obtained by identifying the locations of the sets of hydrogens just discussed. The unfolding model predicts that these hydrogens, if they arise in a true breathing unit, should all be found on a single polypeptide segment (Fig. 1 *a*), or at worst on neighboring segments which share a concerted unfolding reaction. From the point of view of penetration models, the sets observed must be mere artifacts selected by kinetic filtering and should represent somewhat generally distributed hydrogens which share a common rate for more or less accidental reasons.

Fig. 5 shows early results of this test, obtained for the small class of responsive hydrogens characterized in Fig. 3. In this experiment, oxyhemoglobin was labeled for 1 min as before, made deoxy, and exchanged-out in the presence of pyrophosphate (Fig. 3 *a*) for 18 h. At this point the sample, initially at pH 7.4 and 0°C, was quickly readjusted to pH 2.7. This
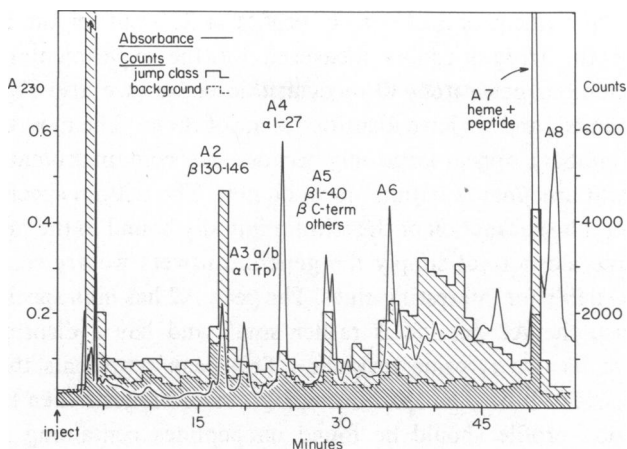
Figure 5   HPLC profile for peptic fragments of hemoglobin labeled at the "jump class" (Fig. 3). Peptide effluent was recorded by its absorbance at 230 nm. The histograms indicate tritium counts carried by deoxyhemoglobin (light toning) and by background (dense toning) samples taken from experiments as in Fig. 3 *a*, after 18 h of exchange-out.

condition denatures the protein, but exchange halftime of the unstructured peptide is here 70 min (30), and this provides time for further manipulations. Pepsin was then added to fragment the protein, the fragments were separated by HPLC (see Methods), peaks were collected, and their carried tritium was measured. Samples corresponding to the background curve of Fig. 3 *a* were handled similarly. (Rosa and Richards (14) have applied a similar approach in a tritium exchange experiment with the ribonuclease-S system).
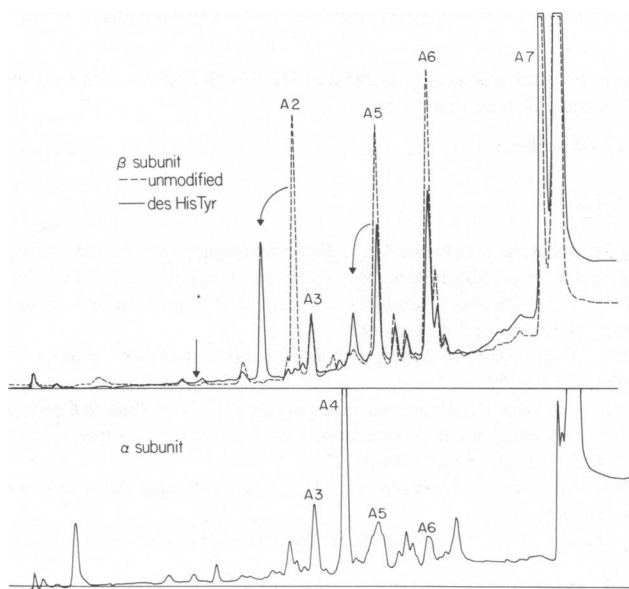

Figure 6   HPLC pattern for peptic peptides of isolated α subunits and for β subunits both unmodified and with the C-terminal His-Tyr residues removed by treatment with carboxypeptidase A. C-terminal peptides occur where the dashed-line profile is not matched by a solid line. For comparison with the hemoglobin pattern in Fig. 5, note that the tritiated hemoglobin sample was injected into an identical HPLC gradient at the arrow.

Fig. 5 registers the effluent optical density profile at 230 nm for the hemoglobin peptic peptides and shows the tritium counts measured for the experimental and background samples. These conditions generate ~30 recognizable peaks (see also Fig. 6 obtained with isolated α and β subunits), and we have identified some of these. The most conspicuous peaks, labeled as A (peak number), appear large only because they contain aromatic residues. A few peaks carry significant amounts of tritium; most do not. The tritium specifically localized in this way accounts for a large fraction of the tritium initially bound to the protein (Fig. 3 a).

These early results do not yet supply the general answers we are seeking, but one can examine the data available for interesting clues. The peak A2 has high specific activity, higher than it appears since the A2 peptide is rather small and has a disproportionately large absorbance owing to its two tyrosine residues. This peak represents the essentially pure C-terminal peptide, $\beta130$–146. The local unfolding model suggests then that all the carried tritium in this elution profile should be found on peptides containing some part of this segment. Is this true? Some information about the identity of the various peaks is contained in Fig. 6, obtained for separated α and β subunits. We attempted to identify those peaks in the β subunit pattern that contain some C-terminal peptide by studying βdes-Tyr 145, His 146. With this material, peaks initially containing any C-terminal peptide will be obviously altered. The results indicate that some β C-terminal peptide is contained in the peaks A2, A5, A6, and A7 (compare differences between dashed and solid curves in Fig. 6 for β subunits). It can be seen that these are just the peaks that carry tritium (Fig. 5). In addition, however, a major amount of tritium is smeared through the A6–A7 region. It appears that some interesting artifact is at work here, since the optical density in this region is not accounted for by the isolated α and β subunit patterns.

One is well aware that dealing with this system is likely to provide its own special challenges. For now, we take these already available results as a demonstration that the important questions at stake can be experimentally asked in this way.

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DISCUSSION

Session Chairman: Hans Frauenfelder   *Scribe*: Donald W. Pettigrew

VON HIPPEL: If one wants to make the unfolding be as much the local manifestation of a global unfolding as possible, then by the time a protein has finished getting all its H out, all the different parts of it should have gone through the complete unfolding process of a whole molecule. Eventually all the $\Delta H\ddagger$ should add up to that observed for unfolding the whole protein. I'm trying to see if one can make this precise by pushing the model to perhaps a ridiculous extreme. Some of the $\Delta H\ddagger$ of some of these processes really don't seem to have much more in them than just the $\Delta H\ddagger$ of the exchange process itself, the chemical exchange. Maybe by focusing on that, one can get closer to the difference between solvent penetration and local unfolding.

ENGLANDER: In the local unfolding model, measured apparent $\Delta H\ddagger$ corresponds to the $\Delta H°$ of individual small opening reactions. The sum of all these should probably not add up to the $\Delta H°$ for total denaturation.

VON HIPPEL: Everything has to get out, eventually.

ENGLANDER: Yes, everything has to get out eventually. The H-exchange curve is the sum of all of them, but the sum of the $\Delta H°$ that you can see for one opening and a neighboring one will overlap. When you open one segment, you break the contacts that happen to be stabilizing against the unfolding reaction. When you make a different unfolding reaction, you can be breaking some of the very same contacts independently, so that, in principle, just adding them up would double count, or worse.

VON HIPPEL: That makes it worse.

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