Hydrogen Exchange in Proteins as a Measure of Solvent Exclusion due to Ligands

NUCLEASE AND MYOGLOBIN

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SUMMARY

The kinetic distribution of hydrogen atoms back-exchanging from tritium-labeled proteins has been estimated from the results of interrupted flow and slow, continuous-flow gel filtration.

Staphylococcal nuclease exchanges almost all of it bound tritium atoms in a few hours at pH 8.1, but in the presence of calcium ions and deoxythymidine 3',5'-diphosphate about 34 hydrogen atoms remain associated with the protein for periods longer than 8 hours. The effect of temperature on the binding of tritium, in the presence and in the absence of these ligands, has been studied.

Sperm whale myoglobin shows an analogous trapping of tritium atoms upon the addition of hematin to apomyoglobin. The hematin causes the retention of about 25 hydrogen atoms for periods longer than 20 hours at pH 7.0; protoporphyrin IX traps fewer hydrogen atoms.

These results indicate that the addition of the ligands to these two proteins causes the exclusion of solvent water from otherwise exchangeable protein hydrogen atoms. The mechanism of this effect probably involves some combination of direct shielding of the protein from the solvent by the ligand, and changes in conformation and motility.

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The investigation of protein conformation by measurement of hydrogen exchange differs significantly from other approaches to the study of conformation in solution in that the extent of exchange reflects the behavior of protein molecules over a period of time or their "history" (1, 2). Methods involving spectroscopic or hydrodynamic measurements give information only about the mean conformation at a specific moment. The potential usefulness of the hydrogen exchange method for supplying information on the "motility" of native proteins in solution and on the relative exposure of various parts of their structures to bulk solvent has, however, not been fully realized. The predominant emphasis on kinetics, until recently, has frequently led to the selection of biologically abnormal environmental conditions (e.g. low pH) at which the rates of exchange of the amide hydrogen atoms of peptide bonds become measurable.

There is much experimental evidence that the three-dimensional structures of native proteins represent minima of conformational free energy determined by the sum of the interactions of amino acid residues with each other, with prosthetic groups, cofactors, substrates or other ligands, and with the solvent (3-6). The selective forces of evolution have presumably acted to optimize these conformations with respect to function under biological conditions.

In the present paper we present results of the measurement of suppression of hydrogen exchange by ligands, under relatively physiological conditions. These studies are intended to emphasize the total number of potentially exchangeable hydrogen atoms in proteins that are essentially completely shielded from solvent. We have recently reported (7) that the addition of calcium ions and deoxythymidine 3',5'-diphosphate to staphylococcal nuclease, previously labeled with tritium, suppresses the exchange of a significant fraction of its hydrogen atoms. The association of the two nonequivalently interesting fragments of nuclease-T, derived from nuclease by trypsin digestion in the presence of the above ligands, also causes a similar "trapping" of tritium atoms in this enzymically active nuclease derivative.

Using modifications of the original gel filtration method, we have now studied this phenomenon further in nuclease and in sperm whale myoglobin. These studies permit the estimation of the number of hydrogen atoms within the protein matrix that are shielded from solvent water as a result of the stabilizing protein-ligand interaction. These trapped hydrogen atoms are virtually inaccessible to the solvent for many hours and constitute a core whose size, we believe, is a useful index of the relative rigidity of the tertiary structure of the protein and its protein-ligand complex. Hydrogen exchange, from this point of view, examines proteins as space filling objects possessing more or less clearly defined "inside" and "outside" compartments. In terms of hydrogen exchange, proteins that attain biologically significant structures only when associated with specific ligands appear to behave, before these are added, as hollow structures into which solvent may penetrate with ease. Interaction with ligands excludes solvent by some combination of change in conformation, motility, and direct shielding of the polypeptide backbone and its side chains.
EXPERIMENTAL PROCEDURE

Materials—Tritiated water (100 mCi per g, lot 380-15) and toluene (lot 140A-102) were obtained from New England Nuclear. Sephadex G-25 was purchased from Pharmacia. Deoxothymidine 3',5'-diphosphate, hematin, and protoporphyrin IX were obtained from Calbiochem.

Staphyloccocal nuclease was isolated from the growth medium of Staphylococcus aureus (Foggi strain) by adsorption onto phosphorylated cellulose (8) and subsequent purification by ammonium sulfate fractionation, chromatography, and gel filtration (8, 9). Nuclease-T was prepared as described by Taniuchi, Anfinsen, and Sodja (10). Sperm whale myoglobin was purchased from Calbiochem and further purified by chromatography on carboxymethylcellulose as previously described (11). The fraction composed primarily of electrophoretic bands IV and V was used for these studies after they had been reoxidized to the metmyoglobin form. Apomyoglobin was prepared from the metmyoglobin by the acid-acetone method as described originally by Theorell and Åkeson (12), and subsequently modified (11, 13).

Labeling—Protein solutions at concentrations of 5 to 10 mg per ml were tritiated by the addition of 5 µl of the tritiated water (100 mCi per g) per ml of protein solution. The tritium radioactivity in the labeling solution was 0.5 mCi per ml. Preliminary experiments were done with tritium concentrations in the labeling mixture ranging from 0.01 to 1.00 mCi per ml, with no significant change in the results. For convenience in counting, the 0.5 mCi per ml concentration was used in standard procedures.

The time necessary for labeling was ascertained, for metmyoglobin, by determining all measurably exchanging hydrogens on a Sephadex G-25 column (2 × 15 cm), by the method described below, after various periods of incubation with tritiated water in 0.05 M borate buffer, pH 8.8. It was found that maximal labeling was achieved at between 24 and 48 hours of incubation at 4°C. Thus, for this protein, as well as the more labile nuclease, the "in-exchange" of tritium atoms proceeded for 2 or more days before back-exchange was studied.

Gel Filtration—Experiments with continuous-flow gel filtration were done on columns of Sephadex G-25, of indicated size and flow rates, as previously described (7). In order to get information about the magnitudes of back-exchange over longer periods of time, the gel filtration procedure was modified by stopping the flow, with the protein in the column, for specified periods of time. In some experiments, several interruptions were employed. When the protein was finally eluted and the subsequent fractions collected, it was possible to ascertain the number of tritium atoms per molecule still associated with the protein and the numbers of tritium atoms that had back-exchanged during each period of interruption of flow. Column flow was interrupted at effluent volumes previously shown to yield good resolution of the "out-exchanged" tritium peaks.

Addition of Ligands—Calcium chloride and pdTp were added to the aliquot of labeled protein and to the buffer equilibrating the gel column as already described (7).

The reconstitution of metmyoglobin was accomplished by adding an equimolar amount of hematin solution, in 0.05 M borate buffer at about pH 8.8, to apomyoglobin in 0.05 M phosphate buffer at pH 7.0. The addition constituted about 10% of the volume of the protein aliquot and was not considered in the calculations since a comparable volume of buffer was added to the aliquots of labeled protein that were gel filtered in the form of apomyoglobin or native metmyoglobin. The hematin solutions used for these additions were prepared by dissolving several milligrams of hematin in 0.1 ml of 1.0 N KOH and then adding 5 ml of the borate buffer. The stoichiometry of the addition was established by titrating a 5 × 10⁻⁴ M solution of apomyoglobin with the hematin solution and measuring the optical density at 409 μm. The extinction at the inflection or equivalence point indicated that, within a few percent, binding in a 1:1 molar ratio had occurred.

The addition of the protoporphyrin solutions to the apomyoglobin was done similarly. Several milligrams of protoporphyrin IX were dissolved in KOH and then diluted into 0.05 M phosphate buffer, pH 7.0. Titration of the protein in phosphate buffer, as described for hematin above, established the amount of the solution necessary for reconstituting all of the labeled protein as protoporphyrin IX-globin (Fig. 1). These procedures, and the gel filtration with this material, were carried out in subdued light or in the dark because of the known photosensitivity of the apomyoglobin protoporphyrin IX complex (14).

Temperature Studies—In order to measure the effect of temperature on the rate of tritium back-exchange in the gel columns, the column was provided with a water jacket. Water, at temperatures controlled to within less than a degree, was circulated through this jacket with a Haake (Gardner Laboratories, Inc., Bethesda, Maryand) thermostated water pump and heater. Temperatures below 25°C were achieved by allowing the returning water to pass through a copper coil suspended in an ice-water mixture.

In all experiments, the protein solution was at room temperature before application to the column, which had been equilibrated with the circulating thermostated water. However, on entering the matrix of the gel, the protein is quickly transferred
to buffer at the specified temperature. Protein yields, as determined by ultraviolet absorbance for unliganded nuclease, were over 90% and did not change with temperature.

**Assay of Radioactivity**—Aliquots from the collected effluent of the gel columns were added to 15 ml of Bray's solution, with enough water to make a final volume of 16 ml, and counted as previously indicated (7). Internal standardization was done with \(^{3}H\)-toluene and the counting efficiency (about 10%) was used to convert counts per min into disintegrations per min.

**Calculations**—Linderström-Lang's (15) original suggestion for the relationship between \(n\), the number of labeled atoms per protein molecule, and the concentrations of protein and label before and after separation was modified by Englander (16) for studies with tritium to the following form,

\[
n = \frac{n \times C}{C_s \times \frac{E_s}{A}}
\]

where \(E_s\) is the extinction coefficient of the protein at some wave length; \(A\) is the optical density per cm light path at that wave length; \(C\) is the tritium activity in the protein; \(C_s\) is the tritium activity in the labeling mixture, and \(n\) is the molar concentration of hydrogen in water.

In our studies, when the ascertainment of labeling was based on optical density in the effluent,

\[
n = \frac{n \times \Sigma dpm}{V_s \times \frac{C}{C_s} \times \frac{E_s}{2A}}
\]

where \(\Sigma dpm\) is the sum of the disintegrations per min in all counting vials corresponding to the protein peak and \(2A\) is the sum of the optical densities per cm light path for all fractions in the peak. \(V_s\) is the volume of the aliquot taken for counting.

When the calculation of labeling was based upon the amount of protein applied to the column,

\[
n = \frac{n \times \Sigma dpm}{V_s \times \frac{C}{C_s} \times \frac{1}{p \times V_s}}
\]

where \(V_s\) is the volume of each fraction collected, \(p\) is the molar protein concentration in the labeling mixture, and \(V_s\) is the volume of the applied sample of labeled protein.

**RESULTS**

We have previously presented evidence that the binding of calcium ions and pdTp to tritium-labeled nuclease suppresses the back-exchange of some of the tritium atoms. This conclusion was drawn from the determination of the specific activity of the free and liganded nuclease after gel filtration under specified conditions. In order to make this increment in specific activity in the liganded protein a useful parameter for studying the tritium exchange of sperm whale myoglobin. The elution pattern from a Sephadex G-25 column (2 X 100 cm) of tritium-labeled metmyoglobin, after interruptions of 6 ml per hour (about 1 hydrogen atom per molecule of protein), whereas the protein with added calcium ions and pdTp is associated with radioactivity corresponding to 37 hydrogen atoms per molecule.

The data in Fig. 2 show that, after back-exchange for about 8 or 9 hours, the unliganded nuclease is devoid of labeled hydrogen atoms. These results allow one to choose conditions for studying molecular interactions, a gel filtration technique using interrupted flow was employed to obtain information about the kinetic distribution of the back-exchanging hydrogen atoms.

Fig. 2B shows that almost all of the tritium atoms back-exchanged from unliganded nuclease during the first interruption of flow (for 1 hour) on the gel column. This peak of radioactivity corresponded to about 42 hydrogen atoms per molecule of protein. The peak corresponding to the 74 hour interruption contained radioactivity corresponding to 5 hydrogen atoms per protein molecule, while less than 1 hydrogen atom per molecule was found with the protein peak. The distribution of the back-exchange of the tritium atoms was different when the gel filtration was done with ligands added to the labeled protein and to the buffer equilibrating the Sephadex G-25 column, as shown in Fig. 2A. The protein peak now retained about 47 hydrogen atoms per molecule even after more than 8% hours of back-exchange. The peaks corresponding to the 1-hour and 74-hour interruptions contained radioactivity equivalent to 23 and 17 hydrogen atoms, respectively, per molecule of nuclease.

These results show that the tritium atoms trapped by the addition of calcium ions and pdTp to nuclease are prevented from back-exchange for periods longer than 8% hours. The number of hydrogen atoms trapped in this experiment is the highest of five experiments with nuclease. The average trapping was equivalent to 34 hydrogen atoms. These tritium atoms which are trapped for more than 8% hours, and most of those back-exchanging in the 74-hour interruption, must originate from the groups of hydrogens in unliganded nuclease which back-exchange during the 1-hour interruption as well as some that exchange "instantaneously."

The data in Fig. 3 shows that, after gel filtration for 7 hours (6 ml per hour), the unliganded nuclease is almost free of tritium (about 1 hydrogen atom per molecule of protein), whereas the protein with added calcium ions and pdTp is associated with radioactivity corresponding to 37 hydrogen atoms per molecule.

The same method of interrupted flow gel filtration was employed for studying the tritium exchange of sperm whale myoglobin. The elution pattern from a Sephadex G-25 column (2 X 100 cm) of tritium-labeled metmyoglobin, after interruptions of 10 min, 5 hours, and 15 hours, is depicted in Fig. 4A. Significant radioactivity is still associated with the protein peak...
Fig. 3. Gel filtration of nuclease at room temperature, with continuous elution at 6 ml per hour, on a fine grade Sephadex G-25 column (2 x 40 cm) in 0.05 M Tris-HCl, pH 8.1. The protein sample and buffers were the same as those in Fig. 2, A and B. Each tube of effluent contained 1.9 ml. The optical density at 280 m\textmu (\bullet--\bullet) and radioactivity (disintegrations per min) (\bullet-\bullet) of unliganded nuclease and the radioactivity (disintegrations per min) (\bullet--\bullet) of liganded nuclease are denoted.

Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Back-exchange interval</th>
<th>$\Sigma$dpm</th>
<th>Hydrogens per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomyoglobin</td>
<td>1 hr$^a$</td>
<td>(~8,100)</td>
<td>(~14)</td>
</tr>
<tr>
<td></td>
<td>5 hrs</td>
<td>3,500</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>15 hrs</td>
<td>2,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>2,500</td>
<td>4</td>
</tr>
<tr>
<td>Metmyoglobin</td>
<td>1 hr$^a$</td>
<td>(~0,000)</td>
<td>(~17)</td>
</tr>
<tr>
<td></td>
<td>5 hrs</td>
<td>3,900</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>15 hrs</td>
<td>2,500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>3,200</td>
<td>5</td>
</tr>
<tr>
<td>Reconstituted metmyoglobin</td>
<td>1 hr$^a$</td>
<td>(~8,400)</td>
<td>(~14)</td>
</tr>
<tr>
<td></td>
<td>5 hrs</td>
<td>4,000</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>15 hrs</td>
<td>3,100</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>17,200</td>
<td>29</td>
</tr>
</tbody>
</table>

$^a$ Values of 1 hour include 10 min of interruption and 50 min of flow time until the 5-hour interruption.

Even after more than 21 hours of back-exchange, the total radioactivity in each peak and its corresponding value in hydrogen atoms per molecule of protein are shown in Table I. When apomyoglobin was labeled and then gel filtered under the same conditions, a very similar pattern was obtained (Fig. 4B). In a third experiment, metmyoglobin was reconstituted by the addition of an equimolar quantity of hematin solution to the labeled aliquot of apomyoglobin; and in C, the protein was labeled as apomyoglobin, reconstituted with hematin as described under "Experimental Procedure," and filtered as metmyoglobin.\(\bullet\), radioactivity (disintegrations per min); \(\Delta\), optical density at 280 m\textmu.

Fig. 4. Gel filtration of myoglobin, apomyoglobin, and reconstituted metmyoglobin at room temperature. Aliquots containing 2 mg of protein labeled at pH 8.8 were filtered on a Sephadex G-25, fine grade, column (2 x 100 cm) in 0.05 M phosphate, pH 7.0. The elution of the protein at 60 ml per hour was interrupted, after the volume of the effluent was 20 ml, 50 ml, and 75 ml, for 10 min, 5 hours, and 15 hours, respectively. Fractions of 2.0 ml were collected, and optical density at 280 m\textmu and radioactivity were assayed. A, the protein was labeled and filtered as metmyoglobin; B, the protein was labeled and filtered as apomyoglobin; and in C, the protein was labeled as apomyoglobin, reconstituted with hematin as described under "Experimental Procedure," and filtered as metmyoglobin.
metmyoglobin. This difference, of about 25 hydrogen atoms per molecule, corresponds to tritium trapped during the reconstitution process and stable to 21 hours of back-exchange. The 10 min, 5 hour, and 15 hour peaks were very similar with all three forms of myoglobin (Table I).

Unlike the studies with nuclease and its inhibitor, it was not necessary to add the ligands to the buffer used to equilibrate the column in these experiments. The dissociation constant of the heme-apomyoglobin complex is about $10^{-13}$ M (11, 17) and no significant dissociation was observed on the gel column in our studies, as indicated by the spectral properties of the protein effluent. In control experiments on the gel filtration of tritium-labeled hematin alone, the radioactivity associated with the eluted hematin aggregates was no higher than the background level.

Effects of Variations of Temperature and Ligand Structure—As shown above, a significant number of hydrogen atoms are "trapped" for periods of many hours by the addition of ligands to apomyoglobin and nuclease. The following experiments illustrate the usefulness of measurements of this class of atoms as an index of the effects of changes in temperature and ligand structure.

Protoporphyrin IX binds strongly to apomyoglobin and is chemically identical with hematin except for the absence of the central atom of iron. The binding of protoporphyrin IX causes many, but not all, of the physical changes in the protein caused by the binding of hematin (14) and it is presumed that the two ligands bind at the same site in the protein.

Apomyoglobin was labeled with tritiated water and identical aliquots of the labeled protein were used to study back-exchange in the apomyoglobin and its complexes with hematin and protoporphyrin IX. Fig. 5 shows the elution pattern of these three aliquots from a column (2 × 40 cm) of Sephadex G-25 with continuous flow at 6 ml per hour (6 to 7 hours of total back-exchange). The free apomyoglobin has back-exchanged to the greatest extent and contains radioactivity equal to about 8 hydrogen atoms per molecule of protein. The reconstituted metmyoglobin (apomyoglobin and hematin) contains radioactivity equal to about 31 hydrogen atoms per molecule of protein. (The trapping effect, thus, involves about 23 hydrogen atoms in this experiment.) Protoporphyrin IX-apomyoglobin, with its 20 bound hydrogen atoms per protein molecule, falls between these two values. The lower degree of trapping observed in
the protoporphyrin IX complex is not due to irreversible dissociation of the protoporphyrin IX on the column because the yields of these two complexes were very similar. We must assume that the protoporphyrin IX-globin complex is more readily dissociable, or more accessible to solvent, than is the heme-globin complex. Possible mechanisms for this difference will be discussed below.

Taniuchi et al. (10) have measured the transition temperatures of nuclease and nuclease-T, and their complexes with calcium ions and pdTp, using the optical rotatory Cotton effect at 233 nm ([m]$_{233}$) as a parameter of structure. We have studied the effect of temperature on tritium trapping in these systems in order to compare the hydrogen exchange results with the optical rotation data.

Gel filtration studies were done on Sephadex G-25 columns (2 x 15 cm) under well specified conditions of temperature and flow rate. Fig. 6 shows the elution patterns of liganded nuclease as a function of temperature. The amount of tritium associated with the protein peak in each of these gel filtrations was summed and this value was plotted as a function of temperature (Fig. 7). The binding of tritium to each form undergoes a temperature-induced transition, but the transition of the liganded nuclease is shifted about 30° higher than that of free nuclease. The two forms seem to be converging at low temperatures to the same value of tritium binding.

In a similar set of experiments with liganded nuclease-T, a temperature-induced transition in the amount of tritium associated with the protein occurred between 40 and 60°. Nuclease-T without ligands binds so little radioactivity, even at 25°, that it was not possible to study the effects of temperature.

When these results are compared to those from the optical rotatory study of temperature effects, it is seen that both methods give similar estimates of stability to temperature for the liganded forms of nuclease and nuclease-T. The midpoint of the temperature transitions for nuclease and nuclease-T in the liganded form, as determined by the optical method, is 63° and 45°, respectively. However, the spectral methods and hydrogen exchange do not correlate well for unliganded nuclease which no longer binds tritium at a temperature at which the optical rotatory transition has not even begun.

The fairly uniform distribution of potentially exchangeable hydrogen atoms throughout protein molecules makes the measurement of rates of hydrogen exchange a useful probe of the conformation of all parts of these macromolecules. This advantage is partially offset by the difficulty in relating distinguishable rate classes of hydrogen exchange to the details of secondary and higher structure. In practice, the method is further limited by the fact that, under physiological conditions (i.e. temperature 25-37°, pH 6 to 8, etc.), the rates of exchange of hydrogen with solvent are usually too rapid to allow the detailed study, by the usual techniques, of any except a small fraction of the total exchangeable hydrogen atoms (1, 2). For this reason, most of the studies of hydrogen exchange kinetics have been done under conditions, such as low pH, at which exchange rates are markedly slowed. The interpretation of such results in terms of protein structure has not been entirely satisfactory (1, 2, 18).

We have chosen to focus our interest on a single aspect: the number of hydrogen atoms that are very slowly exchanging under specified conditions. In some proteins, such as staphylococcal nuclease in the absence of ligands, all of the hydrogen sites which can be labeled after several days of exposure to tritiated water, back-exchange within a few hours at pH 7 or 8.1. There are essentially no "core" hydrogen atoms in this protein, which contains about 15 to 20% helix and no disulfide bridges (19). Myoglobin, in contrast, whether studied in the apo-protein or holo-protein forms, has a significant number of these very slowly exchanging hydrogen atoms at pH 7.0. Benson and Linderstrom-Lang (20) observed this core in metmyoglobin under similar conditions. Classes of very slowly exchanging hydrogen atoms have also been noted in studies of ribonuclease (21), bovine serum albumin (22), and several other proteins. The number of these very slowly exchanging hydrogen atoms is markedly increased upon the addition of ligands to the two proteins that we have studied. This suppression of back-exchange, or trapping, is a sensitive indication of protein-protein or protein-ligand interactions.

Effects on the kinetics of exchange of protein hydrogen atoms have been observed upon interaction of pyridine nucleotides with yeast alcohol dehydrogenase (23) and chicken heart lactic dehydrogenase (24), zinc with insulin (25), trisaccharide with...
lysozyme (25), substrates with luciferase (26), and horse heart cytochrome with the two oxidation-reduction states of its heme group (27). In general, the magnitude of these effects have been variable and not easily related to other physical parameters. Our approach has differed in the following ways. First, the protein is labeled in the unliganded or motile state and back-exchange is measured in both the motile and taut state. Second, the difference in the number of very slowly exchanging hydrogen atoms is used as the single index of the effect of the interaction. Such differences can be estimated with considerable accuracy by slow gel filtration (see, for example, Fig. 4, A, B, and C). On the basis of one interrupted flow chromatogram, it is possible to define conditions under which a difference between unliganded and liganded protein may be studied.

In staphylococcal nuclease back-exchange is complete after several hours at 25° and pH 8.1. In the liganded form, however, about 34 hydrogen atoms are immobilized for a period longer than 8 hours. There is no gross conformational change in nuclease upon the addition of calcium ions and pdTp detectable by spectroscopy (19) or in the A x-ray diffraction pattern. Therefore, this trapping of hydrogen atoms must reflect a local conformational change (or shielding) around the ligand or a change in motility or both. At present, there does not appear to be a way of distinguishing between the contributions of these two factors.

Having selected a certain column length and flow rate under these solution conditions, as in Fig. 6, we were able to measure this very slowly exchanging class of hydrogen atoms as a function of temperature. Unliganded nuclease loses these bound tritium atoms between 8° and 35°.2 Nuclease in the presence of calcium ions and pdTp shows only a slight loss in tritium until about 35°. Loss of bound tritium occurs, for the liganded form of the protein, between 35° and 65° with a slope similar to that of unliganded nuclease. Liganded nuclease-T behaved in a manner similar to liganded nuclease except that the transition was shifted to slightly lower temperatures. The detailed interpretation of these results is complicated by the fact that there are kinetic as well as equilibrium factors determining the observed binding. The increasing temperature undoubtedly increases diffusion of solvent as well as changing protein structure and motility. It is possible that the stabilization caused by the addition of the ligands is due to a cooperative interaction with the protein and that the additional thermal energy is necessary to dissociate the protein-ligand complex in order to allow back-exchange to occur. This would explain the fair correlation of spectral and hydrogen exchange results for the liganded forms of nuclease and nuclease-T. Once ligand interactions are weakened, the effect of temperature on the motility of the protein and the diffusion of the solvent would be the major factors leading to an increased rate of out-exchange of tritium atoms. This mechanism would be of the general EX1 type of Hvidt and Nielsen (1) or, more precisely, of the cooperative, reversible unfolding type of Rosenberg and Chakravarti (25).

The studies of myoglobin with the interrupted flow method indicated that very long periods of back-exchange, corresponding to very slow gel filtration, would be necessary to resolve effects of ligands. The greater retention of labeled hydrogens in myoglobin (found at pH 8.0 as well as at 7.0) as compared to nuclease may be related both to the higher helix content of myoglobin and to lower motility of the protein in solution under these conditions. It is significant that, although apomyoglobin differs from metmyoglobin in many physical properties (29-31), the distribution of back-exchanging kinetic classes of hydrogen atoms is not very different between these two forms. However, the addition of the heme prosthetic group immobilizes 25 hydrogen atoms for periods of 20 hours or more. The similarities of the values obtained for the apomyoglobin and metmyoglobin after several days of labeling in tritiated water implicate that the prosthetic group shields these exchangeable sites from exchange-in for comparable periods. Both phenomena are consistent with the exclusion of solvent from the interior of the protein by the presence of a tightly bound ligand.

Protoporphyrin IX caused the trapping of only about 12 hydrogen atoms when added to the labeled apomyoglobin under conditions of gel filtration which resulted in the trapping of 23 atoms by hematin. The heme and protoporphyrin IX ligands are of similar size and one would not expect a difference in direct shielding upon their binding to the protein. The difference in accessibility to solvent must relate, therefore, to the "tautness" of the protein around the ligand or to differences in the motility of the liganded proteins. Since the forward reaction rates for prosthetic group attachment are roughly equal for hematin and protoporphyrin IX (32), the lower association constant for apomyoglobin with protoporphyrin IX than with hematin (33) would suggest that the dissociation of the complex occurs more frequently with the iron-free ligand. Thus, the lower trapping of tritium in protoporphyrin IX-globin would also be consistent with hydrogen exchange occurring predominantly in the dissociated form.

The approach to the measurement of hydrogen exchange used in the present experiments is, in a sense, transparent to all but those hydrogen atoms whose ability to exchange is directly or indirectly affected by specific attachment of ligands.

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