Effects of Ligand Binding on the Rates of Hydrogen Exchange in Myoglobin and Hemoglobin†

Ellis S. Benson,*‡ Maria Rosaria Rossi Fanelli, Giorgio M. Giacometti, Andreas Rosenberg,§ and Eraldo Antonini

ABSTRACT: Rates of exchange of hydrogens between protein and solvent were used to study the effects of ligand binding on myoglobin and hemoglobin. A tritium exchange technique was used, employing gel filtration to effect separation between protein and solvent water. Ligand-bound and ligand-free derivatives were compared simultaneously under precisely parallel conditions in which only the concentration of ligand was varied. Exchangeable hydrogens could be roughly categorized as rapid (exchanging in less than 2 min) and slow (exchanging in more than 2 min). Myoglobin at 6°, pH 7, had 168 rapid and 100 slow hydrogens per mol, while hemoglobin had 112 rapid and 120 slowly exchanging hydrogens per polypeptide chain. Slowly exchanging hydrogens of equivalent rank order of exchange, when the rates could be observed, exchanged more rapidly in myoglobin than hemoglobin. Binding of ligands such as carbon monoxide had no discernible effect on the hydrogen exchange rates of myoglobin. In hemoglobin, binding of CO was accompanied by an increase in rates of exchange, a shift of the rate spectrum to more rapid rates. These observations suggest that binding of ligand at the heme groups of hemoglobin is associated with rather large conformational changes. Similar changes are not detected by hydrogen exchange in myoglobin on ligand binding. The nature of these changes is not yet clear but they may be related to rearrangements of tertiary and quaternary structure, including dissociation into subunits, allowing exchange sites to become more readily accessible to solvent water.

Ligand binding curves of hemoglobin characteristically are those of a macromolecule displaying multiple equilibria with linked functions (Wyman, 1948, 1964). It is now generally recognized that this behavior is associated with extensive conformational changes accompanying ligand binding (Wyman, 1964; Rossi Fanelli *et al.*, 1964; Antonini and Brunori, 1971). Such conformational changes have been made "visible" by the X-ray diffraction studies of crystalline oxy- and deoxyhemoglobin of Perutz and his associates. The changes in protein conformation involve both tertiary and quaternary structure (Muirhead *et al.*, 1967; Bolton *et al.*, 1968; Perutz, 1970).

In myoglobin, on the other hand, no such complexities are found in ligand binding curves (Taylor and Morgan, 1942; Wyman, 1948; Rossi Fanelli *et al.*, 1964). X-Ray studies have not revealed significant conformational differences between ligand-bound and ligand-free forms of myoglobin, except small ones in the immediate region of the heme group (Kreitsinger *et al.*, 1968; Nobbs and Watson, 1968).

In solution, many structural properties change in hemoglobin but not in myoglobin, on binding of ligand at the heme group in the ferrous form (Antonini and Brunori, 1971).

The present study is one in which isotopic hydrogen exchange was used in an attempt to further clarify the relationship between ligand binding and conformational behavior in myoglobin and hemoglobin. Hvidt and Linderstrøm-Lang (1954) introduced hydrogen exchange as a means of study of secondary and tertiary structures of proteins. In more recent years, it has become clear that hydrogen exchange studies are sensitive probes of the dynamics of protein conformation (Hvidt and Nielsen, 1966). Special attention had been focused in this study on myoglobin and metmyoglobin and their liganded derivatives as a basis for further exploration of the effects of ligand binding on hemoglobin.

Experimental Procedures

Materials. Sperm-whale metmyoglobin, 1 × crystalled, was obtained from Saravac Laboratories, Ltd. Just prior to use it was dissolved in water at a concentration of 2–3%. Aggregated forms of the protein were removed by centrifugation at 6000*g* for 15 min.

Human hemoglobin was prepared from freshly drawn blood by the ammonium sulfate method (Rossi Fanelli *et al.*, 1961). Stock solutions were passed through a column of mixed bed ion exchange resins to remove inorganic and organic ions (Dintzis, 1952) and stored at 4° at a protein concentration of 4–5%.

Tritiated water, lot no. 328-292, 1 Ci/ml, was obtained from New England Nuclear Corporation and Sephadex G-25, coarse and medium, from Pharmacia, Inc.

Hydrogen Exchange Procedures. The hydrogen exchange method used in these experiments was that of Englander (1963), in which separation between protein and water phases is obtained by passage of the protein solution through a column of Sephadex resin. The two-column method of Englander was used exclusively.

Labeling of the protein with tritium ("in-exchange") was accomplished by incubation of the protein in tritiated water at pH 9, 37–38°, for 24 hr. Sodium borate buffer (2%) was added to bring the pH to 9 and the protein concentration to 1–2%. To this solution was added an equal volume of tritiated water to provide an activity of approximately 3.8 × 10^5 dpm/ml. Benson (1959), using a deuterium exchange

† From the Center for Molecular Biology of the Consiglio Nazionale delle Ricerche, Institute of Biological Chemistry, University of Rome, Italy. *Received January 30, 1973.*

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§ Present address: Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minn. 55455.

technique, found that all exchangeable hydrogens of metmyoglobin exchanged within 10 hr at pH 8.8, 38°. In preliminary experiments, exchange between hemoglobin and deuterium oxide was followed using the Linderstrøm-Lang density-gradient technique (Hvidt *et al.*, 1960). These experiments indicated that at pH 9, 37–38°, nearly all (90–95%) of the exchangeable hydrogens of hemoglobin exchanged with those of solvent water in 24 hr.

Deoxyhemoglobin and myoglobin and carboxyhemoglobin and carboxymyoglobin are much more stable than their oxygenated counterparts and much less susceptible to autooxidation (Antonini and Brunori, 1971). Nevertheless, appreciable autooxidation may occur in experiments such as the ones we have described. Raising temperature or extending the time of the preliminary incubation of hemoglobin in the "in-exchange" procedure resulted in conversion of hemoglobin to ferrihemoglobin. For this reason, we accepted an "in-exchange" procedure for hemoglobin which left a small percentage (5–10%) of very slowly exchanging hydrogens unlabeled, while in myoglobin, in all probability, labeling was complete. Under these circumstances, the amount of methemoglobin and metmyoglobin detected was never more than 2% of protein.

After incubation, separation of tritiated protein and tritiated water was achieved by passage of the solution through a Sephadex G-25 column of dimensions 18 × 2.2 cm at 5° equilibrated with sodium phosphate buffer at pH 7, ionic strength 0.2. Filtration time was 2–3 min; zero time of "exchange-out" was taken as the time at which the filtration front had traversed half the column. A 1-ml aliquot of the filtrate was used to estimate the zero-time labeling value of the protein. The remaining filtrate, containing tritiated protein in phosphate buffer, was used in the "exchange-out" experiments. It was incubated at a temperature and under solvent conditions, including ligand concentration, specified for each experiment.

In the back-exchange procedure, 0.5-ml aliquots were withdrawn from the incubated filtrates at timed intervals over several hours. Protein and water phases were again separated by filtration through additional columns of Sephadex, G-25 medium. Column dimensions were either 10 × 2.2 cm or 8 × 1.5 cm and filtration times 1–2 min. The time at which the filtration front of the sample had traversed one-half of the height of the column was taken as the end of the incubation time, t. Two or three eluent protein fractions were obtained at each time interval. The protein concentration of each was estimated by optical density in the Soret spectral region using a Cary Model 14 spectrophotometer. Absorptivities and wavelengths of maximal absorption used for each derivative of myoglobin and hemoglobin studied were those given by Antonini and Brunori (1971).

For scintillation counting, 1 ml of each eluent fraction was mixed with 15 ml of scintillation fluid according to the method of Bray (1960). The activity expressed as distinegrations per minute (dpm) was measured using a Packard Tri-Carb liquid scintillation counter, Model 3380, with an absolute activity analyzer attachment, Model 544. A varying counting time was used to provide a constant counting error of no more than 1.5%. The contribution of color to quenching was less than 2%.

In experiments on deoxymyoglobin and deoxyhemoglobin, granular sodium dithionite was added to the protein solution to provide a concentration of approximately 5 mg/ml. Metmyoglobin was converted to ferromyoglobin by the same means. Sodium dithionite was also then added to the equilibrating and eluting buffers in the gel filtration column to provide concentrations of 5 mg/ml.

In the experiments reported comparing ligand-bound and ligand-free derivatives of hemoglobin, myoglobin, and metmyoglobin, differential measurements of exchange rates were made in each instance: that is, the exchange rates of the ligand-bound and corresponding free protein were observed concurrently under precisely parallel conditions, except for the presence of the ligand. After the first filtration, the sample was divided into two equal portions; to one was added ligand at saturating concentrations and the other was used as the ligand-free control.

In experiments on carbon monoxide hemoglobin and myoglobin, the following procedures were used. Argon was used to thoroughly flush out the air from a 12-ml screw cap vial. Two to three milliliters of the protein solution was placed in the vial and the remaining air space was again flushed out with 20 ml of carbon monoxide. Equilibration of the heme protein with CO was achieved by gently rotating the vial for a few seconds. After an hour of incubation in the exchange-out experiments an additional 10–15 ml of CO was passed through the air space in the vial. The protein solutons were equilibrated again with CO prior to determination of optical density.

In the cyanide and azide experiments, 100 μl of 0.2 M NaCN or 100 μl of 0.25 M NaN_3, respectively, was added to 3 ml of the first filtrate of metmyoglobin to convert these to the cyanide- and azide-liganded derivatives of metmyoglobin.

The use of an isotope exchange method usch as we have employed rests on the assumption that the exchange rates of the isotope are representative of those of hydrogens from the same sites. That there are small kinetic effects is apparent from a number of studies, notably those of Rosenberg and Chakravarti (1968) and Hallaway and Benson (1971).

The presence of equilibrium isotope effects or isotope enrichment is still a question under investigation. A number of authors (Ikegami and Kono, 1967; Rosenberg and Chakravarti, 1968; Hallaway and Benson, 1971; Byrne and Bryan, 1970) find the effects minor and mostly below 5%. However, effects in the neighborhood of 15% have been reported (Englander and Staley, 1969). In parallel comparative studies of the different forms of one protein, such as those reported here, these effects can be safely ignored.

Calculations

The function which we measured in the exchange-out experiments is the size of the hydrogen pool per protein molecule (in number of hydrogens per iron atom) which has been labeled by tritium and which has not yet exchanged with solvent water at time t after the start of the exchange-out period. To obtain this function, we assessed the ratio of activity to optical absorbance using a formula devised by Englander (1963)

$$H/\text{mol} = \frac{\text{dpm} \times 111 \times \epsilon}{\text{dpm}_0 \times A}$$

where dpm is the activity in the sample counted, dpm_0 is the activity in the equivalent volume of in-exchange solution, ϵ is the molar extinction coefficient, and A is the optical absorbancy. The atom concentraton of H in H_2O is 111.

Results

Labeling and First Filtration. Figure 1 illustrates separation

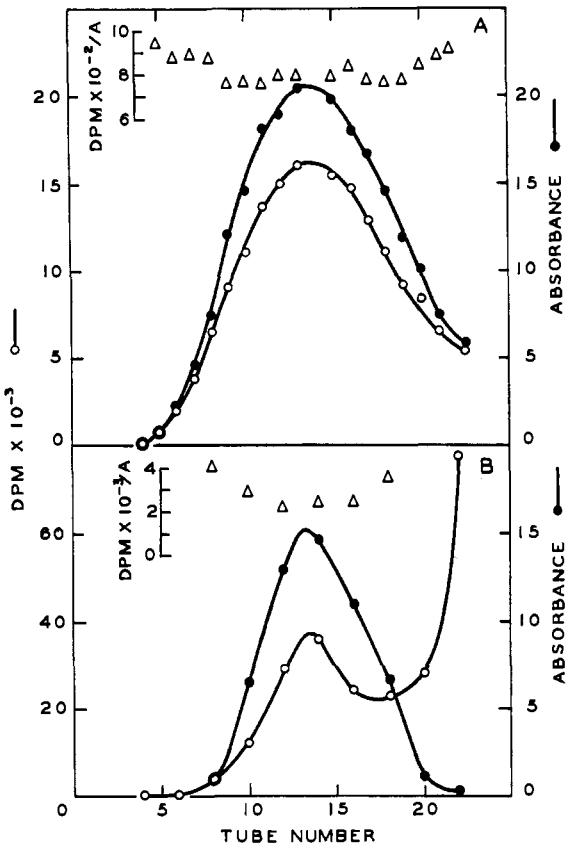

FIGURE 1: Separation between protein and solvent water in first filtration: A, hemoglobin; B, myoglobin; ●, absorbance per ml (at 540 mμ for hemoglobin, 556 mμ for myoglobin); O, activity of tritium in dpm/ml; △, ratio of dpm to absorbance. Tube numbers from left to right in order of elution from Sephadex column, 1 ml of filtrate/tube. (The first 25 ml of eluate was discarded as void volume.)

of labeled protein and labeled water in the first filtration. In both hemoglobin and myoglobin, separation was satisfactory; only the tail of the protein peak was contaminated by tritiated water. On the basis of a constant ratio of activity to optical density (Figure 1), it appeared safe to use the first two-thirds of the protein peaks for the exchange-out experiments.

Hemoglobin. In the following figures, illustrating kinetics of exchange out of tritium from hemoglobin and myoglobin, the curves are drawn to show the rate of loss from the protein of residual labeled hydrogens per protein chain (or per iron atom).

Rates of exchange of deoxyhemoglobin and carbon monoxide hemoglobin at three temperatures (6.5, 20, and 36°) are shown in Figure 2. From the results it can be seen that a significant number of hydrogens exchange more rapidly from the carbon monoxide derivative than from the ligand-free form. The difference in rate between the two derivatives at each of the temperatures is apparent but it appears that the difference is more pronounced at 6.5 than at 36°.

It must be noted at this point that all hydrogens with exchange times of less than 2 min, including in all probability all exchangeable hydrogens at or very near the protein–solvent interface, will exchange during the first filtration and will not be estimated in the exchange-out procedure. Thus, zero time of the exchange-out runs illustrated in Figures 1 and 2 is really approximately 2 min after the beginning of the exchange-out process. In the experiments reported here we are

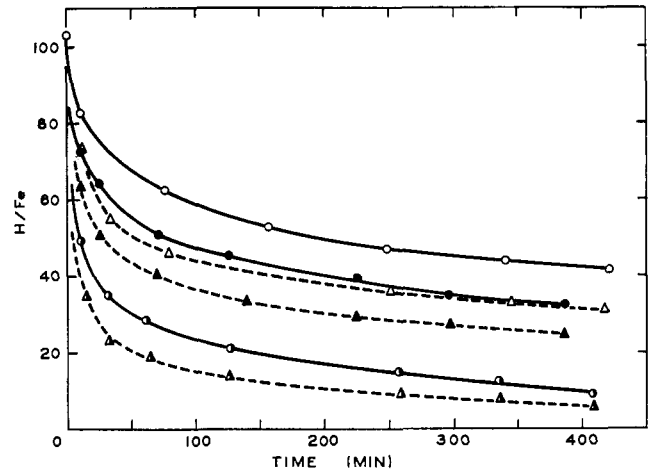

FIGURE 2: Rates of exchange of hydrogen from deoxyhemoglobin (O) and carbon monoxide hemoglobin (△) as a function of temperature ((O, △) 6.5°; (●, ▲) 20°; (◑, ◭) 36°; pH 7, ionic strength 0.2 μ).

only studying the exchange rates of relatively slowly exchanging hydrogens.

Myoglobin. The rates of exchange out of slowly exchanging hydrogens are shown at three temperatures, 6, 20, and 36° (Figure 3). Deoxymyoglobin is compared with myoglobin saturated with carbon monoxide. In these experiments precisely the same conditions apply for both the labeling and "exchange-out" steps as in the case of deoxy- and carbon monoxide hemoglobin, except in instances specifically indicated. In contrast to hemoglobin, no difference between ligand-bound and ligand-free forms was noted at 20 and at 38°.

Because no differences were noted between the exchange rates of deoxy- and carbon monoxide myoglobin, in sharp contrast to hemoglobin, we thought it worthwhile to examine the exchange rates of other derivatives of myoglobin.

To assess the effects on myoglobin of the oxidation state of iron, experiments were performed in which tritium labeling was undertaken at pH 9, 38°, for 24 hr using alternatively metmyoglobin and myoglobin solutions. After the first filtration, metmyoglobin was converted to deoxymyoglobin by the addition of sodium dithionite and the exchange rates of the two protein solutions were compared. No differences in ex-

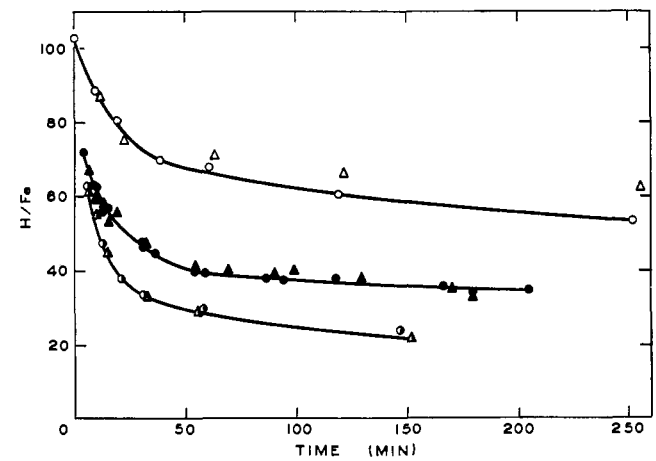

FIGURE 3: Rates of exchange of hydrogen from deoxymyoglobin (circles) and carbon monoxide myoglobin (triangles) as a function of temperature ((O△) 6°; (●▲) 20°; (◑◭)36°; pH 7, ionic strength 0.2 μ).

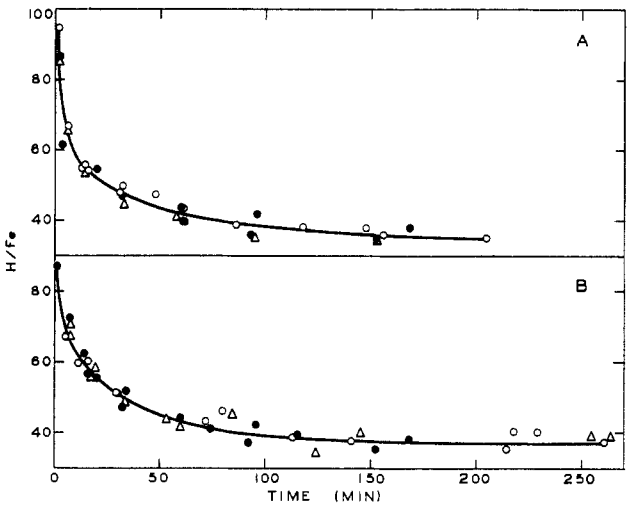

FIGURE 4: (A) Rates of exchange of metmyoglobin and myoglobin compared to 20°, pH 7, ionic strength 0.2 μ: (○) in exchange to metmyoglobin, exchange out from deoxymyoglobin; (△) in exchange to deoxymyoglobin, exchange out from metmyoglobin; (●) in exchange to metmyoglobin, exchange out from metmyoglobin. (In all instances in-exchange labeling was carried out at 37–38°, pH 9, for 24 hr.) (B) Rates of exchange of hydrogen from metmyoglobin (●), cyanometmyoglobin (○), and azidometmyoglobin (△), 20°, pH 7, ionic strength 0.2 μ.

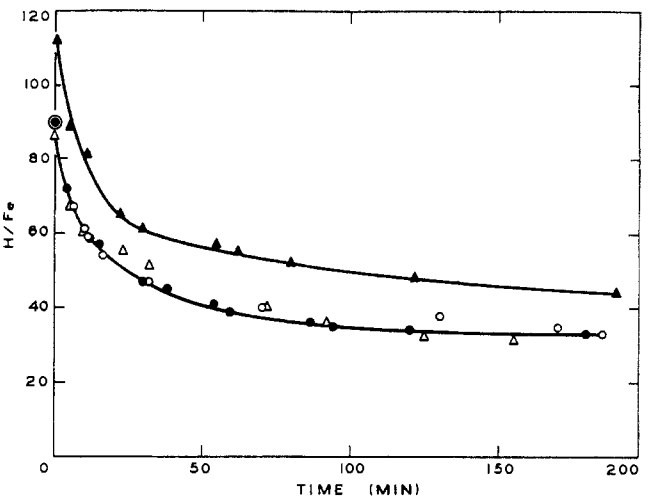

FIGURE 5: Rates of exchange of hydrogen from deoxyhemoglobin (▲) and carbon monoxide hemoglobin (△) compared with those of deoxy- (●) and carbon monoxide myoglobin (○); temperature is 20.5°, pH 7, ionic strength 0.2 μ.

change rates were noted (Figure 4A). When, then, labeling of metmyoglobin (pH 9, 38°) was followed by exchange out of metmyoglobin (pH 7, 20°), comparison of the derivatives could be made. The exchange rates of cyanometmyoglobin and azidometmyoglobin at 20°, pH 7, are illustrated in Figure 4B. In the case of each of these derivatives, differential measurements were made in which the ligand-bound and ligand-free derivatives were compared concurrently under identical conditions. It is obvious that the exchange rate of none of these derivatives differs significantly from that of myoglobin (Figure 4A).

In Figure 5, exchange curves of myoglobin and hemoglobin at 20.5°, pH 7, are compared; both the deoxy and carbon monoxide derivatives of each heme protein are included. It is notable that three of the curves are very similar, probably not significantly different from one another. These curves are those of deoxymyoglobin and carbon monoxide myoglobin and hemoglobin. The only curve that is strikingly different is that of deoxyhemoglobin.

Discussion

Detailed descriptions of possible mechanisms of exchange of hydrogens from sites within protein molecules inaccessible to solvent have been elaborated (Hvidt and Nielsen, 1966; Klotz, 1968; Bryan, 1970). These proposals are in essence variations or elaborations of a mechanism proposed by Linderstrøm-Lang (1955, 1958). Linderstrøm-Lang envisioned proteins as existing in solution in n number of folded states relatively close to each other in free energy. Fluctuations between these conformational states took place and the rates of fluctuation depended upon ambient conditions such as pH, temperature, and ionic strength. The exchange of hydrogens occurred from conformational states in which sites were accessible to solvent. Accordingly, the exchange pathway for hydrogens folded within the protein was

$$\sum_{1}^{n}\text{inaccessible states} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \sum_{1}^{n}\text{accessible states} \xrightarrow{k_3} \text{exchange} \quad (1)$$

In this reaction pathway the exchange rate of hydrogens at inaccessible sites ($k_2 \gg k_1$) will depend on the relative values of the rate constants k_1 (and k_2) and k_3. When k_2 is low compared to k_3, exchange will take place at the rate characteristic of k_1. When k_2 is very much greater than k_3, the rate equation for exchange will be $(k_1/k_2)k_3$ (Hvidt and Nielson, 1966). Rosenberg and Chakravarti (1968) subsequently observed that much of the slow exchange of hydrogens from carbonic anhydrase could not be explained by either of the Hvidt and Nielsen mechanisms, since the temperature dependency of exchange indicated a rate determining process of low activation energy, while the energy of unfolding is known to be high. Also, the temperature dependency is not different from that of most slowly exchanging hydrogens of oxidized ribonuclease. These authors suggested that exchange from inaccessible sites in temperature regions remote from the thermal transition temperature of the protein took place by some type of process of low activation energy. Bryan (1970) has suggested that this low-energy process is a diffusion of water molecules to previously inaccessible sites within the domain of the protein.

In the meantime, Klotz and associates (Leichtling and Klotz, 1966; Scarpa *et al.*, 1967) in studies on acid and base catalysis of the exchange of hydrogens of small peptides found that exchange was both general acid and base catalyzed and suggested that the intrinsic exchange rate of each hydrogen was a function of its local environment. Each site was influenced by the charged groups around it and the concentration and state of water in its vicinity (Klotz, 1968). Woodward and Rosenberg (1970), as well as Molday *et al.* (1972), have found that in oxidized ribonuclease, in which presumably there are no inaccessible sites, exchange took place through reaction pathways having a continuous distribution of first-order rates, in support of the Klotz proposal.

In summary, it seems likely that exchange from inaccessible sites (slow exchange) in proteins takes place over a reaction pathway like that described by Linderstrøm-Lang but not by unfolding except in the region of thermal transition. At temperatures away from this latter region exchange takes place from the folded protein through pathways having a continuous distribution of first-order rate constants. The physical process by which this exchange takes place is unknown but it may be the movement of water molecules into the protein

core (Bryan, 1970). All hydrogens exchanging at rates too fast to measure are lumped together as "rapid" and all hydrogens exchanging at very slow rates are lumped as "core"; we observe only those exchanging at intermediate rates but these form a continuous distribution of first-order rate constants and it is reasonable to assume that this is true of the "rapid" and "core" ones, as well. When the rates are varied by temperature of pH, it is probable that one moves along this distribution spectrum: the whole spectrum is shifted to faster or slower rates. The rank order of exchanging sites is probably not altered (Woodward and Rosenberg, 1971). Recent studies of pH and temperature transition in bovine serum albumin (Benson and Hallaway, 1970) and ribonuclease (Woodward and Rosenberg, 1971) support these conclusions.

In our studies, our attention has focused on the exchange rates of slowly exchanging hydrogens, that is, ones which have exchange times of 2 min or more. Again, on the basis of studies on a large number of polypeptides and proteins (Hvidt and Nielsen, 1966; Klotz, 1968) it seems reasonably safe to predict that these hydrogens are predominantly ones which are buried in structure within the protein molecule, for example, helical segments but not necessarily exclusively in such segments, and thus relatively inaccessible to bulk solvent.

Of 268 exchangeable hydrogens/mol of deoxymyoglobin, all but approximately 100 exchange so rapidly under the conditions we studied that their rates of exchange could not be measured. In deoxyhemoglobin, of the 232 exchangeable hydrogens per iron atom, all but 120 exchange more rapidly than we could measure by the method used. Without attempting an exact assignment of sites of rapid and more slowly exchanging hydrogens, it is reasonable to suggest that the 168 rapidly exchanging hydrogens in myoglobin and the 112 rapidly exchanging ones in hemoglobin are predominantly side-chain and end-group hydrogens at the surface of the protein molecule and readily accessible to solvent. A large body of evidence from studies of other proteins and polypeptides supports this generalization (Hvidt and Nielsen, 1966). The observed difference between deoxymyoglobin and deoxyhemoglobin in a number of rapidly exchanging hydrogens therefore may be attributed to a more extensive surface exposed to solvent than is the case with the equivalent unit of hemoglobin, the individual chain.

Because, as noted above, the unlabeled core of hemoglobin may be larger than that of myoglobin, the differences in number of rapidly and slowly exchanging hydrogens between the two proteins may be even greater than estimated in these studies.

Perhaps because of the detailed elaboration by X-ray diffraction of the three-dimensional structure of metmyoglobin by Kendrew and his associates (Kendrew, 1962), several studies of hydrogen exchange of metmyoglobin have been made using deuterium and tritium methods (Benson, 1959; Beychock *et al.*, 1961; Englander and Staley, 1969; Abrash, 1970a). Taking into account differences in pH and temperature, our results on metmyoglobin are in reasonable accord with those of these authors. Our study appears to be the first on the exchange properties of deoxymyoglobin and the ligand-bound derivatives of ferri- and ferromyoglobin.

Englander and Mauel (1972) studied hydrogen exchange rates of deoxyhemoglobin and oxyhemoglobin in intact red cells at 0°. The results we have obtained on solutions of deoxy- and carbon monoxide hemoglobin at 6° are very similar to the rates Englander and Mauel observed for the ligand-bound and ligand-free derivatives of the protein in red cells.

On the basis of the observed differences in hydrogen exchange kinetics between liganded and unliganded hemoglobin, we conclude that there are conformational differences between these two forms. Englander and Mauel (1972), on the basis of their hydrogen exchange analysis, suggested that the conformational change induced by ligand is a local one involving only 11 exchangeable hydrogens. As a result of the ligand-induced conformational change, the rate of exchange of these susceptible hydrogens goes from very slow to very fast.

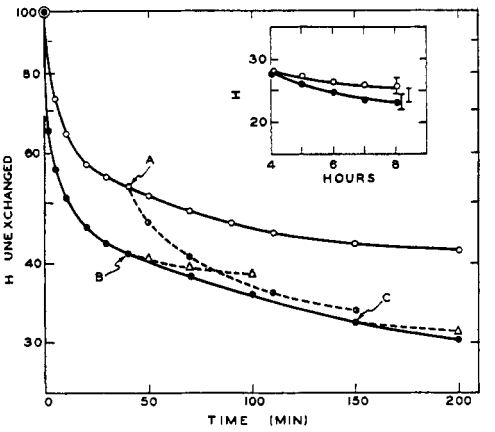

FIGURE 6: The calculated exchange out of 100 hydrogens from a model of hemoglobin with an assumed rate distribution, described in detail in text. Exchange from the unliganded form (○—○); exchange when all rate constants are increased by a factor of 5 (corresponding to the liganded form) (●—●). At point A the nonliganded form is assumed to convert instantaneously to the liganded form (◑---◑); at points B and C the liganded form is converted to nonliganded form (△---△). The inset shows such a divergency experiment with conversion from liganded (●) to nonliganded (○) form on a more extended time scale.

The key experimental observations are the following. In back-exchange from isotope-labeled hemoglobin there is a constant parallel shift in the exchange rate when one goes from the unliganded to the liganded form. This difference persists over a 30° temperature range. If, at some point after exchange from the unliganded form had begun, ligand is introduced (point A in Figure 6), the exchange curve should drop smoothly to the exchange curve characteristic of the liganded form without overshoot (Englander and Mauel, 1972). When ligand is removed any time after exchange of the liganded form has begun, the exchange curve should change to the slower rate of the unliganded form (points B and C in Figure 6). This change becomes increasingly small and vanishes the further along one goes in time along the exchange curve (point C and inset, Figure 6).

The observation that the difference in rate curves between the liganded and unliganded forms persists over the entire temperature range studied suggests that the 11 susceptible hydrogens of Englander and Mauel must be among the 10–20 slowest exchanging in the molecule. Yet, at low temperature (6°), their exchange rate changes drastically in only a few minutes representing a rate change of at least 100-fold. Experiments in which ligand is removed after back-exchange has started from liganded hemoglobin should reveal no change in rate, since the susceptible hydrogens have already exchanged. According to the actual observations of Englander and Mauel, however, a gradually diminishing rate was seen on removal of ligand.

In order to assess the validity of alternate explanations we have performed a simulated experiment shown in Figure 6. Hemoglobin here is represented by six groups of hydrogens

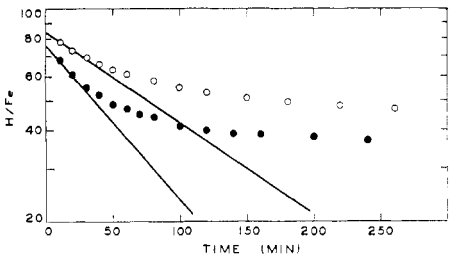

FIGURE 7: First-order rate plots of slowly exchanging hydrogens of deoxyhemoglobin (○) and deoxymyoglobin (●). Note the difference in rate between the two proteins of hydrogens of equivalent rank order of exchange (for example, hydrogens 45–60).

per Fe atom, with a total of 100 sites: $H_{rem} = 10e^{-k_1t} + 15e^{-k_2t} + 20e^{-k_3t} + 20e^{-k_4t} + 15e^{-k_5t} + 20e^{-k_6t}$, with $k_1 = 4.95 \times 10^{-5}$ min^{-1}, $k_2 = 1.98 \times 10^{-4}$ min^{-1}, $k_3 = 1.118 \times 10^{-3}$ min^{-1}, $k_4 = 1.98 \times 10^{-2}$ min^{-1}, $k_5 = 1.386 \times 10^{-1}$ min^{-1}, and $k_6 = 0.3466$ min^{-1}.

The distribution was patterned according to the rate distribution determined for native ribonuclease (Woodward and Rosenberg, 1971). This represents an arbitrary decision, of course, and the distribution could be expanded, which means that the slowest groups could be represented by additional subgroups. If we now conduct calculated experiments with this model, as shown in Figure 6, we can see that if we assume that *all* the rate constants are slowed by a factor of 5 when the ligand is removed, we can reproduce all the observed experimental curves. A constant difference of 13 apparent sites appears. When the ligand is introduced at point A, the curve drops smoothly to the curve of the liganded form. In experiments in which ligand is removed, the exchange rate shows a gradually diminishing change (points B and C). The only difference is that in the insert a final difference of 3.5 hydrogens appears while Englander and Mauel (1972), under these conditions, observed no difference. This difference is probably within the limits of experimental error. We conclude, therefore, that while Englander and Mauel's suggestion of 11 susceptible sites is a possible mechanism, an alternative mechanism can also explain the difference in exchange rate between liganded and unliganded hemoglobin, a mechanism represented by a shift in the entire rate spectrum of exchange, implying a rather widespread and not a local change in conformation on ligand binding.

In deoxymyoglobin and deoxyhemoglobin as in other globular proteins, slowly exchanging hydrogens exchange by pathways having a distribution of first-order rate constants (Figure 7). Sites in myoglobin of equivalent rank order of exchange from slowest to fastest exchange more rapidly than those of hemoglobin; the distribution of first-order rate constants is shifted to the left, to higher rates, when one goes from hemoglobin to myoglobin. The exchange of these families of hydrogens in the two proteins may be by the low-activation energy mechanisms described by Woodward and Rosenberg (1971) and Bryan (1970). At any rate, the differences are in all likelihood structurally related; myoglobin may have a looser structure, one more easily penetrated by water. It seems most reasonable to suggest, however, that whatever the immediate structural reasons for the differences, the ultimate basis for the differing behavior of the two proteins lies in their quaternary structure: deoxymyoglobin is a monomer in solution while deoxyhemoglobin exists primarily as a tetramer under the conditions of these experiments.

The occurrence of interchain and intrachain conformational changes in crystalline hemoglobin on interaction with ligands

TABLE I: Activation Energies of Slowly Exchanging Hydrogens of Deoxyhemoglobin.

H/Fe	t_1 (min)	t_2 (min)	t_3 (min)	E^* 6.5° → 20° (kcal/mol)	E^* 20° → 36° (kcal/mol)
70	38	15		11	
60	90	36	5	11	22
50	204	77	12	17	21
40	465	215	22		25.5
30		420	50		24

at the heme group is now well documented (Perutz *et al.*, 1969). These changes involve not only interchain rearrangements but also apparently the interchain interfaces and are especially pronounced at the $\alpha_2\beta_1$ contact region (Perutz *et al.*, 1968; Perutz, 1970). Evidence of conformational changes in hemoglobin on ligand binding has also come from studies of the protein in solution (see Antonini and Brunori, 1971).

On the basis of studies of the temperature dependence of spin–lattice relaxation of the water protons of solutions of metmyoglobin, Atanasov (1970) concluded that the native state of myoglobin consists of at least two distinct conformers. There is a rapid fluctuation between the two states, which are close to each other in free energy, and the equilibrium between the two is markedly influenced by the presence of ligand. The transition from one state to the other markedly influences the spatial arrangement of the F-helical region. It is known that this region is at interchain faces in hemoglobin where changes in quaternary structure are detected on oxygenation of hemoglobin (Muirhead *et al.*, 1967).

At this stage, we cannot state with assurance what type of structural or conformational changes account for the differences in exchange rates between ligand-bound and ligand-free hemoglobin. In light of the considerations of possible mechanisms of exchange of slowly exchanging hydrogens, based on studies of polypeptides and other proteins, several possible explanations can be considered. There may be an increase in k_1, the rate constant of unfolding, of protein segments containing exchange sites inaccessible to solvent and a shift in the equilibrium distribution of conformational isomers in favor of unfolded forms. The structure of the protein may show increased motility, without actual unfolding, so that sites within the protein become more readily hydrated, the low-activation energy mechanism of Woodward and Rosenberg (1971) and Bryan (1970). Finally, there may be a change in the equilibrium distribution between associated (tetramer) and dissociated states (dimers and single chains), the shift favoring the dissociated forms.

If k_1, the rate of unfolding, is the rate-determining process of exchange of these hydrogens (β_1) and an increase in this rate is responsible for the increase in exchange rates in the ligand-bound protein, then the temperature dependency of exchange should reflect this circumstance. Apparent activation energies (E^*) can be calculated for this rate process ($\beta_1 = k_1$) (Rosenberg and Chakravarti, 1968; Rosenberg and Enberg, 1969). Activation energies for the exchange process for deoxyhemoglobin and carbon monoxide hemoglobin have been calculated from our data (Tables I and II). The expected E^* of a cooperative thermal unfolding process in proteins is considerably higher (E^* = 70–100 kcal) (Eisenberg and Schwert, 1951; Lumry *et al.*, 1966; Pohl, 1969) than those we have observed for the

exchange rate. Seemingly the change in exchange rates on ligand binding is not the result of an increase in k_1 and a shift in the equilibrium distribution of folded and unfolded conformational isomers.

Much the same argument can be used in considering the second alternative of the unfolding scheme, namely the case where k_1 is not rate determining. In this case, the EX_2 mechanism of Hvidt and Nielsen (1966), the overall rate of exchange (β_1), is given by the equation

$$\beta_1 = (k_1/k_2)k_3 \qquad (2)$$

Apparent activation energies (E^*) for this process can also be derived (Rosenberg and Chakravarti, 1968; Rosenberg and Enberg, 1969). Again, the calculated activation energies for ligand-bound and ligand-free hemoglobin (Tables I and II) are much too low to be consistent with a cooperative unfolding process and thus an EX_2 mechanism dependent on unfolding seems unlikely. The observed low-activation energies of exchange take place from folded conformations (Woodward and Rosenberg, 1970, 1971).

We are left, thus, with two remaining possibilities: that of a low-activation energy loosening of structure, without unfolding, and a ligand-dependent change in quaternary structure, such as a dissociation into subunits. At this point, we cannot choose between these possibilities. Spectroscopic studies of ligand binding make it attractive to suggest that the shift in distribution of rates of slowly exchanging hydrogens on ligand binding in hemoglobin is based on a shift in distribution of associated–dissociated forms of the protein toward dissociated isomers (single chains, dimers) Antonini *et al.* (1968). This explanation has the advantage that it would also account for the difference in the rates of exchange of this class of hydrogens between myoglobin and deoxyhemoglobin.

These observations, therefore, suggest to us that the differences in the effect of ligand binding on the exchange of hydrogen from hemoglobin and myoglobin are a consequence of the association of single-chain units in hemoglobin into tetramers while no such association is found in myoglobin in which the functional unit is a monomer.

In Figure 2, it may be seen that the temperature dependency of the exchange process is different in deoxy- and carbon monoxide hemoglobin. The exchange curves of carbon monoxide and deoxyhemoglobin are closer to each other at 37° than at 6°. Furthermore, the apparent energy of activation (E^*) of the rate process governing exchange of these hydrogens in deoxyhemoglobin is higher than that of carbon monoxide hemoglobin.

The suggestion, that the difference between exchange rates in ligand-bound and ligand-free hemoglobin is the result of dissociation into subunits will be the subject of further investigation under conditions in which hemoglobin tetramer is known to dissociate (Anderson *et al.*, 1970; Antonini and Brunori, 1971).

In this connection, it is of interest to note that Abrash (1970b) compared hydrogen exchange rates of metmyoglobin and apomyoglobin. The exchange rate of apomyoglobin was much greater than the parent protein. The differences in exchange between the two protein derivatives were minimal after both underwent identical conditions of acid denaturation.

Steinhardt and his associates (1963) have shown that cyanometmyoglobin and azidometmyoglobin are more resistant to acid denaturation than unliganded metmyoglobin. No hint of a conformational basis for this behavior is found in our comparative exchange rate data (Figure 4B). However, a more thorough study in which especially the pH dependency of exchange in the ligand-bound and ligand-free derivatives is studied should be performed to assess further a possible conformational basis for this behavior.

TABLE II: Activation Energies of Slowly Exchanging Hydrogens of Carbon Monoxide Hemoglobin.

H/Fe	t_1 (min)	t_2 (min)	t_3 (min)	E^* 6.5° → 20° (kcal/mol)	E^* 20° → 36° (kcal/mol)
45	95	50		8	
32	375	165	18	14	25
25		365	20		30

Our observations strongly suggest that ligand binding has associated long-range conformational effects in hemoglobin, but not in myoglobin. These observations parallel ones obtained by other methods, notably X-ray diffraction and optical rotatory dispersion.

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Inhibition by Heparin of Globin Messenger Ribonucleic Acid Translation in a Mammalian Cell-Free System†

Alan A. Waldman and Jack Goldstein*

ABSTRACT: Cell-free extracts of Krebs ascites cells, preincubated to decrease endogenous protein synthesis, translate rabbit globin mRNA with the production of both α- and β-globin chains. The addition of low levels of heparin, but not of chondroitin sulfate, hyaluronic acid, Sulfon (a sulfonated polystyrene), or dextran sulfates of varying size, results in a decrease in the mRNA-dependent stimulation of amino acid incorporation into protein. In the presence of 20 μg/ml of rabbit globin mRNA the addition of 4–5 μg/ml of heparin causes approximately 50% inhibition of globin mRNA translation, while 40 μg/ml of heparin completely prevents globin mRNA translation. Over the concentration range studied, the heparin effect is not reversible by mRNA. Synthesis of both α- and β-globin chains is inhibited by the presence of heparin to approximately the same extent. The distribution of ribosomal subunits is changed in the presence of high levels of heparin, but not in the presence of low levels of heparin. The data indicate that heparin acts at the level of initiation to inhibit translation of a natural mammalian mRNA in a mammalian cell-free system.

A Krebs ascites cell-free system, originally described by Mathews and Korner for translation of encephalomyocarditis (EMC)[1] viral RNA (Mathews and Korner, 1970), has recently been used to support translation of several other heterologous mRNAs (Mathews *et al.*, 1971, 1972; Housman *et al.*, 1971; Forget and Benz, 1971; Jacobs-Lorena and Baglioni, 1972a; Metafora *et al.*, 1972; Jacobs-Lorena and Baglioni, 1972b). Translation of rabbit reticulocyte globin mRNA has been the most extensively studied (Housman *et al.*, 1971; Jacobs-Lorena and Baglioni, 1972a,b; Metafora *et al.*, 1972), and it has been shown that the globin mRNA is translated with the production of both rabbit α- and β-globin chains.

Heparin, a naturally occurring polyanion found in many tissues (Freeman, 1964), has been shown at high levels to inhibit protein synthesis in rat liver cell extracts (Berlinguet and Normand, 1968) and to effect the distribution of ribosomal subunits in HeLa cell extracts (Miller, 1968). Heparin has also been reported to inhibit binding of Phe-tRNA to 40S ribosomal subunits isolated from rat lymphosarcoma (Koka and Nakamoto, 1972), to inhibit translation of poly(U) by extracts of *Escherichia coli* (Wacker *et al.*, 1967), and to bind to 30S ribosomal subunits and to 70S ribosomes isolated from

† From the Nucleic Acids Laboratory, The New York Blood Center, New York, New York 10021, and the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received November 9, 1972.* This work was supported by Grant No. HL-09011-09 from the National Institutes of Health.

[1] Abbreviations used are: EMC virus, encephalomyocarditis virus; S-30, 30,000*g* supernatant; PS-30, preincubated derivative of 30,000*g* supernatant.