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Study of Hydrogen Exchange in Hemoglobin as a Function of Fractional Ligand Saturation†

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ABSTRACT: We have studied the hydrogen-exchange kinetics of hemoglobin A_0 as a function of ligand, CO, saturation. In the noncooperative system, azide binding to methemoglobin, the alterations in exchange kinetics are proportional to the average degree of ligation. However, in the case of CO binding to deoxyhemoglobin the changes in hydrogen-exchange pattern run ahead of the degree of ligation. The data can be best fitted assuming that all the liganded species, regardless of the number of ligands, show the same exchange properties. This two-state behavior must be the consequence of the fact that all the conformational changes leading to increased solvent accessibility take place when the first ligand is bound. Studies of the effect of pH changes and carbamoylation on the relationship between ligand binding and hydrogen exchange show that the observed differences of hydrogen exchange between deoxy and the liganded state are linked to the alkaline Bohr effect and to the state of the α-N-termini. As a consequence, at pH 9 despite a highly cooperative ligand binding isotherm the differences in hydrogen exchange between the deoxy and fully liganded species have vanished. We have to conclude that the hydrogen exchange is mirroring only the first part of the overall R to T transition. In all the experiments with pH changes and carbamoylation it is the liganded form that shows changes becoming more like the deoxy state. This is not consistent with a model where ligand binding removes a structural restriction in the less accessible deoxy state.

The cooperative binding of ligands such as oxygen and carbon monoxide to ferrohemoglobin is characterized by a sigmoidal binding isotherm and a large difference in the first and fourth binding constants (Adair constants). This cooperative binding behavior has been a central area of research in hemoglobin chemistry. The two-state thermodynamic model of Monod–Wyman–Changeux (1965) and the X-ray structural studies of Perutz and others have been both popular and instrumental in attempting to describe the sequential binding of ligands to ferrohemoglobin.

Unfortunately, direct structural studies and thermodynamic anlysis of the partially liganded intermediates are difficult due to their transient nature as dictated by the binding equilibria. Therefore, few studies of ligand-induced conformational changes have been done as a function of ligand saturation, and the results have been somewhat contradictory. Early studies by spin-label methods (Ogawa & McConnell, 1967; Ogata & McConnell, 1972, 1971) and by cicular dichroism (Simon & Cantor, 1969) indicated that conformational changes were linear with respect to fractional saturation. More recently, kinetic rebinding measurements following flash photolysis of the carboxyhemoglobin complex have shown that the conformational change lags behind the fractional change in ligation (Sawicki & Gibson, 1978). Finally, several recent studies by a variety of physical methods have revealed significant structural changes which occur with the binding of the first ligand at physiological pH. These observations are as follows: the thiol reactivity of the β-93 SH group runs well

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ahead of the fractional saturation (Makino & Sugita, 1982); the dimer–tetramer equilibrium is greatly shifted toward the oxy state when only one oxygen is bound as measured by hemoglobin–haptoglobin association (Romeo et al., 1982); the largest change in heat capacity between deoxy- and carboxyhemoglobin occurs at the initial ligation step (Battistel & Lumry, 1983); release of carbamates from all four N-termini of hemoglobin as well as Bohr proton release from both α and β chains probably occurs with the binding of the first oxygen (Poyart et al., 1978).

The thermodynamic model of Monod–Wyman–Changeux has recently been expanded on by Johnson & Ackers (1981) and by Pettigrew et al. (1982) in order to partition the energy at each step in the ligation process and the dimer–tetramer assembly, with the goal of correlating the thermodynamic and structural data. The energy calculations of this linkage scheme predict a large difference in the free energy of dimer–tetramer assembly between the unliganded and singly liganded molecule, accounting for about half the total free energy change which is observed with full ligation.

It is quite reasonable to assume that many of these methods, if not all, see only very localized areas in the macromolecule and thus do not give a consistent picture of the sequence of steps leading from the deoxy structure to the liganded one. Hydrogen-exchange kinetics has the advantage that it measures the behavior of the whole molecule represented by hundreds of exchanging sites distributed quite uniformly over the whole molecule. However, it can also by use of partial exchange-in techniques look at any section of the distribution of rate constants. This allows one to see very small changes against a reduced background (Barksdale & Rosenberg, 1982).

The results of our experiments using hydrogen-exchange techniques indicate profound changes in the fluctuation behavior of hemoglobin occur at the first oxygenation step rather than at the later stages of oxygenation when the R to T switch is postulated to occur. These results will be discussed in terms of cooperativity, pH, and modification of the N-termini of the hemoglobin molecule.

Materials and Methods

The main component of human hemoglobin, HbA_0,[1] was prepared from outdated red cells as described by Barksdale et al. (1975). The carbamoylated derivative of HbA_0 with all four N-termini modified was prepared by the method of Williams et al. (1975). Tritiation of the hemoglobin to isotopic equilibrium was accomplished by addition of 150 μL of approximately 50 mCi/mL tritiated water to 25 mL of 0.52 mM hemoglobin and 0.06 M sodium borate solution, pH 9.8, followed by incubation at 32 °C for 24 h. All tritiated water came from the same stock, and volumetric measurements were made carefully so that the tritium activity could be later duplicated.

Outexchange of the Methemoglobin–Azide System. After tritiation, the pH was lowered from 9.8 to 7.0 by addition of 4 M sodium monobasic phosphate having the same tritium activity as the sample. This solution was then stored at 4 °C until needed.

Each outexchange used 1 mL of solution and was done in duplicate. Therefore, for a particular azide concentration, 2 mL of inexchange was oxidized with 15 μL of 1 M potassium ferricyanide having the same tritium activity as the sample. Oxidation was considered to be complete after 1 h at room temperature. Enough sodium azide was then added to yield the desired degree of saturation. Calculation of the amount of sodium azide to be added was based on the azide binding isotherm of Barksdale et al. (1975). The sodium azide was added either as a 0.1 or 1 M solution to 0.1 M sodium phosphate buffer, pH 7.0, $I = 0.2$, having the same tritium activity as the sample. The volume of azide solution added was therefore generally between 2 and 20 μL. Equilibration was allowed to continue for 2 h at 6 °C, after which the true degree of saturation and free azide were determined spectrally at 6 °C in a 1-mm path-length cuvette. This determination presents an experimentally difficult problem. The customary spectral analysis of the degree of ligation (Barksdale et al., 1975) is, for a large number of small samples, cumbersome and prone to error due to uncertainty in total protein concentration. Consequently, the degree of saturation and the corresponding free ligand concentration were determined indirectly. Separate experiments relating the free ligand concentration and the degree of saturation to spectral changes provided a calibration curve of the spectral ratio R (the ratio of absorbance at 572 nm to that at 632 nm) as a function of the degree of ligation.

At our conditions the average degree of ligation, $\bar{Y}$, was given by

$$\bar{Y} = 7.90 \times 10^{-2} + (5.10 \times 10^{-1})(\ln R) + (1.14 \times 10^{-1})(\ln R)^2 - (4.60 \times 10^{-2})(\ln R)^3$$

where R stands for the absorbancy ratio. This type of analysis provides for rapid determinations that are independent of the total protein concentration in the sample. The sample was then split into two 950-μL aliquots and dialyzed against tritiated, pH 7.0, sodium phosphate buffer having the same azide concentration as the free concentration in the sample. Dialysis was carried out against three 10-mL volumes of tritiated azide buffer at 6 °C, allowing 3 h between changes. This produced a tritiated hemoglobin solution equilibrated at a known degree of saturation, without having changed the isotopic equilibrium.

Removal of the solvent tritium and initiation of outexchange were accomplished by applying the contents of one of the dialysis bags to a Sephadex G-25 column (2.2 × 11 cm), thermostated to 6 °C and equilibrated with pH 7.0 sodium phosphate buffer, $I = 0.2$, having the same azide concentration as the sample. The protein peak, about 4.5 mL, was collected and incubated at 6 °C. The outexchange was followed by taking six time points between 15 and 400 min. At each time point 500 μL of exchanging solution was chromatographed on a Sephadex G-25 column equilibrated with the same buffer used for the initial tritium separation, in order to remove the tritium which had exchanged out since the beginning of the experiment. The protein peak was collected in five fractions of about 500 μL each. The three most concentrated fractions were retained for analysis of the number of hydrogens remaining unexchanged at each time point, according to standard methods (Englander, 1963). The heme concentration was determined from the absorbance at 419 nm by using 111 mM^{-1} cm^{-1} as the heme extinction coefficient for the cyanomet derivative obtained by dilution into Drabkin's reagent. Radioactivity was measured on a Beckman LS7500 liquid scintillation counter with each sample corrected for quenching by H# standardization.

Outexchange of the Deoxy–Carbonmonoxy System. One milliliter of the hemoglobin solution exchanged in as described above was used for each experiment. However, in order to achieve partially saturated solutions for outexchange, two aliquots were used. One aliquot was converted to the HbCO derivative in a glass vial with several flushes of CO. Residual unbound CO was removed from the vial with a flush of ni-

[1] Abbreviations: Hb, hemoglobin; HbCO, (carbonmonoxy)-hemoglobin.

trogen gas. The other aliquot of exchanged in hemoglobin was then added and the solution swirled and allowed to stand at room temperature for 1 h to allow redistribution of the ligand. The approximate degree of saturation was determined by the relative volumes of the two aliquots. Just prior to the initiation of outexchange a small amount of sodium dithionite was added to the sample, in order to reduce any methemoglobin present to the ferro form and to convert all non-CO-liganded hemes to the deoxy form. The sample was then applied to a Sephadex G-25 column equilibrated with nitrogen-purged, pH 7.0, sodium phosphate buffer containing 0.5 mg/mL sodium dithionite. We are well aware of the increased scatter in experiments utilizing sodium dithionite. However, under conditions of partial ligation the presence of small amounts of methemoglobin are unacceptable and difficult to correct without introducing additional uncertainty into the data. There is good agreement between our previous work without dithionite and the present investigation with respect to the linearity of the plots. The columns were thermostated to the desired temperature. The eluate was collected in a 5-mL glass syringe fitted with a three-way stopcock, to allow anaerobic transfer of the outexchanging solution. Second separations were done on Sephadex G-25 columns equilibrated with the oxygen-free buffer used in the first separation. The second eluates were collected in CO-filled test tubes, thereby converting all the Hb to the CO form. The number of hydrogens remaining was determined as described above, except that the heme concentration was determined by dilution into CO-saturated buffer and reading the absorbance at 419 nm by using a heme extinction coefficient of 202 mM^{-1} cm^{-1}.

The true degree of saturation was again determined indirectly from spectral ratios. However, in this case we used three independent pairs of wavelengths:

$$\bar{Y} = 2.99(A_{538}/A_{547}) - 2.45$$

$$\bar{Y} = (-8.68 \times 10^{-1})(A_{568}/A_{554})^2 + 4.35(A_{568}/A_{554}) - 3.15$$

$$\bar{Y} = (4.35 \times 10^{-1}) \ln (A_{554}/A_{480})^2 - 2.47 \ln (A_{554}/A_{480}) + 2.53$$

The form of the calibration curves above represents the best fit to the data with the least deviation from linearity. The mean of these three values was taken to be the degree of saturation.

Partial Exchange-In. Partial exchange-in experiments were carried out by incubating 1 mL of oxyhemoglobin, pH 9.0, and 0.09 M sodium borate with 50 μL of tritiated water having an activity of approximately 6 mCi/mL for only 1 min. This partially trititated sample was then quickly deoxygenated by addition of a few crystals of sodium dithionite and applied to a Sephadex G-25 column equilibrated with nitrogen-purged 50 mM sodium borate, pH 9.0, containing 1 mg/mL sodium dithionite. The eluate was collected, and the number of hydrogens remaining as a function of time was calculated in the same manner as for the fully tritiated experiments. For an estimate of nonspecific retention of label the first and second separations were carried out without the addition of dithionite.

Analysis of Hydrogen-Exchange Data. All our data represent the kinetics of the loss of tritium label from hemoglobin previously exchanged in either partially or fully. Full exchange-in represents isotopic equilibrium for all exchangeable sites.

The data are obtained as pairs consisting of time and the amount of label retained. The results appear as kinetic curves illustrated in Figure 1. We have used a logarithmic time scale because the time range is large. The linearity of such plots over the time range used is the consequence of the form of the

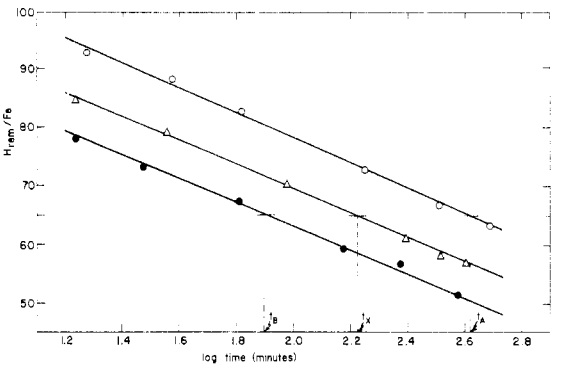

FIGURE 1: Typical exchange plot of the number of hydrogens remaining unexchanged per subunit vs. log time for deoxyHb (○), HbCO (●), and a partially saturated intermediate (△). Exchange was carried out at pH 7.0, 6 °C, and 0.2 ionic strength sodium phosphate buffer. Log time values to reach 65 hydrogens remaining for HbCO, the partially liganded intermediate, and deoxyHb are represented by t_B, t_X, and t_A, respectively.

distribution function for rate constants (Barksdale & Rosenberg, 1982). It can, over relatively narrow intervals of time, be approximated by a boxcar function (Shenkin, 1978).

The curves are sections of the overall exchange curve represented by a sum of exponentials

$$H(t) = \sum_i e^{-k_i t} \quad (1)$$

and remain parallel over a remarkably broad time range. This indicates that the distribution function for the rate constants does not change its shape appreciably but rather is shifted by ligand binding (Barksdale & Rosenberg, 1982). This property of the rate constant distribution function, regardless of its physical origin, allows us to simplify the kinetic analysis provided we realize its limitations.

In such a case we can characterize the exchange by an average rate constant, $\langle k \rangle$, for each of the exchanging species. The well-known equation (Will & Damaschun, 1973)

$$\phi = \frac{t_X - t_B}{t_A - t_B} \quad (2)$$

linking a fractional change, ϕ, to rate constants or times is valid in the case of hemoglobin when the times required for equal degree of exchange in the different forms are compared (Barksdale & Rosenberg, 1978, 1982). The kinetic considerations are by no means unique for hydrogen exchange; isotope-exchange kinetics is but one way of utilizing the general concept of differential reactivity (Christen & Gehring, 1982). Equation 2 is another form of the well-known case for differential reactivity (Citri, 1973)

$$k_{app} = k_A\phi + (1 - \phi)k_B$$

where k_{app} is the measured rate constant and k_A and k_B are the rate constants for the unliganded and liganded states. The symbol ϕ has the same meaning as in eq 2. For this equation and eq 2 to be valid, the ligand binding and dissociation rates must be much faster than the rates k_A and k_B.

The problem in the case of hydrogen exchange, where we observe a large family of exchanging sites each with a first-order rate constant, is to ascertain that the rates we observe in all cases from unliganded to fully liganded states represent exchange from the same sites. Further, as we have argued previously (Rosenberg & Chakravarty, 1968), eq 2 is valid only if the rank and order of the exchange are not altered by ligation. In such a case, the instantaneous average rate constant, $\langle k \rangle$, characterizing the exchange of each H_{rem} value, is describing the exchange from a group of sites whose rate

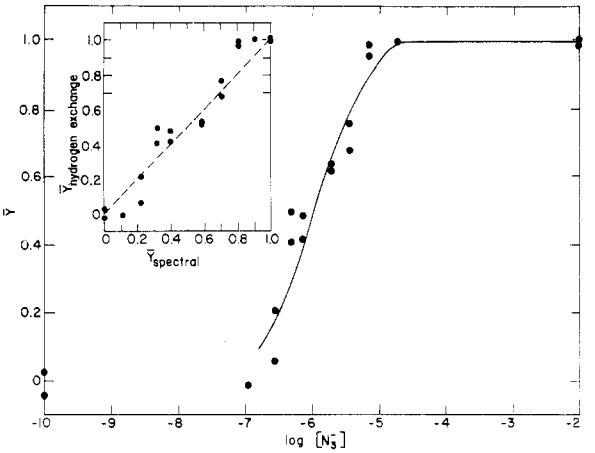

FIGURE 2: Fractional change in hydrogen exchange (●) vs. the free azide concentration and the fractional spectral change (—) vs. the free azide concentration. The binding isotherm is from Barksdale et al. (1975). Both are at pH 7.0, 6 °C, and 0.2 ionic strength sodium phosphate buffer. The insert is a plot of the fractional change in hydrogen exchange vs. the fractional spectral change for the methemoglobin–azidomethemoglobin system. Perfect correlation is represented by the broken line.

constants contribute the same fraction in all cases. The criteria for such fortunate behavior are that the times, t_A and t_B, required to reach the same H_{rem} value in the unliganded and fully liganded state always obey the equation

$$\log t_A - \log t_B = \text{constant} \quad (3)$$

Although a difference in hydrogen exchange kinetics upon ligation can be due to either small effects on many sites or a larger effect on a single group of hydrogens, it is easy to see that a large change from slow to rapid for a group of hydrogens would violate the conditions outlined above, and eq 3 would not hold. The validity of our argument in this case rests on the measured parallelism of our curves over a respectable time range.

We have reasons to believe that a small group of hydrogens is present in hemoglobin, a group whose rate changes so radically that it seems to violate the rank and order of the rate constants (Liem et al., 1980). The number of such hydrogens, in this case, is small enough as not to influence the behavior of the large group of hydrogens changing their rate upon ligand binding. The experiments using partial exchange-in described under Materials and Methods are designed to study the behavior of this group of hydrogens. The interpretation is straightforward. We are looking only at a very small segment of the rate constant distribution, and the increased resolution is due to the absence of a large background.

Results

Azidomethemoglobin System. The hydrogen-exchange data for azidomethemoglobin at various degrees of saturation were fitted as described under Materials and Methods, and values of log (t) at 40 hydrogens remaining per subunit were used to calculate the fractional change in hydrogen exchange. Azidomethemoglobin has already been shown to exchange more slowly than methemoglobin (Benson et al., 1974). In this work the average of two determinations gave log (t) values at 40 hydrogens remaining of 2.3962 and 2.9128 for metHb and fully saturated azidometHb, respectively. The fractional change in hydrogen exchange is plotted in Figure 2 as a function of the free azide concentration, i.e., the azide concentration of the equilibrating buffer. The inset in Figure 2 represents the fractional spectral change vs. the free azide concentration at the same conditions as determined by

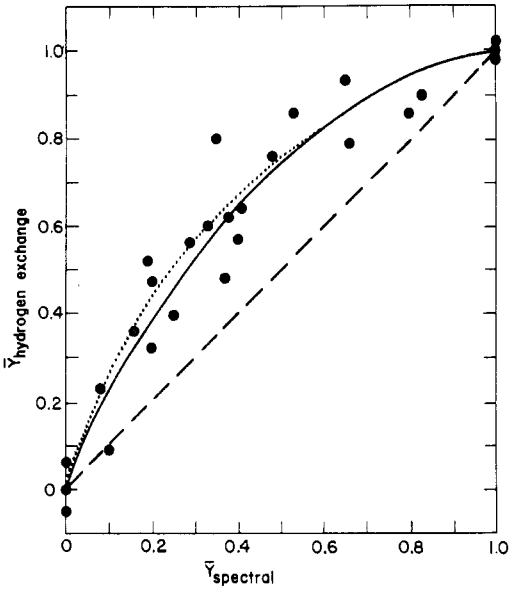

FIGURE 3: Plot of the fractional change in hydrogen exchange vs. the fractional spectral change for the deoxy–(carbonmonoxy)Hb system (●) at pH 7.0, 6 °C, and 0.2 ionic strength sodium phosphate. The broken line (- - -) represents the perfect correlation. The solid line is the best fit to the data and the dotted line (···) is the sum of all liganded species as a function of Y as explained in the text.

Barksdale et al. (1975). The high degree of correlation between the fractional change determined spectrally and by hydrogen exchange is reflected in the inset. The solid line represents the perfect correlation.

(Carbonmonoxy)hemoglobin System. The hydrogen-exchange data for ferroHb as a function of thee CO saturation was handled in the same manner as the azidometHb system. In this case the liganded form exchanges more rapidly at neutral pH (Benson et al., 1973; Englander & Mauel, 1972; Hedlund et al., 1978). The values of log (t) at 65 hydrogens remaining per subunit were used to calculate the fractional change in hydrogen exchange. The mean of two determinations was 2.6319 and 1.9135 for deoxyHb and the fully CO saturated form, respectively. The fractional change in hydrogen exchange as a function of the CO concentration, i.e., pCO, was not plotted since the binding isotherm is not known at these conditions. It is unfortunate that we cannot use oxygen as a ligand, since the binding curves are very well characterized. However, the partially oxygenated hemoglobin solutions are not stable for the relatively long time periods necessary for hydrogen-exchange experiments. Because it is assumed that the shape of the isotherm is the same as that of oxyhemoglobin, with the partition constant being invariant as a function of saturation (Wyman, 1964), the relative contributions of the five species in solution at any fractional saturation should be the same for (carbonomonoxy)Hb as for oxyHb. In addition, the analysis presented here is quite insensitive to small variations in the concentrations of two and three liganded species.

A correlation plot of the fractional change in hydrogen exchange vs. the fractional change in ligation determined spectrally is shown in Figure 3. The correlation is very poor, with the fractional change in hydrogen exchange proceeding ahead of the fractional spectral change.

Effect of Carbamoylation and pH on the Exchange Properties of (Carbonmonoxy)Hb. The difference in hydrogen-exchange rates between the liganded and unliganded species of ferroHb has been shown to be pH dependent, with the maximal difference at about pH 7.4 (Hedlund et al., 1978). Extrapolation of these data would predict a very small dif-

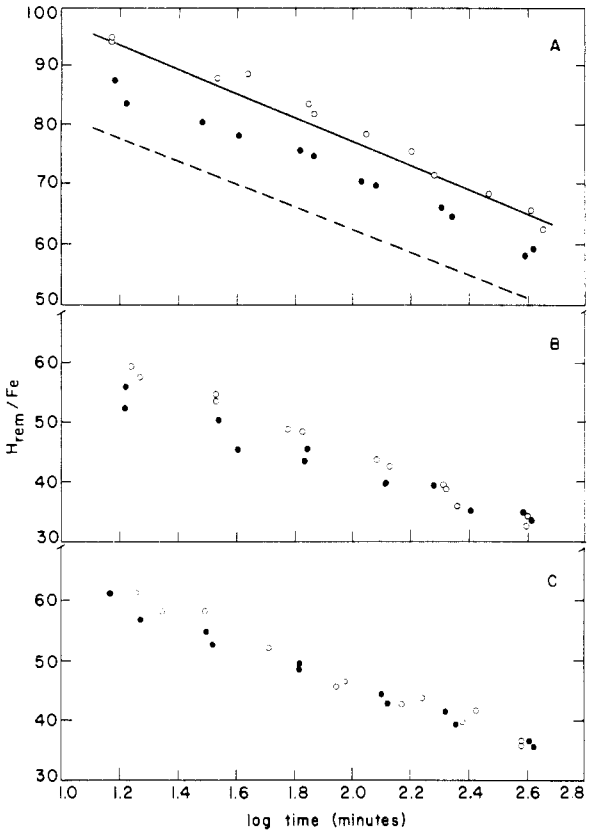

FIGURE 4: Exchange plots of the following: (A) Deoxy carbamoylated Hb (○) and carbamoylated HbCO (●) at pH 7.0, 6 °C, and 0.2 ionic strength sodium phosphate. The solid line represents the best fit for normal deoxyHb, and the broken line represents the best fit for normal HbCO carried out at the same conditions as the carbamoylated derivaties. (B) DeoxyHb (○) and HbCO (●) at pH 9.0, 6 °C, 0.05 M sodium borate buffer. (C) Deoxy carbamoylated Hb (○) and CO carbamoylated HbCO (●) at pH 9.0, 6 °C, 0.05 M sodium borate.

ference at pH 9. Figure 4B corroborates this prediction. Figure 4A shows the effect of carbamoylation of the four N-termini on the deoxy and oxy forms of Hb at pH 7.0, 6 °C, and Figure 4C shows the effect of carbamoylation at pH 9.0, 6 °C.

Partial Exchange-In. The results of the partial exchange-in studies in which hemoglobin is exchanged in as oxy and outexchanged as deoxy are shown in Figure 5, together with the appropriate control. The difference has been plotted in the inset to Figure 5 on a semilogarithmic scale. This represents the "jump class" of hydrogens (Liem et al., 1980). The extrapolated value of 1.5–1.7 hydrogens is not a very firm value considering the magnitude of the background subtraction. It probably represents an overestimate.

Discussion

The relationship between the average degree of ligation and the fraction of change in the hydrogen exchange properties presented under Materials and Methods has to be expanded to the degree that the observations can be related to the Adair description of ligand binding and the models describing the cooperativity of the system.

The observed exchange represented, at a given degree of exchange, by an average rate constant $\langle k \rangle$ contains contributions from all the species in solution. The fractional saturation of a site, $\bar{Y}$, is given at any free ligand concentration by

$$\bar{Y} = \frac{1}{4}\sum_{i=1}^{4} i f_i; \quad \sum_{i=0} f_i = 1$$

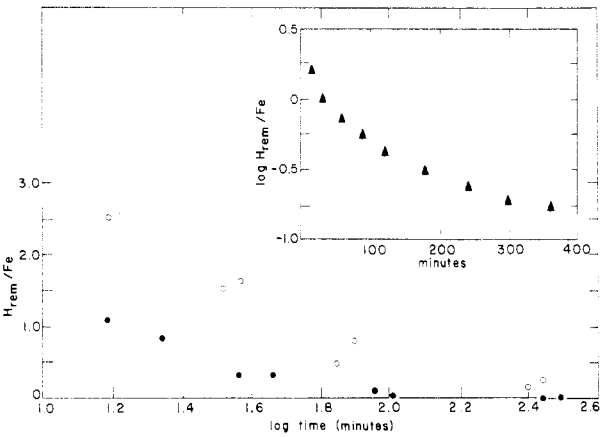

FIGURE 5: Exchange plot of the "jump class" at pH 9.0, 6 °C, 0.05 M sodium borate. Exchange-in was carried out for 1 min as oxyHb and outexchanged as deoxyHb (○) and as oxyHb (●). The inset is a difference plot of the lower plot.

where i represents the number of ligand molecules bound to the hemoglobin molecule and f_i the fraction of molecules carrying i ligands. The fraction f_i is a function of Adair constants and the free ligand concentration and can be calculated for CO binding by using in our case the Adair constants given by Imai & Yonetani (1975). The apparent hydrogen-exchange rate constant $\langle k \rangle$ is given by

$$\langle k \rangle = \sum_i (f_i)(\langle k_i \rangle)$$

where $\langle k_i \rangle$ is the average apparent rate constant for the ith species at a given degree of exchange. The above equation can be normalized to let $\langle k_0 \rangle$ (deoxyHb) equal 0 and $\langle k_4 \rangle$ (fully CO saturated Hb) equal 1 in order to analyze the relationship of the apparent rate constants of the intermediates to the end states and thermselves.

In order to attempt an analysis of the structural and dynamic changes which occur in ferroHb as a function of ligand binding, we began with an examination of the hydrogen-exchange behavior of methemoglobin as a function of azide saturation. Azide binding to methemoglobin has been shown to be noncooperative (Epstein & Stryer, 1968; Barksdale et al., 1975; Anusiem & Beetlestone, 1976). Therefore, it represents a tetrameric reference model to which the cooperative system will be compared. The fractional change in hydrogen exchange in this system shows a good correlation with the spectrally determined degree of binding (Figure 2). If k_i represents the average exchange rate from the species with i ligands and i can be 0, 1, 2, 3, and 4, then the linear correlation between the degree of ligand saturation and increase in hydrogen exchange rate implies

$$\langle k_i \rangle - \langle k_{i-1} \rangle = 0.25(\langle k_4 \rangle - \langle k_0 \rangle)$$

Therefore, the change in fluctuation behavior is probably propagated only in the subunit to which the ligand is bound and strictly proportional to the number of ligands bound. Since this is a noncooperative system, the linear correlations cannot be ascribed to the paucity of intermediate species as has been proposed for a cooperative system (Sawicki & Gibson, 1978).

The hydrogen-exchange behavior of hemoglobin as a function of the fractional CO saturation reveals a strikingly different profile from that observed for azidomethemoglobin. The solid line in Figure 3 is the best fit to the data. In terms of the Adair scheme, an excellent fit to the data is obtained when $\langle k_1 \rangle = \langle k_2 \rangle = \langle k_3 \rangle = \langle k_4 \rangle$, which is equivalent to the dotted line in Figure 3. Therefore, the dynamic behavior of any liganded molecule is very similar regardless of the number of ligands, but very different from the unliganded molecule.

The importance of the first ligation step in causing large disruptions in the hemoglobin molecule has supportive evidence from several lines of experiments. Perutz (1976) has suggested that binding of one oxygen molecule to the deoxy quaternary structure may loosen all the constraining hydrogen bonds in the molecule, prior to a change in the quaternary structure. The work of Poyart et al. (1978) at very low levels of oxygen saturation is in agreement with Perutz's view that the entire molecule "feels" the first oxygenation step with concomitant structural changes. Physical methods such as SH reactivity and heat capacity measurements may be seeing the same phenomenon as hydrogen-exchange data (Makino & Sugita, 1982; Battistel & Lumry, 1983). Also, as mentioned previously, the dimer–tetramer equilibrium is affected more by the first ligand bound than any subsequent ligand (Valdes et al., 1978; Pettigrew et al., 1982) with an accompanying quaternary change in the $\alpha_1\beta_2$ interface (Romeo et al., 1982).

Next we tried to identify in as much detail as possible the structural changes caused by the binding of the first ligand. Our strategy was to use perturbations of ligand binding of known structural origin and observe their effect on the correlation between ligand binding and hydrogen-exchange changes. The first variable used was pH as to determine the relationship of our observations to the alkaline Bohr effect. The results were more drastic than expected.

The large difference in hydrogen exchange between the deoxygenated and the oxygenated state at pH 7.0 (Figure 4A) vanishes at pH 9.0 (Figure 4B). After considering the accumulated evidence concerning the change in the hydrogen-exchange pattern of hemoglobin following full ligation, we have previously concluded that approximately 25%, and possibly more, of the 120–130 observable hydrogens in a hemoglobin chain are influenced to a varying degree by full ligation (Barksdale & Rosenberg, 1982). This major change seems to be lost at pH 9. However, there might be some small group of hydrogens, the exchange of which could be influenced by ligation at pH 9, and which we failed to see since the number is small compared to a large background. The background in this case is about 70 hydrogens. Such a class, the "jump class", represented by three hydrogens per hemoglobin dimer, has been suggested to reflect a structural, cooperative motion associated with the T to R transition (Liem et al., 1980). We have carried out the appropriate jump experiments at pH 9. The results in Figure 5 are surprisingly consistent but indicate that only about one hydrogen per monomer could possibly relate to the T to R transition. This extremely small difference is, of course, not discernible except by the method of partial inexchange. It is not known whether this change will vanish also upon the binding of the first ligand since the experiments with partial ligand saturation involving a total change of only one hydrogen are at present not experimentally feasible. Considering that the T to R transition exists at pH 9 and the Hill coefficient, n, is 2.86 and 2.7 at pH 7 and pH 9, respectively (Imai & Yonetani, 1975), one must conclude that the majority of changes seen by hydrogen exchange are not necessarily correlated to the T to R transition per se. It seems, instead, that hydrogen exchange sees only the first part of the overall change of structure resulting from ligand binding. The hydrogen-exchange transition seems to be more closely correlated to the alkaline Bohr effect.

Our knowledge about the Bohr effect has recently been changing, and a relatively clear picture has become more clouded. The classical picture of the localized alkaline Bohr effect is presented perhaps most concisely by Kilmartin (1976). In the deoxy structure, weak positively charged bases form salt bridges with negatively charged partners. These bonds stabilize and restrict the deoxy state, causing its hydrogen-exchange pattern to be shifted toward a slower average exchange. Ligand binding results in the breaking of these bonds, and the hydrogen exchange of the oxygenated, relaxed structure is more rapid, showing an increased solvent accessibility (Barksdale & Rosenberg, 1982). At pH 9 these weak bases are deprotonated, and the salt bridges cannot form. The Bohr effect vanishes after pH 9. The pK_a values of the weak bases are altered in the salt bond. When the salt bond is broken, the pK_a is normalized. Thus, ligand binding results in the release of the so-called Bohr protons. Our knowledge about the groups participating in the Bohr effect is based on measured shifts in pK_a values when the liganded and unliganded forms are compared. The weak bases identified as alkaline Bohr groups are the α-amino-terminal valine, the β-146-histidine and possibly the α-122-histidine (Nishikura, 1978). The α-amino end group does not form a direct salt link, as previously envisioned, but a two-step salt bridge where the amino group interacts with arginine through an anion (O'Donnel et al., 1979). Our findings seem, at first glance, to fit well to this classical picture of the Bohr effect. The differences between the effects of ligation on hydrogen exchange at pH 7.0 and 9 can be explained by assuming that the increased freedom in the oxy state is linked to the breaking of the first α-terminal salt bond. We know from analysis of the allosteric free energy, as defined by Ackers' group (Pettigrew et al., 1982; Chu & Ackers, 1981), that of the total free energy of 6.3 kcal per dimer nearly 3 kcal vanishes when it is measured at pH 9. A similar reduction of the linkage energy is achieved by carbamoylation (Table I). The conclusion is that about 3 kcal of linkage energy is stored in a delocalized manner, since the restrictions imposed on the deoxy state and released by the first ligand result in a change of hydrogen-exchange rates for approximately 25% of all exchangeable hydrogens. The similarity in the changes of allosteric linkage energy produced by carbamoylation and by pH change focuses our attention to the α-N-termini. Reaction of the four N-termini with cyanate, i.e., carbamoylation, reduces the Bohr effect (Kilmartin, 1976) and abolishes the pH-dependent alterations in the hydrogen-exchange pattern we previously reported (Hedlund et al., 1978; unpublished observations in this laboratory). The carbamoylation has no effect on the exchange kinetics of either the liganded or unliganded form at pH 9, but it affects the behavior at pH 7 (Figure 4A) by altering interactions which are based on charge. Removal of charge differences by either carbamoylation or pH has a similar effect on the difference in exchange behavior between the liganded and unliganded species.

In addition, the release of four carbamino adducts and Bohr

Table I: Free Energies of Dimer–Tetramer Association, the Calculated Linkage Free Energy, and Hydrogen Exchange

species	dimer–tetramer association (kcal)[a]		linkage (kcal),	deoxy–oxy hydrogen exchange differences
	$^0\Delta G_2$	$^4\Delta G_2$	$\delta\Delta G_{04}$	
HbA_0, pH 7.4[b]	−14.3	−8.0	6.3	reference state, large
carbamoylated HbA_0, pH 7.4[b]	−14.4	−9.4	5.0	decreased 50%
HbA_0, pH 9[c]	−12.3	−9.0	3.3	abolished

[a] $^0\Delta G_2$ and $^4\Delta G_2$ represent association of the deoxy and the liganded form, respectively. [b] The values for HbA_0, pH 7.4, and for carbamoylated are from Pettigrew et al. (1982). [c] Values for HbA_0, pH 9, are from Chu & Ackers (1981).

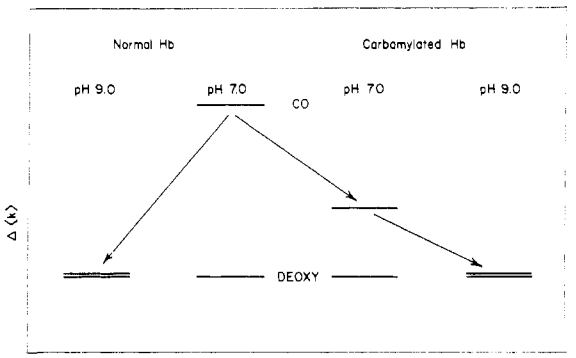

FIGURE 6: Schematic representation of the effect of pH and carbamoylation on $\langle k \rangle$, the average rate constant of the distribution function of hydrogen exchange.

protons from more than one subunit with only one ligand bound (Poyart et al., 1978) supports the linkage between the N-termini and the structural perturbation.

Also, a considerable fraction of the Bohr protons are released after binding of the first ligand molecule (Imai & Yonetani, 1975). At this point complications appear. First, the classical Bohr effect assignments have been challenged, most recently by studies of the chemical reactivity of the alleged Bohr groups (Kaplan et al., 1982). The results raise the question of whether instead of highly localized storage of Bohr energy we should not consider a very general charge model similar to those encountered in colloidal chemistry (Blank, 1975, 1980).

Hydrogen exchange has problems because, although changes appear in the exchange patterns as predicted by the classical Bohr effect model, the direction of these changes is unexpected. First, if going from pH 7 to pH 9 implies that the α-amino terminal cannot form a bond stabilizing the deoxy structure and if, as we have previously observed, the breaking of the stabilizing bond leads to the increased solvent accessibility seen in the liganded state, then the exchange pattern of deoxyHb at pH 9 should change and approach that of the oxy form. Careful examination of the previously published data on the pH dependence of hydrogen exchange in oxy- and deoxyhemoglobin (Hedlund et al., 1978), however, shows that deoxyhemoglobin does not show a change in exchange behavior between pH 7.5 and pH 8, whereas the exchange pattern of oxyHb in the same region shifts toward the behavior of the more restricted deoxy form. These data are confirmed in a far more convincing manner when one examines the behavior of the carbamoylated forms of hemoglobin as seen in Figure 4A. Cabamoylation has no effect on the exchange properties of the deoxy form whereas the properties of the oxy, R, form change and shift toward a more restricted T-like state. This agrees well with the shift in the dimer–tetramer association constant of carbamoylated Hb toward that of deoxyHb (Pettigrew et al., 1982). The fact that the carbamoylated forms of both deoxy- and oxyhemoglobin remain unchanged at pH 9 (Figure 4C) serves as an excellent control. This makes it improbable that carbamoylation introduces some de novo change not associated with the Bohr groups.

The behavior of hemoglobin hydrogen exchange as a function of pH and carbamoylation can be summarized by a single scheme (Figure 6) where the distance along the ordinate is proportional to the shift in the average rate of hydrogen exchange, $\langle \bar{k} \rangle$ (Barksdale & Rosenberg, 1982). If we assume that the shifts in the rate function are proportional to the allosteric free energy changes, we conclude the following. First, part of the allosteric free energy, approximately 2–3 kcal, is released upon binding of the first oxygen, and that energy is stored in the form of restricted motion involving a substantial segment of the molecule (Hopfield, 1973). It is only this part of the overall T to R change that hydrogen exchange sees, a part that is controlled by the forces responsible for the alkaline Bohr effect. Other methods are sensitive to other events in the sequence of structural changes that lead from the crystallographically determined T to R state (Sawicki & Gibson, 1978). Second, it is the interaction of the α-terminal amino groups that controls the structural Bohr effect linked change seen by hydrogen exchange. However, whatever interactions the terminal amino group is involved in, it is the R structure that seems to be stabilized.

Registry No. HbA_0, 54651-57-9; azidometHbA_0, 87901-12-0; (carbonmonoxy)HbA_0, 87901-13-1; metHbA_0, 77538-47-7; deoxycarbamoylated HbA_0, 63091-34-9; oxyHbA_0, 61912-95-6; carbon monoxide, 630-08-0; azide, 14343-69-2; hydrogen, 1333-74-0.

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Kinetics of Activation and Autoactivation of Human Factor XII†

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ABSTRACT: The kinetics of the enzymic reactions that participate in the contact activation system of human plasma were examined. These reactions are potentiated by dextran sulfate, a negatively charged solute that mimics many of the effects of glass or kaolin on this system. The reactions of reciprocal activation, consisting of activation of factor XII by kallikrein and of prekallikrein by activated factor XII, follow Michaelis-Menten kinetics; values of k_{cat} and K_m for each of these reactions were determined in the presence of dextran sulfate and in its absence. In the presence of dextran sulfate, the catalytic efficiency for factor XII activation was increased 11 000-fold, and that for prekallikrein was increased 70-fold. Autoactivation of factor XII in the presence of dextran sulfate also follows Michaelis-Menten kinetics with $k_{cat} = 0.033\ s^{-1}$ and $K_m = 7.5\ \mu M$. This finding supports the concept that autoactivation is an enzymic process, initiated by traces of activated factor XII which are invariably present in factor XII preparations. At prekallikrein and factor XII levels equal to those in plasma, reciprocal activation is ~2000-fold more rapid than autoactivation. Thus, reciprocal activation is the predominant mode of factor XII activation in normal plasma.

The contact activation system of human plasma consists principally of the three glycoproteins factor XII (Hageman factor), prekallikrein (Fletcher factor), and high molecular weight (M_r)[1] kininogen (Fitzgerald factor) [for a review, see Griffin & Cochrane (1979)]. The first two are zymogens which are converted to the serine proteinases factor XIIa[2] and kallikrein by limited proteolysis. High molecular weight kininogen is a nonenzymic cofactor which circulates as a complex with prekallikrein (Mandle et al., 1976) and with factor XI (Thompson et al., 1977). It may function by enhancing the binding of both prekallikrein and factor XI to negatively charged surfaces (Wiggins et al., 1977) whereupon their proteolytic activation by factor XIIa is facilitated. Activation of factor XI results in initiation of the intrinsic coagulation pathway, whereas the activation of prekallikrein impinges on a number of systems in plasma. Substrates of kallikrein include kininogen, plasminogen (Colman, 1969), prorenin (Derkx et al., 1979), and factor XII (Cochrane et al., 1973). The ability of kallikrein to activate factor XII, coupled with the activation of prekallikrein by factor XIIa (i.e., reciprocal activation), thus affords a positive-feedback amplification system which may be extremely efficient in effecting activation once initial "triggering" levels of either enzyme are achieved.

The mechanism for generating this triggering enzymic activity is controversial. Griffin & Cochrane (1979) have suggested that a low level of enzymic activity may be inherent in zymogenic prekallikrein and/or factor XII. Others have proposed that adsorption of factor XII to a negatively charged surface produces a conformational change that results in generation of enzymic activity in single-chain factor XII (Ratnoff & Saito, 1979) or that such adsorption produces a specific, nonenzymic cleavage to form factor XIIa (Ratnoff & Saito, 1982). It has also been suggested that initiation involves substrate-induced catalysis by single-chain factor XII (Heimark et al., 1980).

The observation that factor XIIa can cleave surface-bound factor XII to form additional factor XIIa (i.e., autoactivation) led Miller et al. (1980) and Silverberg et al. (1980a) to propose that this mechanism may be an important facet of contact activation, particularly in prekallikrein-deficient plasma. Although autoactivation does not explain the origin of the initial triggering level of factor XIIa, it nonetheless provides an alternate mechanism for factor XIIa formation once such triggering is achieved.

In the present work, we have addressed the question of the relative importance of autoactivation, as opposed to reciprocal activation, by studying the kinetics of the individual reactions involved in each mode of activation. These studies have employed dextran sulfate as a soluble, negatively charged

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[1] Abbreviations: M_r, molecular weight; S-2302, H-D-prolyl-L-phenylalanyl-L-arginyl-*p*-nitroanilide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

[2] In this work, α-factor XIIa is the M_r 80 000 form of activated factor XII, and β-factor XIIa is the M_r 28 000 form (also known as factor XII_f or Hageman factor fragment); factor XIIa (without prefix) is used to refer to either α- or β-factor XIIa, or both.