Proton Magnetic Resonance Study of the Influence of Chemical Modification, Mutation, Quaternary State, and Ligation State on Dynamic Stability of the Heme Pocket in Hemoglobin As Reflected in the Exchange of the Proximal Histidyl Ring Labile Proton†

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Received January 14, 1988; Revised Manuscript Received September 12, 1988

ABSTRACT: Proton nuclear magnetic resonance spectroscopy has been utilized to investigate the rates of exchange with deuterium of the proximal (F8) histidyl ring protons in a series of chemically modified and mutated forms of Hb A. Differences in rates of exchange are related to differences in the stability of the deformed or partially unfolded intermediates from which exchange with bulk solvent takes place. Each modified/mutated Hb exhibited kinetic subunit heterogeneity in the reduced ferrous state, with the α subunit exhibiting faster exchange than the β subunit. Modification or mutation resulted in significant increases in the His F8 ring NH exchange rates primarily for the affected subunit and only if the modification/mutation occurs at the allosterically important $\alpha^1\beta^2$ subunit interface. Moreover, this enhancement in exchange rate is observed primarily in that quaternary state of the modified/mutated Hb in which the modified/substituted residue makes the intersubunit contact. This confirms the importance of allosteric constraints in determining the dynamic properties of the heme pocket. Using modified or mutated Hbs that can switch between the alternate quaternary states within a given ligation state or ligate within a given quaternary state, we show that the major portion of the enhanced exchange rate in R-state oxy Hb relative to T-state deoxy Hb originates from the quaternary switch rather than from ligation. However, solely ligation effects are not neligible. The exchange rates of the His F8 ring labile protons increase dramatically upon oxidizing the iron to the ferric state, and both the subunit kinetic heterogeneity and the allosteric sensitivity to the quaternary state are essentially abolished. Some plausible molecular links between the site of modification and the unfolding of the F helix are discussed.

It is now abundantly clear that the complete understanding of the mechanism of hemoglobin (Hb)[1] function requires information on not only the ground-state structure but also its dynamic properties (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; McCammon & Karplus, 1983). Relevant dynamic properties range from simple amino acid side chain motions that allow facile penetration of molecular oxygen into the heme cavity (Case & Karplus, 1979) to large-scale cooperative motions involved in the allosteric transition (Gelin et al., 1983).

One productive area for investigating protein dynamics in Hb involves the characterization of the exchange behavior of buried labile protons (Hvidt & Nielsen, 1966; Englander et al., 1972; Abaturov et al., 1976, 1977; Hedlund et al., 1978; Woodward & Hilton, 1979; Woodward et al., 1982; Hallaway et al., 1984). Such protons exchange with water only because structural fluctuations transiently expose a site to bulk solvent. Hence, such proton exchange can be used to assess the flexibility of a region of a protein. Extensive $^1H/^3H$ exchange studies have revealed (Englander et al., 1980, 1983; Malin & Englander, 1980; Liem et al., 1980; Ray & Englander, 1986) that there are several subsets of labile protons in Hb A whose exchange rates are remarkably accelerated in oxy Hb as compared to deoxy Hb. These faster exchange rates in R-state oxy Hb relative to T-state deoxy Hb have been interpreted in terms of the decreased free energy for partially unfolding Hb in the latter state that is characteristic for the T → R transition (Englander et al., 1980, 1983; Malin & Englander, 1980; Liem et al., 1980; Ray & Englander, 1986). Tryptic digestion of Hb has located two of the allosterically sensitive subsets of labile proton on the F–FG helical section of the β subunit (Englander et al., 1983) and near the amino terminus of the α subunit (Ray & Englander, 1986).

We have initiated ^1H NMR studies of the exchange behavior of the proximal His F8 labile ring protons in order to probe the dynamic stability of individual heme pockets in a variety of states of Hb A (Jue et al. 1984; Han & La Mar, 1986). The advantage of following those protons is that they are located in the heme cavity on one of the residues (His F8) involved in the control of ligand affinity, and the signals of the individual subunits of deoxy Hb are both resolved and have been unambiguously assigned (La Mar et al., 1977, 1980; Takahashi et al., 1980). We have shown (Jue et al., 1984; Han & La Mar, 1986) that their exchange behavior follows the EX_2 mechanism and that the rates are allosterically sensitive and differ between the two subunits in both deoxy and oxy Hb. Both the exchange rates and extent of acceleration in oxy relative to deoxy Hb suggest that the β subunit His F8 is a member of the subset of protons, "slow I", characterized by Englander et al. (1983).

† This research was supported by grants from the National Science Foundation (CHE84-15329) and the National Institutes of Health (HL16087).

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[1] Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; T, tense; R, relaxed; DPG, 2,3-diphosphoglyceric acid; IHP, inositol hexaphosphate; NEM, *N*-ethylmaleimide; ppm, parts per million.

0006-2960/89/0428-2169$01.50/0

The exchange rate of buried labile proton in Hb can be considered to be influenced individually by ligation (Englander, 1974; Malin & Englander, 1980; Liem et al., 1980; Jue et al., 1984; Hallaway et al., 1984; Han & La Mar, 1986), quaternary state, oxidation state (Hedlund et al., 1978), modification/mutation (Liem et al., 1980; Hallaway et al., 1984; Craescu et al., 1985), and pH or binding of allosteric effectors (Hedlund et al., 1978; Liem et al., 1980; Hallaway et al., 1984; Han & La Mar, 1986), as well as by any combination of effects. The influence of tetramer dimer equilibrium would also be expected to exert an influence (Schaeffer et al., 1984; Shih et al., 1985). However, under the high protein concentration utilized for the ^{1}H NMR experiment, dissociation into dimers will be negligible. The most important solution condition for a given state of Hb is pH, with both subunits exhibiting base-catalyzed exchange by an EX_2 mechanism in both oxy Hb and deoxy Hb (Han & La Mar, 1986). The detailed mechanisms, however, differ for subunits in both oxy Hb and deoxy Hb, and between oxy Hb and deoxy Hb for the α subunit. The rate expressions operative in the pH range 6.2–8.9 have been presented for each subunit in both oxy and deoxy Hb A. The influence of allosteric effectors for a given quaternary state was found to be small for both 2,3-diphosphoglycerate, DPG, and inositol hexaphosphate (IHP) (Han & La Mar, 1986). Unraveling the other contributions to the exchange rate, however, is more difficult, since native Hb undergoes the T $\rightleftharpoons$ R allosteric transition only when accompanied by ligation.

In this report we attempt to assess the influence of these individual factors and, in particular, seek to determine if the major contribution to the enhanced exchange rates for His F8 ring proton in oxy Hb (R) relative to deoxyHb (T) is indeed the quaternary transition. This requires the measurement of exchange rates in a hemoglobin for both quaternary states *in the same ligation state*, as well as in a hemoglobin that undergoes ligation in the same quaternary state. The influence of iron oxidation state on the allosteric sensitivity of their His F8 ring proton will also be probed. Since for native Hb A the allosteric transition is inextricably linked with ligation [deoxy Hb (T) $\xrightarrow{O_2}$ oxy Hb (R)], we resort to chemically modified or mutant Hbs that have been demonstrated to undergo the T $\rightleftharpoons$ R allosteric transition within a given ligation state or ligate in a fixed quaternary state. Since a chemical modification or a mutation can affect the exchange rates of the T and R state of Hb to a different degree, we first explore how a modification/mutation influences the exchange rates within a fixed quaternary or ligation state. This will allow us to understand how accurately we can estimate the changes in the exchange rates induced by the allosteric transition within a given ligation state or by ligation within a given quaternary state. The only assumption which has to be made is that the NMR exchange mechanism in the modified/mutant Hbs is the same as that of native Hb A and that changes in exchange rates reflect changes in the dynamic stability of the heme pocket. The limited data on pH dependence of exchange rates for the modified/mutant Hbs are similar to the Hb A profile and support our assumption that the exchange mechanism has not significantly changed.

The Hbs available to us include mutations within the heme cavity of one subunit, Hb M Boston [α57 His(E7) → Tyr] (Fermi & Perutz, 1981), in which the α subunit is permanently in the ferric state, and mutation at the $\alpha^1\beta^1$ interface, Hb San Diego [β109 Val(G11) → Met] (Nute et al., 1974; Bellingham, 1976). The $\alpha^1\beta^1$ interface does not change with quaternary structure. Perturbations at allosterically important interfaces are represented by chemically modified Hbs (Kilmartin & Hewitt, 1971; Kilmartin et al. 1975) which have the terminal amino acid removed in either or both chains: des-His(β146)-Hb, des-Arg(α141)-Hb, des-His(β146)-des-Arg(α141)-Hb, NES-des-Arg(α141)-Hb [the latter derivative has the Cys(β93) reacted with *N*-ethylmaleimide (NEM) in addition to the removal of the C-terminal Arg(α141)] (Kilmartin et al., 1975), and the two $\alpha^1\beta^2$ interface mutants, Hb Kempsey [β99 Asp(G1) → Asn] (Reed et al., 1968; Bunn et al., 1974) and Hb Kansas [β102 Asn(G4) → Thr] (Bonaventura & Riggs, 1968).

Des-His(β146)-des-Arg(α141)-Hb and NES-des-Arg(α141)-Hb exist in the R structure in the deoxy state in the absence of organic phosphates at low pH but can be readily switched to the T state by the addition of IHP (Kilmartin & Hewitt, 1971; Ogawa et al., 1974; Kilmartin et al., 1975; Miura & Ho, 1982; Nagai et al., 1982). The mutant Hb Kempsey [β99 Asp(G1) → Asn] also exists in the R state when stripped and converts to the T state in the presence of IHP (Bunn et al., 1974; Nagai et al., 1982; Perutz et al., 1974; Takahashi et al., 1982). Hb Kansas [β102 Asn(G4) → Thr], on the other hand, when stripped exists in the normal T state in the deoxy form and in the R state in the oxy form, but the latter form can be switched to the T state upon addition of IHP (Ogawa et al., 1972). Another convenient comparison will be the separated Hb A β chain tetramer, deoxy β_4 (Chatterjee et al., 1982; Nagai et al., 1982). The influence of oxidation state on the allosteric sensitivity of the His F8 ring proton exchange rates can be assessed by using fluoromet Hb A, whose R structure when stripped can be converted to the T state at low pH in the presence of IHP (Fermi & Perutz, 1977).

The ^{1}H NMR spectra of the deoxy forms of these modified and mutant Hbs and separated chains have been reported, and the His F8 ring exchangeable proton has been located and assigned by their close analogy to Hb A (La Mar et al., 1977, 1980; Takahashi et al., 1980). While the ^{1}H NMR signals for the His F8 ring NHs are not resolved in either oxy or met Hb derivatives, we take advantage of both the slower exchange rates and ready detectability of the signals in the deoxy forms to monitor the exchange processes in other Hb forms by rapid conversion to the deoxy derivative, as discussed in detail previously (Han & La Mar, 1986).

Materials and Methods

Materials. Des-His(β146)-Hb, des-Arg(α141)-Hb, des-His(β146)-des-Arg(α141)-Hb, NES-des-Arg(α141)-Hb, and the isolated β chain tetramer, β_4, were prepared in the CO forms as previously reported (Nagai et al., 1982). Fluoromet Hb was obtained by adding sodium fluoride to aquomet Hb which was prepared by oxidizing oxy Hb A by sodium fericyanide according to the standard procedure (Antonini & Brunori, 1971). Hb San Diego was obtained from Dr. J. Rosa and purified by the same procedure as for Hb A. Literature methods were used for purifying Hb Kansas (Bonaventura & Riggs, 1968) and Hb Kempsey (Bunn et al., 1974).

Methods. To study the exchange of proximal histidyl ring NH protons in deoxy Hb, concentrated (25–30%) oxy Hb was placed in a NMR tube and deoxygenated by repeated evacuation as previously reported (Jue et al., 1984; Han & La Mar, 1986). Complete conversion of oxy Hb to deoxy Hb was achieved by adding 10-fold molar excess of sodium dithionite. At t = 0, deaerated ^2H_2O was added to deoxy Hb to make final ^2H_2O of 40–50% and time evolution of the NH peak intensity was immediately followed, as described in detail previously (Han & La Mar, 1986). It was shown in the

previous reports (La Mar et al., 1980; Takahashi et al., 1980) describing the assignment of individual NH resonances that the area under each NH peak corresponds to a single proton. This is supported by the fact that the height of each NH obtained in 50% H_2O/50% ^2H_2O after the exchange has reached an equilibrium is half of that obtained in 100% H_2O; the width of each NH peak remains invariant during exchange (Han & La Mar, 1986). Peak intensity was used to monitor the amount of NH unexchanged and plotted against time for each subunit (Han & La Mar, 1986). Intensities of NH peaks in T-state deoxy Hb produced in this manner were measured within 10 min (time typically required to obtain a spectrum, see below) after the introduction of sodium dithionite in order to minimize, if any, harmful effects of sodium dithionite on deoxy Hb. It was shown earlier (Han & La Mar, 1986) that, even after several hours following the addition of sodium dithionite, there was no indication of deterioration of deoxy Hb such as into met Hb. In addition, intactness of heme methyl peaks relevant to the T-state deoxy Hb also seems to suggest that the effect of sodium dithionite seems to be minimal as far as measurement of exchange rates is concerned. Experiments for the T state of des-His(β146)-des-Arg-(α141)-Hb, NES-des-Arg(α141)-Hb, and Hb Kempsey were carried out in the presence of 33 mM IHP near pH 6.5 to ensure that the Hbs stay in the T state (Nagai et al., 1982). Due to the limited amount of samples, the exchange experiments for des-His(β146)-Hb and Hb Kansas were performed in ~40% ^2H_2O, and the rates were extrapolated to those in 50% ^2H_2O by assuming no contribution of isotope effect on rates.

Exchange rates for R-state fluoromet Hb and R-state oxy Hb Kansas were measured by the difference hydrogen exchange method described for oxy Hb A (Han & La Mar, 1986): concentrated (25–30%) Hb with an appropriate buffer in a NMR tube was deoxygenated by repeated evacuation and flushed with nitrogen under gentle shaking. ^2H_2O deaerated in the same manner was then added to the NMR tube and allowed to exchange with ligated Hb in 50% ^2H_2O for a certain mixing period, t_m, before sodium dithionite was added to quickly yield T-state deoxy hb. Rates for T-state fluoromet Hb and T-state oxy Hb Kansas were measured by first adding inositol hexaphosphate (IHP) prior to adding ^2H_2O.

Exchange rates in deoxy T-state Hb Kempsey, the β subunit of deoxy T-state des-His(β146)-des-Arg(α141)-Hb, isolated deoxy β chain tetramer, β_4, R-state deoxy Hb Kempsey, and R-state deoxy des-His(β146)-des-Arg(α141)-Hb were sufficiently slow to be measured by the standard procedure for obtaining exchange rates in the deoxy form described above. The NH resonance from the α subunit of R-state deoxy Hb Kempsey is broad and ill-defined in its ^1H NMR spectrum (Nagai et al., 1982) so that it was not possible to follow the small changes in this peak area; thus we report rate data only in the β subunit. The exchange reaction in the α subunit of deoxy T-state des-His(β146)-des-Arg(α141)-Hb, deoxy NES-des-Arg(α141)-Hb, R-state oxy Hb Kansas, and deoxy des-Arg(α141)-Hb at pH 7.25 was too fast to measure by ^1H NMR under the conditions of interest, so that only lower limits to the exchange rates could be obtained by taking the advantage of the fact the $I(\alpha) = 1.0$ at $t = 0$.

The NH resonances from two subunits of R-state deoxy des-His(β146)-des-Arg(α141)-Hb are essentially degenerate, as reported previously (Nagai et al., 1982). Hence, the normalized intensity at time zero in this case represents two protons, $I_0 = 2.0$, and at equilibrium represents two half-protons, i.e., $I_\infty = 1.0$. The decay of the intensity with time therefore represents two rate processes, one for the α and the other for the β subunit. The plot of residual intensity versus time in Figure 5B exhibits a very rapid decrease for the first few points and then decays more slowly. A least-squares fit to two exponentials is shown by the solid line. The first rate can be safely attributed to the α subunit, and the slow one to the β subunit (see below). Very similar rates are obtained if single-exponential fits are made separately only to the first two data points and to the data points after I has decreased to less than 1.0. Consequently, the rate for the α subunit has a larger uncertainty than usual (~30%), as only two points and $I = 2.0$ at $t = 0$ are used to obtain the rate data.

NES-des-Arg(α141)-Hb is a valuable sample that could be prepared in sufficient purity for only a single NMR sample (~100 mg). Hence the exchange rates for both the R and T states had to be determined with this single sample. It was necessary, therefore, to allow the exchange in the R state without IHP for a short period of mixing time (35 s), during which the NH of the α subunit has reached its equilibrium and that of the β subunit has undergone ~13% exchange before IHP was added to convert the quaternary state from R to T: the rates for the R state were obtained from a single point for each subunit at $t = 35$ s. Since there are not enough points to define the exchange profile in the plot of intensity versus time (as in other typical cases) in the α subunit where the exchange is practically completed during the 35-s mixing time, only a lower limit for the exchange rate was estimated by taking the advantage of the fact that $I = 1.0$ at $t = 0$. In the β subunit, the initial slope measurement was used. The rates in the T state were obtained as follows: an apparent exchange rate for the β subunit was first calculated by a simple linear regression method with exchange time scales (t' in min) counted from the addition of IHP (insert in Figure 6B). The best fit in the insert yielded $I(\beta) = (-4.42 \times 10^{-4})t' + 0.867$. The intercept at $t' = 0$ is 0.87 since 13% exchange has already occurred for the β subunit in the R state (note that the α subunit has undergone complete exchange in this time). To define the true zero time ($t = 0$) for the exchange in the T state, a value of t' at $I(\beta) = 1.0$ was calculated by extrapolation, yielding $t' = -300$ min. Finally, true exchange rates for both subunits in the T state were obtained by setting $t' = -300$ as $t = 0$ (time scale in Figure 6B).

The pH of samples was read directly from a Beckman 3550 pH meter equipped with an Ingold microcombination electrode. The temperature of a sample was maintained either by a variable-temperature control unit of the NMR spectrometer or by a Haake KT33 constant-temperature bath. The NMR spectra were collected on a Nicolet NT200 FT spectrometer, and a typical spectra required 2000–5000 scans (<10 min), 4096 data points, and a 20-kHz spectral window. For further details, see Han and La Mar (1986).

Error Analysis. Rates are determined from the plots of absolute mode intensities versus time, either by a nonlinear, least-squares line-fitting procedure, in cases where the exchange is fast and data could be collected over several exchange half-lives, or by a simple linear regression method (initial slope measurement) when the exchange is slow and data could be collected only over a fraction of an exchange half-life (Han & La Mar, 1986). In both cases, the abscissa at $t = 0$ is used to normalize the peak intensities, which were plotted against time as shown in Figures 2–7.

Uncertainties in rates depend upon the number of data points as well as the reproducibility of each point used in the line-fitting procedure. Because of widely different sets of experimental conditions, rates, and sample availability, there

are no "typical" uncertainties associated with the rates presented in this study, and hence we classify the rates into three different classes on the basis of different ranges of uncertainties involved. In this regard, the rates of individual subunits of the same Hb may belong to different classes.

Class A. This is an ideal case where only one NMR sample of deoxy Hb is used for the experiment and many (15–40) data points are collected and used in the line-fitting procedure. Here, the uncertainties associated with obtaining each point, which originate from the variations in temperature (±0.1 °C), phase of the NH peak, and sample composition, are less than 10%, and the resulting uncertainties in rates belonging to this class are less than 15% (Han & La Mar, 1986). Rate data belonging to this class are for both subunits of deoxy des-His(β146)-Hb (Figure 2A), deoxy Hb San Diego (Figure 3A), and deoxy Hb Kansas (Figure 7A), the β subunit of deoxy des-Arg(α141)-Hb (Figure 2B), deoxy Hb Boston (Figure 3A), T-state deoxy des-(Hisβ146)-des-Arg(α141)-Hb (Figure 5A), and the β chain tetramer (data not shown).

Class B. This class encompasses the rates that are determined with overall uncertainties $\lesssim$30%. A few different subclasses exist depending on the origin of the uncertainties. (1) Only one NMR sample of deoxy Hb is used, and the reproducibility of each point as good as in class A. However, due to fast exchange only limited data points are used to define the exchange profile before equilibrium is reached. This subclass includes the rates for the α subunits of deoxy des-Arg(α141)-Hb (Figure 2B) and R-state deoxy des-His-(β146)-des-Arg(α141)-Hb (Figure 5B). (2) The sample whose exchange is being monitored must be first converted to the deoxy form by addition of dithionite (Han & La Mar, 1986). Thus several different NMR samples are used to generate the plot of intensity versus time; each point in this case corresponds to a different sample. This introduces additional uncertainties in pH (<0.05 pH unit) from one sample to another, larger variations in temperature (±1.0 °C; samples are incubated outside the NMR probe before the measurement), and small variations in sample composition (<2%). Moreover, the total number of data points used in the line-fitting procedure is also less than in class A [at most 11; see Han and La Mar (1986)]. The rates for oxy Hb A (Han & La Mar, 1986), both T- and R-stage fluoromet Hb (Figure 4), and T-state oxy Hb Kansas (Figure 7B) belong to this subclass. (3) The scatter of data points is large because of either more limited signal to noise due to severe limitations in sample availability [both T- and R-state deoxy Hb Kempsey (Figure 6A) and the β subunit of deoxy NES-des-Arg(α141)-Hb (Figure 6B)] or degeneracy of the α and β subunit signals necessitating simultaneous analysis of two rates [R-state des-His(β146)-des-Arg-(α141)-Hb (Figure 5B)].

Class C. This class includes cases where the exchange rates cannot be determined because the exchange is too fast to monitor by our method; hence, only lower limits of rates are estimated. These data include both subunits of R-state oxy Hb Kansas (Figure 7C), the α subunits of T-state deoxy des-His(β146)-des-Arg(α141)-Hb (Figure 5A), and both T- and R-state deoxy NES-des-Arg(α141)-Hb (Figure 6B).

Each rate shown in Tables I and II is labeled by the class to which it belongs. Also, in the following comparisons of rates (see Discussion), unless specifically mentioned, we consider the difference between the two rates significant only when the rates differ by more than a factor of 2, which is well outside of the uncertainty limits described above.

Results

Figure 1 displays the low-field portions of the 200-MHz ^{1}H NMR spectra of the two chemically modified deoxy Hbs, des-His(β146)-Hb (A) and des-Arg(α141)-Hb (B), as a function of time after diluting an ~15% protein solution in H_2O with an equal volume of ^2H_2O. The two peaks at 76–78 and 62–65 ppm arise from the β and α subunit, respectively, and are found with these characteristic shifts in all T-state deoxy Hbs (La Mar et al., 1977; Nagai et al., 1982; Takahashi et al., 1982). The two peaks have the same line width and height for deoxy Hb A in pure H_2O (Han & La Mar, 1986). The preferential loss of intensity of the α subunit peak at early times is apparent in each case. The plots of the normalized residual NH intensities as a function of time for individual subunits for these singly modified Hbs are displayed in Figure 2.

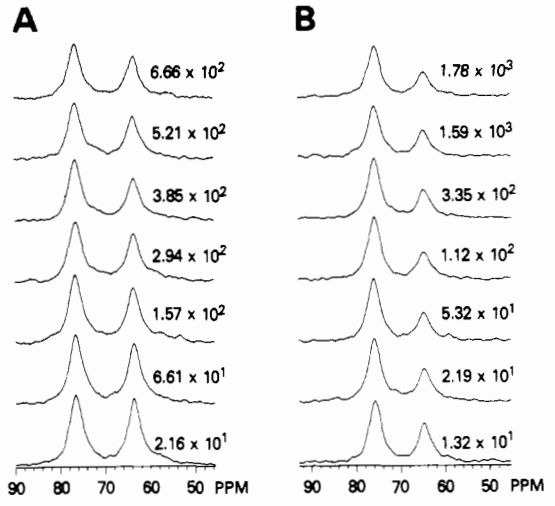

FIGURE 1: Time evolution of the ^{1}H–^{2}H exchange of the proximal histidyl ring NH resonances in the α (64 ppm) and the β (76 ppm) subunits of chemically modified Hbs in their T-state deoxy forms after mixing deoxy Hbs (~15%) in H_2O with ^2H_2O. Time scales in minutes are shown on the right for each trace. The solution condition was 0.1 M Bis-Tris and 0.2 M NaCl at 25 °C. (A) Des-His(β146)-Hb at pH 6.69 diluted to 42% ^2H_2O. (B) Des-Arg(α141)-Hb at pH 6.65 diluted to 50% ^2H_2O.

The plots of residual normalized NH intensity versus time for deoxy Hb M Boston and Hb San Diego are illustrated in Figure 3. With the α subunit permanently in the ferric state in Hb M Boston (Fermi & Perutz, 1981), only the β subunit NH peak is detected, and hence no information is available for the mutated subunit. In both Figures 2 and 3, the solid lines represent the least-squares computer fits of the data points to the equation for reversible first-order kinetics (Han & La Mar, 1986). In each case, the solid markers are due to the α subunit, and the open markers the β subunit, peak. The His F8 ring NH resonances of fluoromet Hb and oxy Hb are not detected in their ^{1}H NMR spectra. The time evolution of the F8 His ring NH signal intensity is followed in the deoxy Hbs obtained from fluoromet Hb and oxy Hb Kansas by reducing these ligated Hbs with sodium dithionite after they have undergone exchange with 50% ^2H_2O for a certain period of mixing time, t_m, as described in detail earlier for oxy Hb A (Han & La Mar, 1986). The residual intensities of NH peaks reflect the amount of protons left unexchanged at t_m, since the exchange is much slower in deoxy Hb than in ligated Hb (Jue et al., 1984; Han & La Mar, 1986).

The time plots of the normalized absolute-mode α and β subunit His F8 ring NH intensities for the R and the T state of fluoromet Hb are reproduced in Figure 4. Figure 5 presents the plots of residual NH intensity versus time for deoxy des-His(β146)-des-Arg(α141)-Hb in the presence (T state) and in the absence (R state) of IHP. The α and β subunit

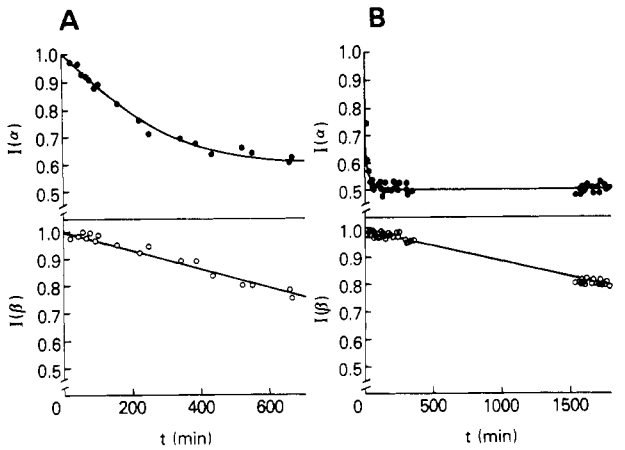

FIGURE 2: Plots of the normalized intensities of the His F8 ring NH peaks, which amount to the fractions of protons unexchanged at time, t, plotted against exchange time for chemically modified T-state deoxy Hbs. The solution condition was 0.1 M Bis-Tris and 0.2 M NaCl at 25 °C. (A) Des-His(β146)-Hb at pH 6.69 in 42% ^2H_2O. (B) Des-Arg(α141)-Hb at pH 6.65 in 50% ^2H_2O. In each trace, the closed symbol (●) represents the α subunit, and the open symbol (○), the β subunit. All points were obtained from a single deoxy Hb sample. The solid lines represent the least-squares fit to the reversible first-order reaction.

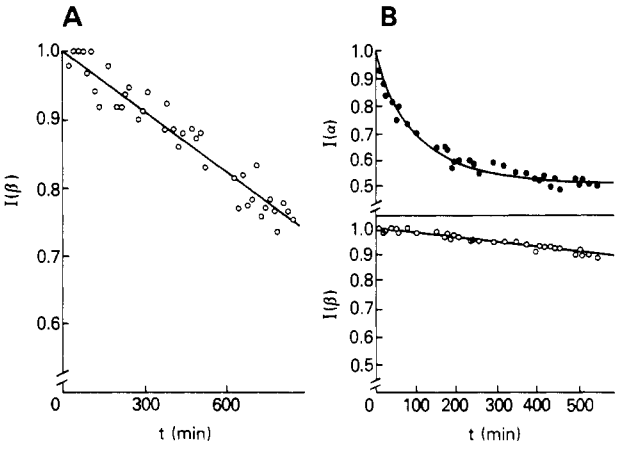

FIGURE 3: Plot of the normalized intensities of His F8 ring NH peaks, which amount to the fractions of protons unexchanged at time, t, plotted against time for two mutant T-state deoxy Hbs. The solution condition was 0.1 M Bis-Tris/Tris, 0.2 M NaCl, and 50% ^2H_2O at 25 °C. (A) Hb M Boston at pH 7.25 (shown only for the β subunit). (B) Hb San Diego at pH 6.85. In each case, the closed symbol (●) represents the α subunit, and the open symbol (○), the β subunit. All points were obtained from a single deoxy Hb sample. The solid line represents the least-squares fit to reversible first-order reaction.

His F8 ring NH peaks are degenerate in the deoxy R state of this derivative (Nagai et al., 1982), so that the intensity at time zero corresponds to two protons. The decay is analyzed on the basis of two-exponential fits (see Methods), which yields the exchange rates for both subunits. We assume the faster exchange rate is due to the α subunit on the basis that this has been the case in all other Hb samples studied to date (Jue et al., 1984; Han & La Mar, 1986; Craescu et al., 1985).

Figure 6 shows the time plots of the normalized intensities of His F8 ring NH peaks for deoxy Hb Kempsey and deoxy NES-des-Arg(α141)-Hb. In Figure 6A, circles represent T state and squares represent R state, respectively. The poorly resolved α subunit peak in the R state of Hb Kempsey (Nagai et al., 1982) prevented obtaining rate data for this subunit. The insert in Figure 6B illustrates the extrapolation scheme used to calculate the exchange rate for the subunit of T-state NES-des Arg(α141)-Hb described under Methods.

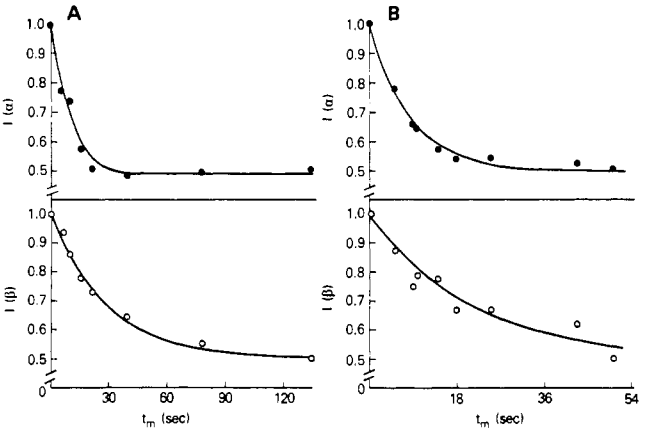

FIGURE 4: Plots of the normalized intensities of His F8 ring NH peaks monitored in the deoxy spectrum after reduction with dithionite, which corresponds to the fractions of the proximal histidyl ring NH protons unexchanged after a certain mixing period, t_m, plotted against t_m during which fluoromet Hb was allowed to exchange with 50% ^2H_2O. (A) At pH 6.19 (R state). (B) At pH 6.24 in the presence of 33 mM IHP (T state). Solution conditions were 0.1 M Bis-Tris and 0.2 M NaCl at 25 °C. Each point in the plot was obtained from a different sample. Closed (●) and open (○) symbols represent the α and the β subunit, respectively. The solid line represents the least-squares fit to the reversible first-order reaction.

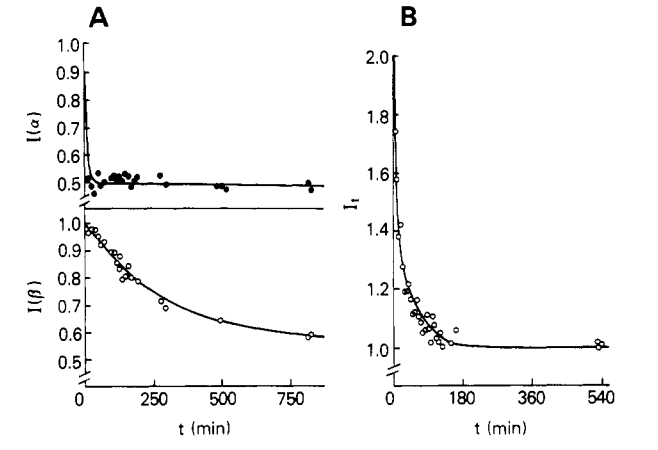

FIGURE 5: Plots of the normalized intensities of His F8 ring NH peaks, which amount to the fractions of the His F8 ring NH unexchanged at time, t, plotted against time for deoxy des-His(β146)-des-Arg-(α141)-Hb. (A) T state at pH 6.55 in the presence of 33 mM IHP. Complete exchange had occurred in the α subunit prior to recording data, so that the fit yields only the *lower limit* to the exchange rate for that subunit. The closed symbol (●) represents the α subunit, and the open symbol (○), the β subunit. (B) R state at pH 6.54. The solid line in this case represents a double-exponential fit (see Methods). The solution condition was 0.1 M Bis-Tris and 0.2 M NaCl at 25 °C. All points were obtained from a single deoxy Hb sample.

Figure 7 shows the plots of residual NH intensity versus time for deoxy Hb Kansas, T-state oxy Hb Kansas, and R-state oxy Hb Kansas. In the R state, oxy Hb Kansas exhibits such a rapid exchange rate that the NH protons were completely exchanged by the time the first experimental point was recorded. Hence the fit to the data points yields only lower limits to the exchange rates. Exchange rates for the T- and R-state Hbs are summarized in Tables I and II, respectively. In Table I, ratios of exchange rates of modified/mutant Hbs to those of deoxy Hb A are also presented. Table II lists the ratio of exchange rates in the R-state Hb to those of the T-state Hbs.

Discussion

The rate of the His F8 ring NH exchange in Hb A has been shown to proceed by the EX_2 mechanism, with some form of base catalysis dominant for both subunits and in both deoxy

Table I: His F8 Ring NH Exchange Rates of T-State Mutant/Modified Hbs and Ratio of Exchange Rates to Those of T-State Deoxy Hb A

	pH	exchange rates[a] k_α(Hb*)	exchange rates[a] k_β(Hb*)	subunit ratio, k_α(Hb*)/k_β(Hb*)	exchange rate ratio[b] k_α(Hb*)/k_α(Hb)	exchange rate ratio[b] k_β(Hb*)/k_β(Hb)
deoxy Hb*						
des-His(β146)-Hb	6.69	3.2	0.78	4.1	0.8	3.2
	7.22	10	1.6	6.3	2.0	3.2
des-Arg(α141)-Hb	6.65	56†	0.22	2.5×10^2	13	1.0
	7.25[c]	$>3.3 \times 10^2$	0.39	$>8.5 \times 10^2$	>40	0.6
NES-Hb[d]	7.20	8.9	8.9	1.0	2.0	18
des-His(β146)-des-Arg(α141)-Hb	6.55[c]	>89	2.0	>45	>40	16
NES-des-Arg(α141)-Hb	6.54[c]	>5.5	0.87†	>6.3	>1.3	7.9
Hb M Boston	7.25	[e]	0.55			1.0
Hb San Diego	6.85	6.2	0.33	19	1.2	1.4
Hb Kempsey	6.47[c]	21†	1.5†	14	5.0	20
Hb Kansas	6.32	6.9	0.28	25	1.6	3.2
Hb A[f]	6.50	4.5	0.12	39	1.0	1.0
ligated Hb*						
oxy Hb Kansas[g]	6.30[c]	98†	2.5†	40	14[g]	8.9[g]
	7.15[c]	2.0×10^2†	8.5†	23		
fluoromet Hb A	6.24[c]	7.1×10^3†	1.9×10^3†	3.7	1.6×10^3	1.6×10^4

[a] Rates × 10^5 s^{-1} at 25 °C. Rates are labeled according to what has been described under Error Analysis. Rates with no labels belong to class A (<15% uncertainties). Rates belonging to class C (only lower limits) are shown with ">" symbols. Rates with the superscript † belong to class B (<30% uncertainties). [b] Ratio of rate for individual subunit in modified/mutant Hb* to that of deoxy Hb A at the same pH (Han & La Mar, 1986). [c] Obtained in the presence of IHP. [d] Data taken from Craescu et al. (1985). [e] Mutated subunit resonance not detectable. [f] Data taken from Han and La Mar (1986). [g] Reference in this case is deoxy Hb Kansas.

Table II: His F8 Ring NH Exchange Rates of R-State Modified/Mutant Hbs and Ratios of Exchange Rates of Individual Subunits to Those of Corresponding T-State Hbs

	pH	exchange rates[a] k_α(Hb*)	exchange rates[a] k_β(Hb*)	subunit ratio, k_α(Hb*)/k_β(Hb*)	quaternary state ratio k_α[Hb*(R)]/k_α[Hb*(T)]	quaternary state ratio k_β[Hb*(R)]/k_β[Hb*(T)]
deoxy Hb*						
des-His(β146)-des-Arg(α141)-Hb	6.54	30†	1.9†	16	<3.4	9.1
NES-des-Arg(α141)-Hb	6.54	$>2.1 \times 10^2$	39†	>5.4	[c]	4.5×10^2
Hb Kempsey	6.51	[d]	1.4†			9.5
β_4[b]	6.50		0.47			39[b]
	7.38		2.8			39[b]
ligated Hb*						
oxy Hb Kansas	6.81	>50	>8.7		>3.5	>17
oxy Hb A[b,e]	6.50	28†	0.69†	40	61[b]	58[b]
fluoromet Hb A	6.19	5.0×10^2†	1.6×10^2†	3.2	0.7	0.8

[a] Rates × 10^4 s^{-1} at 25 °C. Rates are labeled according to what has been described under Error Analysis. Rates with no labels belong to class A (<15% uncertainties). Rates belonging to class C (only lower limits) are shown with ">" symbols. Rates with the superscript † belong to class B (<30% uncertainties). [b] For deoxy β_4 and oxy Hb A, reference for the T state in the ratios is deoxy Hb A. [c] Could not be determined since only lower limits to exchange rates are available in both states. [d] Peak not sufficiently well resolved to obtain data. [e] Data taken from Han and La Mar (1986).

and oxy Hb A at pH >7 (Han & La Mar, 1986). The EX_2 mechanism yields an observed exchange rate $k_{obsd} = K_{op}k_{ex}$, where k_{ex} is the intrinsic exchange rate of the iron-bound His imidazole if it were exposed to bulk solvent and reflects the pH dependence of the exchange process. K_{op} is the equilibrium constant for the formation of an intermediate, I, which allows solvent access to the site of interest through either local unfolding (Englander et al., 1980, 1983; Liem et al., 1980; Malin & Englander, 1980) or forming channels (Woodward & Hilton, 1979; Woodward et al., 1982).

We assume in the following that the same exchange mechanisms are maintained in modified Hbs as found earlier for Hb A, and hence changes in k_{obsd} reflect changes primarily in K_{op}. Thus increases in k_{obsd} must reflect increases in K_{op}, increased stability of I, or decreased dynamic stability of the proximal heme pocket (Englander et al., 1980, 1983; Liem et al., 1980; Malin & Englander, 1980). Limited pH studies of exchange rates in modified T-state deoxy Hb support an unaltered exchange mechanism for des-His(β146)-Hb and des-Arg(α141)-Hb, T-state oxy Hb Kansas, and R-state deoxy β_4, and rate data at two pH values in each case reflect base catalysis in a manner very similar to that reported for Hb A (Tables I and II). Thus changes in observed rates can be qualitatively interpreted in terms of changes in the dynamic stability of the proximal heme pocket.

The data in Tables I and II reveal that the proton exchange is faster in the α than β subunit for every ferrous Hb derivative. It is interesting to note that this ratio of exchange rates at pH 6.5 is strongly reduced in the ferric Hb derivative, with $k_\alpha/k_\beta \sim 3$–4. Thus the strong kinetic subunit heterogeneity for Hb in the reduced state is essentially abolished in the oxidized state. It is obvious from the data in Tables I and II that the exchange rates are influenced by all factors, and hence we consider them in sequence.

Influence of Modification/Mutation in Fixed Quaternary State. Interpretation of the influence of the modification/mutation first requires a brief discussion of the major quaternary interactions that define the two affinity states of Hb. Of the four types of interfaces (Perutz, 1976; Baldwin & Chothia, 1979; Fermi & Perutz, 1981; Fermi et al., 1984), the $\alpha^1\beta^1$ has the most extensive network of contacts. This interface, however, does not experience significant changes in the T → R transition (Baldwin & Chothia, 1979). The two β subunits do not make direct contact in either quaternary state except for the mutual contact with the inorganic phosphate that binds in the space between the two β subunits in the T state (Arnone, 1972; Arnone & Perutz, 1974; Kilmartin, 1976). The two α subunits make weak contact with each other

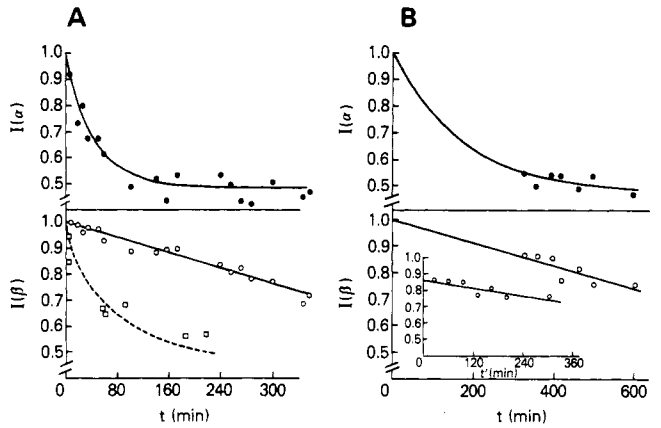

FIGURE 6: Plots of the normalized intensities of the His F8 ring NH peaks, which amount to the fractions of the NH protons unexchanged at time, t, plotted against time. The solution conditions were 0.1 Bis-Tris, 0.2 NaCl, and 50% ^2H_2O at 25 °C. (A) Circles represent T-state Hb Kempsey at pH 6.47 in the presence of 33 mM IHP. Squares, shown only for the β subunit, represent R-state Hb Kempsey at pH 6.51. All points were obtained from a single sample. The solid line represents the least-squares fit to the reversible first-order reaction. (B) Deoxy NES-des-Arg(α141)-Hb at pH 6.54. The insert at the bottom of Figure 6B displays the extrapolation scheme for calculation of an apparent rate in the β subunit of the T-state deoxy NES-des-Arg(α141)-Hb and time scale correction used to calculate the true exchange rates in the T state. The best fit for the points in the insert yielded $I(\beta) = (-4.42 \times 10^{-4})t' + 0.867$, where t' is the time scale counted from the addition of IHP to convert the quaternary state from R to T. The intercept at $t' = 0$ is 0.867 since 13% of the exchange has already occurred in the R state for the NH of the β subunit. To define the true zero time ($t = 0$) for the exchange in the T state, a value of t' at $I(\beta) = 1.0$ was calculated by extrapolation, which gave $t' = -300$ min at $I(\beta) = 1.0$. Finally, true exchange rates in both subunits of the T-state deoxy NES-des-Arg(α141)-Hb were calculated with time scales obtained by setting $t' = -300$ min as $t = 0$ (time scale in Figure 6B). For further details, see Methods.

in both states but to a different degree (Arnone & Perutz, 1974; Fermi & Perutz, 1981). It is the $\alpha^1\beta^2$ interface that experiences the most significant structural changes during the allosteric transition (Ogawa & Shulman, 1972; Ho et al., 1973; Baldwin & Chothia, 1979; Fermi & Perutz, 1981; Gelin et al., 1983).

There are several important contacts at the allosterically important $\alpha^1\beta^2$ interface which stabilize the T quaternary structure. Two such contacts are at the interface edges and involve the terminal residues: the C-terminal Arg(HC3) and the C-terminal His(HC3). More centrally located in the $\alpha^1\beta^2$ interface and closer to the heme pocket is the hydrogen bond between $\beta_2$99 Asp(G1) and the opposing α subunit residue, $\alpha_1$42 Tyr(C7). All of the above bonds are ruptured upon converting Hb from the T to the R state. The $\alpha^1\beta^2$ interface has fewer contacts unique to the R state, of which the most important is the hydrogen bond between $\beta_2$102 Asn(G4) and $\alpha_1$94 Asp(G1) of the opposing α subunit (Perutz, 1970; Bellingham, 1976).

(A) Perturbations at Allosterically Nonimportant Sites. Hb M Boston has its α subunit heme permanently oxidized (Fermi & Perutz, 1981). The data in Table I reveal that the unperturbed β subunit exhibits His F8 ring NH exchange in the deoxy T state unaltered from that in deoxy Hb A. The α subunit cannot be monitored by the NMR method. This major perturbation at the heme of one subunit does not detectably affect the exchange rate of the other subunit.

Hb San Diego has the mutation [β109 Val(G11) → Met] in the β subunit at the $\alpha^1\beta^1$ subunit interface (Nute et al., 1974; Bellingham, 1976; Pettigrew et al., 1982). The rate data indicate a negligible change in the unperturbed α subunit (ratio 1.2, which places the two class A rates within experimental uncertainty). The β subunit rate is enhanced in Hb San Diego relative to Hb A by a factor of 1.4 which just exceeds the experimental uncertainty for two class A rates (30%). Important to note is the fact that the effect is very small, consistent with the lack of importance of the $\alpha^1\beta^1$ interface in influencing the stabilities of the alternate quaternary states.

(B) Perturbations of Allosterically Important Sites. When β146 His is specifically removed by enzymatic digestion, one of the three important $\alpha^1\beta^2$ interface T-state links is abolished. We observe for T-state deoxy des-His(β146)-Hb a factor of ~3.2 increase over deoxy Hb A in the exchange rate for the modified β subunit and a much smaller increase for the α subunit. Similarly, if the α subunit terminal arginine is removed as in deoxy des-Arg(α141)-Hb, the modified α subunit exchange rate increases dramatically over that in deoxy Hb A (factor of ~13–40), while the β subunit rate is essentially not affected. Another example of a singly modified Hb is T-state deoxy NES-Hb, whose exchange rates at pH 7.2 have been reported by Craescu et al. (1985) and are included in Table I. The unmodified α subunit exchange rate is again increased only slightly (factor ~2), while the modified β subunit experiences a factor of ~18 increase in the NH exchange rate as compared to deoxy Hb A. Thus we conclude that modifications of quaternary contacts involved in the al-

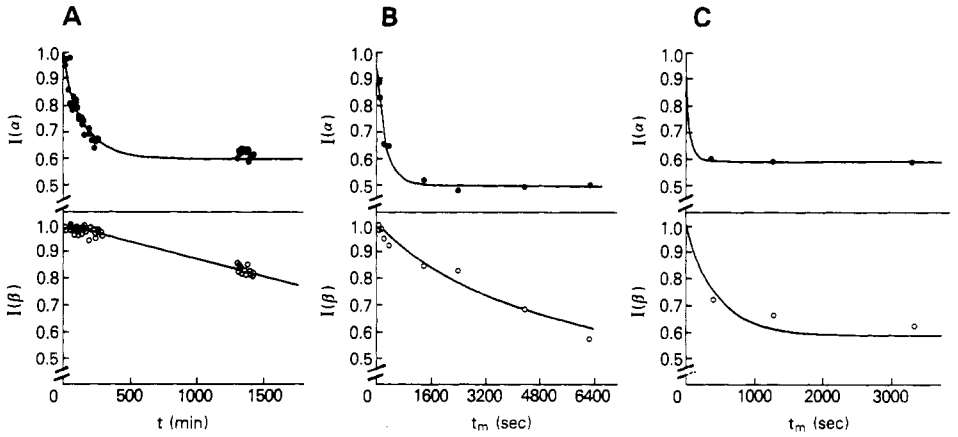

FIGURE 7: Plots of the normalized intensities of His F8 ring NH peaks, which amount to the fractions of protons unexchanged after a certain mixing period, t_m, plotted against time for Hb Kansas. (A) T-state deoxy Hb Kansas at pH 6.32 with 40% ^2H_2O; all points were obtained from a single sample. The solid line represent the least-squares fit to the reversible first-order reaction. (B) T-state oxy Hb Kansas in the presence of 33 mM IHP at pH 7.15 with 50% ^2H_2O; each point was obtained from a different sample. (C) R-state oxy Hb Kansas in the absence of IHP at pH 6.81 with 40% ^2H_2O; each point was obtained from a different sample. The solution conditions were 0.1 M Bis-Tris/Tris and 0.2 M NaCl at 25 °C. The closed symbol (●) represents the α subunit, and the open symbol (○), the β subunit.

losteric transition do indeed strongly influence the dynamic stability of the heme pocket in the T quaternary state of deoxy Hb, but primarily for the subunit possessing the modification.

When two modifications are incorporated into a single T-state deoxy Hb tetramer, significant exchange rate increases over deoxy Hb A are observed for both subunits. The considerably reduced dynamic stabilities of the heme pockets of the doubly modified relative to both the singly modified and native deoxy Hbs parallels the reduced thermodynamic stability of their deoxy T versus R quaternary structures (Kilmartin et al., 1975; Nagai et al., 1982). Thus both des-His-(β146)-des-Arg(α141)-Hb and NES-des-Arg(α141)-Hb exist in the R state even at acidic pH, with their T states becoming the dominant form only in the presence of organic phosphates.

Hbs Kempsey and Kansas represent two contrasting mutants at the central section of the $\alpha^1\beta^2$ subunit interface. In Hb Kempsey, the substitution $\beta_2$99 Asp(G1) → Asn abolishes the T-state hydrogen bond to $\alpha_1$42 Tyr(C7), making the protein unstable and raising the oxygen affinity (Reed et al., 1968; Bunn et al., 1974; Fermi & Perutz, 1981). The exchange rates for T-state deoxy Hb Kempsey in Table I indicate that both subunits are affected, with the rates relative to deoxy Hb A increasing by factors of ~5 and 20 for the α and β subunits, respectively. Again, the mutated subunit exhibits the larger change, although for this protein the exchange rate of the unmodified subunit is also increased significantly. This more symmetric influence on the two subunits may be the result of the greater importance of this $\alpha^1\beta^2$ interface contact.

In contrast, the mutation at the center of the $\alpha^1\beta^2$ subunit interface in T-state deoxy Hb Kansas results in much smaller increases in exchange rates (factors of 1.6 and 3.2 for the α and β subunits, respectively), compared to deoxy Hb A, than observed for deoxy Hb Kempsey. Although the difference between the rates in the α subunits of Hb Kansas and Hb A is less than a factor of 2, it is considered significant because the rates for deoxy Hb Kansas and Hb A belong to class A (<15% uncertainties; see Error Analysis for details). The smaller influence of this mutation on the dynamic stability of the heme cavities in deoxy Hb Kansas (T state) is most likely due to the fact that the substituted group ($\beta_2$102 Asn → Thr) does not participate in a quaternary link in the T state (Fermi & Perutz, 1981). The data in Tables I and II, however, clearly show that the exchange rates of the mutated β chains are increased much more compared to Hb A in the R-state oxy (factor of ~10) than in the T-state deoxy (factor of ~3) forms. This is consistent with the loss of a $\alpha^1\beta^2$ intersubunit hydrogen bond solely in the R state of Hb Kansas. This indicates that removal of a quaternary constraint which stabilizes one quaternary state significantly enhances the exchange rate for that particular state more than those of the other quaternary state. This supports a strong quaternary control of the dynamic stability at the heme pocket of Hb.

(*C*) *Control of Heme Pocket Dynamics.* Ultimately any increase in exchange rate for the His F8 ring NH in a modified/mutated Hb must arise from a destabilization of the tertiary structure of the subunit(s), since the subunit must partially unfold or form channels for allowing the exchange catalyst access to the subunit interior in the intermediate, I. Since the nature of this intermediate is not understood, the detailed basis for altering its stability is obscure at this time. However, the available structural data on the alternate quaternary states of Hb A (Shannan, 1983; Fermi et al., 1984), together with the strain-minimization treatment of cooperativity (Gelin et al., 1983), suggest a possible mechanism for the strong link between the dynamic stabilities of the subunit C-termini and the proximal heme pockets within a given subunit.

Such a connection between the C-terminus of each subunit and the heme cavity exists via hydrogen bonds between the side chain hydroxyl of the residues next to the deleted HC3 residues (the penultimate tyrosines HC2) and the carbonyl group of Val FG5, which is in contact with the proximal His F8. A possible role for these penultimate tyrosines in Hb allostery had been originally proposed by Perutz (1970), although the recent high-resolution X-ray structure of oxy Hb (Shannan, 1983) indicates that they are not expelled from the "pockets" between the F and H helices in the ligated R structure. The rupture of the intersubunit hydrogen bonds involving the C-terminal residues (α141 Arg, β146 His) would increase the motional freedom of the neighboring tyrosines HC2, which in turn would disrupt the hydrogen bond to Val FG5, and thus impart increased flexibility to the proximal side of the heme pocket. Those same series of residues have been implicated in the current molecular model of cooperativity as described by strain-minimization calculations (Gelin et al., 1983). This link could rationalize the observed dramatic influence of the modification in des-Arg(α141)-Hb and des-His(β146)-Hb on the dynamic stability of solely the perturbed subunits. More extensive data on the influence of various mutations on the His F8 ring NH exchange may shed additional light on the nature of the intermediate forms from which exchange takes place.

(*D*) *Correlation with O_2 Affinity.* Modified Hbs are known to have higher oxygen affinities than Hb A, impaired cooperativity, and reduced Bohr effect (Kilmartin & Hewitt, 1971; Kilmartin et al., 1975; Perutz, 1976; Pettigrew et al., 1982). At similar experimental conditions, log P_{50} values in mmHg of the T-state modified Hbs increase in the following order: des-His(β146)-des-Arg(α141)-Hb (0.82) < NES-des-Arg-(α141)-Hb (1.0) < des-Arg(α141)-Hb (1.1) < des-His-(β146)-Hb (1.3–2.2) < NES-Hb (3.2). Heme pocket dynamic stability obtained by the current study for these modified T-state Hbs follows a similar order: des-His(β146)-des-Arg(α141)-Hb < NES-des-Arg(α141)-Hb < des-Arg-(α141)-Hb < NES-Hb < des-His(β146)-Hb. Another qualitative correlation between His F8 ring NH exchange rates and oxygen affinity has also been noted by Craescu et al. (1985). However, the limited available data for this qualitative correlation make it premature to speculate on a molecular structural basis for the relationship.

Influence of Quaternary Transition/Ligation. (*A*) *Quaternary Switch for Fixed Ligation State.* The ratios of exchange rates for individual subunits in the R state to those in the T state for various Hb derivatives are given in Table II. The previously reported ratios of rates for R-state oxy Hb A to those of T-state deoxy Hb A (Han & La Mar, 1986) are included for comparison. The first three entries represent comparisons within the deoxy Hb state in the alternate quaternary states, as switched solely by IHP binding. It is apparent that in each case the exchange rates in both α and β subunits, where determinable, are significantly greater in the R than the T quaternary state. However, it is also obvious that the T → R quaternary switch affects exchange differentially among the various available deoxy Hb examples, and hence there is no simple factoring of the influence of quaternary switch and ligation on the enhanced exchange rate of oxy Hb relative to that of deoxy Hb A. An important fact is that, for the first three Hbs listed in Table II, the quaternary constraints stabilizing the T state are removed, and hence the exchange rates of these Hbs would be enhanced more in the

T than in the R state (vide supra). *Therefore, k[Hb*(R)]/[Hb*(T)] values for these Hbs would necessarily underestimate the contribution of the pure quaternary transition.* Thus both the effect of modification/mutation within a given quaternary state and the effect of the quaternary transition in fixed ligation state strongly support dominant quaternary control of the dynamic stability of the F helix in Hbs.

The single example of the R → T switch solely within ferrous ligated Hb is for oxy Hb Kansas, as modulated by IHP, which yields a similarly significant increase in exchange rate in the R versus T state of Hb (Table II). One other potentially relevant comparison involves the exchange rates of the deoxy β chain tetramer which is in the R state (Chatterjee et al., 1982; Nagai et al., 1982), to that of the β subunit in T-state deoxy Hb A; a ratio is observed which is very similar to that previously reported when oxy Hb (R) is compared to deoxy Hb (T) (Han & La Mar, 1986). Thus the quaternary transition appears to enhance the exchange rate significantly for both subunits in either the unligated or ligated state of reduced Hb (see below).

The last entry in Table II, however, points out the importance of the oxidation state of the iron in determining the sensitivity of the exchange rates to the allosteric transitions. Within experimental uncertainty, *the His F8 ring NH exchange rates are independent of quaternary state in ferric Hb*, and hence the allosteric sensitivity characteristic of ferrous Hb is abolished upon oxidation (see below).

(B) Ligation within Fixed Quaternary State. Hb Kansas provides an example which demonstrates that ligation effects are not negligible. The data on T-state oxy Hb Kansas, compared with the data on T-state deoxy Hb Kansas (Table I), yield $k_\alpha(\text{oxy})/k_\alpha(\text{deoxy}) \sim 14$ and $k_\beta(\text{oxy})/k_\beta(\text{deoxy}) \sim 9$. Thus ligation alone can significantly increase His F8 ring NH exchange rate within the fixed T-state quaternary structure. In this case, the mutation involves a quaternary contact solely in the R-state $\alpha^1\beta^2$ interface and hence should not differentiate significantly between ligated and unligated T-state Hb Kansas. Nevertheless, since ligation does enhance rates, it is likely both factors contribute to the enhancement in rates in oxy Hb A (R) relative to deoxy Hb A (T) (Han & La Mar, 1986). However, since the effect of the quaternary transition is capable of causing an effect larger than observed in the relevant deoxy Hb A (T) → oxy Hb A (R) transition, we conclude that the quaternary structural transition is the major factor in the enhancement in rates observed for the latter reaction.

It remains to be determined if the larger subsets of labile proton previously demonstrated to exhibit significant enhancement due to the deoxy Hb A (T) → oxy Hb A (R) transition (Englander et al., 1980, 1983; Malin & Englander, 1980; Liem et al., 1980; Ray & Englander, 1986) exhibit any influence of solely ligation in a fixed quaternary state.

Influence of Oxidation State. More dramatic than the decrease in the differences in α and β subunit exchange rates upon oxidation of Hb A is the very sizable increase in exchange rate in both subunits. When comparing fluoromet Hb in the T state to deoxy Hb, we find the rates faster in the former form by $\sim 10^3$ in the α and $\sim 10^4$ in the β subunit. A similar comparison for R-state fluoromet Hb to oxy Hb at the same pH yields faster rates for the met derivative by factors of 20–400. It is thus apparent that the kinetic lability at the heme pocket is significantly enhanced in ferric versus ferrous Hbs even within the same quaternary structure. Similar large enhancements of exchange rates of other unidentified labile protons in oxidized versus reduced Hb have been reported by other methods which monitored bulk labile proton distribution (Hedlund et al., 1978). Also noteworthy is the present observation that the allosteric sensitivity of the His F8 ring NH exchange rates, observed in every ferrous Hb derivative, is abolished in the fluoromet Hb, as shown by the data in Table II (see above).

Acknowledgments

We are grateful to H. F. Bunn, D. L. Rousseau, and J. Rosa for gifts of the mutant Hbs Kempsey, Kansas, and San Diego, respectively.

Registry No. Hb M Boston, 39340-61-9; Hb San Diego, 51609-42-8; Hb Kempsey, 37248-11-6; Hb Kansas, 9034-98-4; Hb A, 9034-51-9; oxy Hb Kansas, 39320-94-0; fluoromet Hb A, 54577-85-4; O_2, 7782-44-7; Fe, 7439-89-6; heme, 14875-96-8.

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A Proton Nuclear Magnetic Resonance Study of the Antihypertensive and Antiviral Protein BDS-I from the Sea Anemone *Anemonia sulcata*: Sequential and Stereospecific Resonance Assignment and Secondary Structure†

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Received June 23, 1988; Revised Manuscript Received September 22, 1988

ABSTRACT: The sequential resonance assignment of the ^{1}H NMR spectrum of the antihypertensive and antiviral protein BDS-I from the sea anemone *Anemonia sulcata* is presented. This is carried out with two-dimensional NMR techniques to identify through-bond and through-space (<5 Å) connectivities. Added spectral complexity arises from the fact that the sample is an approximately 1:1 mixture of two BDS-I isoproteins, (Leu-18)-BDS-I and (Phe-18)-BDS-I. Complete assignments, however, are obtained, largely due to the increased resolution and sensitivity afforded at 600 MHz. In addition, the stereospecific assignment of a large number of β-methylene protons is achieved from an analysis of the pattern of $^3J_{\alpha\beta}$ coupling constants and the relative magnitudes of intraresidue NOEs involving the NH, C$^\alpha$H, and C$^\beta$H protons. Regular secondary structure elements are deduced from a qualitative interpretation of the nuclear Overhauser enhancement, $^3J_{HN\alpha}$ coupling constant, and amide NH exchange data. A triple-stranded antiparallel β-sheet is found to be related to that found in partially homologous sea anemone polypeptide toxins.

Sea anemones contain a number of small pharmacologically active polypeptides of molecular weight around 5000. Some of these, such as ATX-I and ATX-II from *Anemonia sulcata*, AP-A from *Anthopleura xanthogrammica*, and RP-II from *Radianthus paumotensis*, are cardiotoxic and neurotoxic. They act by binding specifically to the Na^+ channel, thereby delaying its inactivation during signal transduction. Recently, a small polypeptide known as BDS-I has been isolated from

† This work was supported by the Intramural AIDS Targeted Antiviral Program of the Office of the Director, NIH (G.M.C. and A.M.G.).
‡ National Institutes of Health.
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0006-2960/89/0428-2178$01.50/0