We have investigated the rates of monomer \( \rightleftharpoons \) tetramer self-association of oxygenated \( \beta^s \) subunits of human hemoglobin A as well as the influence of self-association on the binding kinetics for \( O_2 \) and CO. A \( 4\beta \rightleftharpoons 2\beta_2 \rightleftharpoons \beta_4 \) assembly pathway can be used to describe the association equilibria and kinetics. We have determined all four elementary rate constants for this assembly pathway at 15 °C in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM Na$_2$EDTA, pH 7.4. These data imply that a significant amount (\( \approx 17\% \)) of \( \beta_4 \) can be present.

Laser photolysis kinetic studies of \( O_2 \) binding indicate that the \( \beta_4 \) association rate constant is unaffected by the degree of self-association. In contrast, photolysis of \( \beta^s \)CO solutions shows an overall rate of \( O_2 \) binding that increases at higher protein concentrations. These data are consistent with a concentration-dependent equilibrium between two protein species with CO association rates differing by a factor of 2.5, but they do not appear to be compatible with a direct assignment of different CO binding rates to the different assembly states. Rather, we believe the data imply that \( O_2 \) binding to \( \beta \) oligomers is heterogeneous, with both a fast binding and a slow binding form being present in single association states. The fast binding form predominates (\( \approx 87\% \)) in \( \beta_4 \), while the \( \beta_4 \) monomer has very little or none of the fast binding form. We propose that the slow binding component within \( \beta_4 \) may be those subunits with rotationally disordered hemes (La Mar, G. N., Yamamoto, Y., Jue, T., Smith, K. M., and Pandey, R. K. (1985) Biochemistry 24, 5826–5831)

The implications of these findings for the use of isolated subunits as models for the subunits within “R state” hemoglobin tetramers are discussed.

The essence of cooperativity in hemoglobin is the variation in \( O_2 \) affinity through ligand-linked changes in intersubunit contacts. Ackers and co-workers have shown that studies of the coupling between oxygenation and subunit assembly can provide valuable information about the energetics of cooperativity in both normal and mutant hemoglobins (see, for example, Chu et al., 1984 and references therein). Among the interesting phenomenon revealed by their studies is “quaternary enhancement,” i.e., an increase in oxygen affinity upon subunit association as opposed to the more familiar “quaternary restraint,” e.g., the lowering of affinity when deoxy subunits associate to form deoxyhemoglobin A. The quaternary enhancement phenomenon was first found in isolated hemoglobin \( \beta \) subunits when it was observed that the oxygenated subunits self-associate to tetramers more strongly than do the deoxy subunits (Valdes and Ackers, 1978a, 1978b). This data implies that the \( O_2 \) affinity of \( \beta_4 \) is about 4 times greater than that of \( \beta \) monomers, and the overall linkage between oxygenation and subunit association for \( \beta \) subunits is about half that in Hb A, but of opposite sign. Later hemoglobin A itself was also found to exhibit quaternary enhancement; the affinity of triply liganded tetramers was reported to be significantly higher than the mean affinity of isolated \( \alpha \) and \( \beta \) subunits (Mills and Ackers, 1973; Chu et al., 1984), although recently this data has been questioned (Gibson and Edelstein, 1987).

This \( O_2 \) affinity difference between \( \beta \) and \( \beta_4 \) raises a number of interesting questions. First, given that there have been so many studies of Hb subunits, often aimed at using them to model the properties of the subunits in the oxy “R state” hemoglobin A tetramer, it is somewhat surprising that this difference went unnoticed for so long. More importantly, it is now unclear which form (if either) should be used as a model for an R state \( \beta \) subunit. Second, while it is now clear that the \( \beta_4 \) subunit changes ligand binding properties upon assembly of a tetramer, it remains unclear whether there is any heme-heme interaction within a \( \beta \) tetramer. That is, the oxygen binding equilibria data are not sufficiently accurate to detect whether there are small (2–4-fold) differences in affinity between intermediate ligation states in \( \beta \). In an earlier study of the absorption spectra of oxy-Hb and its subunits, we discovered a tetramer-monomer difference spectrum for both oxy and carbonmonoxy \( \beta \) subunits. The oxy \( \beta \) tetramer-monomer difference spectrum has features unlike other difference spectra for oxyhemoglobins (Philo et al., 1981). These spectral studies suggested (but certainly did not prove) that the \( \beta_4 \) subunits in the HbO$_2$ tetramer have properties closer to the \( \beta_4 \) monomer than to the \( \beta \) tetramer. Moreover, the unique nature of the oxy \( \beta \) tetramer-monomer difference spectrum suggested that the mechanism leading to a change in the affinity of \( \beta \) tetramers may differ from that producing the low affinity of deoxy “T state” Hb.

The association-dissociation kinetics of \( \beta \) subunits also may be relevant to the assembly of Hb A tetramers in vivo. Several investigations have shown that the rate-limiting step for in vitro assembly of Hb from \( \alpha \) and \( \beta \) subunits is the dissociation of \( \beta \) tetramers to monomers (Antounni et al., 1966; McGovern et al., 1976). It also appears that in some cases the proportion of mutant and variant hemoglobin tetramers in the red cell is governed by their assembly kinetics rather than by the rate of synthesis of the variant subunits (Shaeffer, 1980).

Therefore, we decided to extend the earlier equilibrium studies and to explore the kinetic aspects of the \( \beta \) tetramer-monomer differences. In the present work we have measured the effect of the \( \beta \) subunit association equilibrium on the
kinetics of CO and O₂ binding. Further, we have used the tetramer-monomer difference spectrum as a marker to study the kinetics of the association reactions.

MATERIALS AND METHODS

The β^H subunits of Hb were prepared as described previously (Philo et al., 1981), stored under liquid nitrogen, and dialyzed against the "Acker's buffer" (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4) prior to use. The kinetics of the oxy β association-dissociation reactions were measured using a temperature jump instrument producing 5 °C jumps in less than 10 μs and by rapid dilutions with a Durrum stopped-flow instrument. The temperature jump instrument and data acquisition and analysis procedures have been described previously (Pennathur-Das and Schuster, 1982). The dissociation reaction was measured by hand mixing a small portion of concentrated material into a cuvette, using the optics of the temperature jump instrument. As an aid to interpretation of the association kinetics, a small perturbation relaxation computer simulation of a monomer-dimer-tetramer reaction scheme was performed. The program was derived from that by Ilgenfritz (1977).

Kinetic data for CO and oxygen binding were obtained by laser photolysis. The optical monitoring was done using the optics and detection system of the temperature jump instrument. A 3-cm photolysis pulse at about 590 nm from a Candela SLL625 flashlamp-pumped dye laser using rhodamine 6G was brought into a sealed cuvette at right angles to the monitoring beam. For each sample, data were recorded for photolysis levels of ±10, ±30, and 100%. For all the kinetic data the changes in optical transmission were digitally recorded. During subsequent analysis the data were converted to changes in absorbance and fit to sums of exponentials by a nonlinear least squares routine. For the CO binding kinetics typically 3000 data points were included in the least squares analysis in order to resolve multiple exponentials. For these CO binding kinetic studies, it was necessary to rigorously exclude oxygen, and two different methods were used. In some samples an enzyme system consisting of 0.1 unit/ml glucose oxidase (Behring Diagnostics 346558), 1 unit/ml catalase (Behring Diagnostics 219261), and 0.3% glucose was added. At the lowest β subunit concentrations this system caused some interference, and a trace of sodium dithionite (Virginia Smelting) was used instead (at higher β concentrations the enzyme system or dithionite gave essentially identical results). The concentration-dependent changes in the visible absorption spectrum of βCO were measured using methods described previously (Philo et al., 1981), except that a Varan 2200 spectrophotometer was used. Protein concentrations were determined relative to the standard extinction for the cyanomet form at 540 nm of 11 mM -1 cm⁻¹ (Tenfori and Salvati, 1981). Oxygen and CO concentrations were based on Henry's law coefficients of 1.77 and 1.32 μM/mm Hg, respectively.

RESULTS

Association-Dissociation Kinetics of Oxy β Subunits—In principle, there are two distinct experimental approaches to study the self-association kinetics of β subunits. Given the coupling between association and ligand binding, it is possible to use ligand binding as the indicator for association. An example of this is shown in Fig. 1, a temperature jump experiment on a partially O₂-saturated β sample. A qualitative interpretation of the two reaction phases is that the faster phase is due to oxygen release (driven by the large negative enthalpy for O₂ binding), while the slower phase is due to association to tetramers (driven by the large positive enthalpy for association), which in turn raises the affinity and leads to a net binding of oxygen. (This slower phase might have been seen in earlier temperature jump studies of β subunits, but at lower protein concentrations it would probably be so slow that it would be obscured by cooling within the temperature jump cell (Brunori and Schuster, 1969; Nakamura et al., 1974).) However, to quantitatively treat the coupled system one must consider both the association and ligand binding kinetics to oxy, deoxy, and partially ligated monomers and tetramers (and dimers also, if present). Even with simplifying assumptions, such a system is too complex to extract elementary rate constants.

The second approach is to work only with fully deoxy or oxygenated samples and to rely on spectroscopic differences to monitor the association reaction. In our earlier study we found small association-dependent changes in absorption spectra of both oxy- and CO-β subunits but were unable to identify a spectral change for deoxy β (Philö et al., 1981). Therefore, in this work we have concentrated on measuring the assembly kinetics of oxy β using the peaks in the difference spectrum near 408, 422, or 582 nm to follow the reaction. We have chosen the buffer system used by Valdes and Ackers (1978a, 1978b) in their equilibrium gel permeation association-dissociation studies so that we may make direct use of, and comparisons with, their data. Since we have found oxy β subunits to be significantly more stable below room temperature, these experiments were done at 15 °C (the final temperature for temperature jump experiments).

In treating the equilibrium association of β subunits, it was found that a simple monomer-tetramer stoichiometry was adequate to describe the data for oxy, CO, and deoxy forms but that for cyanomet-β a substantial amount of dimer is also present (Valdes and Ackers, 1977, 1978b). From a kinetic point of view, a direct monomer to tetramer association would be extremely slow, since four-body collisions are rare. The most reasonable way to treat the self-association kinetics is to formulate a monomer-dimer-tetramer system.

$4\text{O}_2 + 2(\beta\text{O}_2) \rightleftharpoons 2(\beta\text{O}_2) + 2(\beta\text{O}_2)_2$

With corresponding equilibrium association constants

$K_1 = \frac{k_1}{k_{-1}}$, $K_2 = \frac{k_2}{k_{-2}}$, $K_4 = (K_5)^2 \times K_2$

where $K_i$ is the overall monomer → tetramer association constant.

We have measured the self-association kinetics over a range of concentration from about 1 to 100 μM (heme); i.e. from...
where the sample is predominantly monomer to where it is predominantly tetramer. For these studies we have used a combination of temperature jump and stopped-flow techniques. The former is generally preferable, but at the lower concentrations the reaction becomes too slow for conventional temperature jump because of cooling within the cell. Therefore, at the lower concentrations we used a stopped flow to mix samples at two different concentrations to perturb the assembly equilibrium. These experiments are rather difficult because the total change in absorbance is typically only 0.1%.

In all these experiments what we observe in the time range from 100 µs to 20 s is a single exponential relaxation process.\(^1\) The exponential decay time for this process drops from about 7 s at the lowest concentrations to 0.5 s at the highest. These rate data are summarized in Fig. 2. We also did large perturbation experiments where a high concentration stock was diluted 100-fold by hand mixing, giving essentially a complete dissociation from tetramers to monomers. Again a single exponential phase is observed with a rate of 0.1 ± 0.01 s\(^{-1}\). In these latter experiments the observed rate will be essentially determined by \(k_{-1}\) or \(k_{2}\), whichever is slower.

In principle, one would expect to see two exponential relaxations for a monomer-dimer-tetramer system. In a small perturbation analysis, if the monomer-dimer and dimer-tetramer reactions were entirely uncoupled they would give relaxation rates:

\[
\tau_1^{-1} = 4k([\beta O_2]_0 + k_{-1}
\]

\[
\tau_2^{-1} = 4k([\beta O_2]_0 + k_{2}
\]

For the coupled reactions it is expected that there would always be two relaxations, \(\tau_1\) and \(\tau_2\), with rates:

\[
\tau_1^{-1} = \frac{(\tau_1^{-1} + \tau_2^{-1})}{2} \pm \frac{1}{2} \sqrt{(\tau_1^{-1} - \tau_2^{-1})^2 + 16[O_2][k_3k_4]}
\]

While, in theory, two relaxations should always be present, experimentally only one relaxation may be seen if one of the relaxation amplitudes is very small and/or if \(\tau_1\) and \(\tau_2\) are too similar to be distinguished.

Since, in fact, we can distinguish only a single relaxation in our data, how can we tell whether this is \(\tau_1\) or \(\tau_2\)? One clue comes from the fact that at higher protein concentrations the rate we observe is approximately proportional to the square root of the total concentration. At high total [\(\beta\)] the dimer concentration should have this concentration dependence, which suggests (from Equation 2) that the phase we observe is dominated by the dimer → tetramer step. Furthermore, if we use the known assembly equilibrium constants for these conditions (Valdes and Ackers, 1978; Philo et al., 1981) and assume that the association rate constants are within an order of magnitude of that for dimer-tetramer association of Hb, then our small perturbation relaxation simulation predicts that 1) the faster relaxation should be in the range of tens of milliseconds, should consist primarily of the monomer-dimer reaction, and should have a very small amplitude; and 2) the slower relaxation should be in the range of hundreds of milliseconds to seconds, should consist primarily of the dimer-tetramer reaction, and should have an amplitude about an order of magnitude larger than that of the fast relaxation.

We, therefore, believe the relaxation we observe and show in Fig. 2 is the slower one, \(\tau_2\), as given by Equation 3, and that the 0.1 s\(^{-1}\) rate observed in the 100-fold dilution experiments can be assigned to \(k_{2}\). With these assignments, we have done a least squares fit of the relaxation rate data in Fig. 2 to Equation 3 with \(k_1\), \(k_2\), and \(K_4\) as adjustable parameters. We find that we can obtain good unique fits to this scheme. All attempts to interpret the observed relaxation as the fast phase and/or \(k_1\), as the slow dissociation step were incapable of fitting the experimental data.

A further constraint on the rate constants determined from the fitting procedure is that they should be consistent with the equilibrium association data and in particular with our earlier measurements of the concentration dependence of the spectral changes (Philo et al., 1981). As might be expected, the optimum fit to the kinetic data is not the optimum fit to the spectral data, but there is in fact a region of overlap between the confidence intervals of the independently determined parameters. From this overlap region we take a "consensus best fit," whose values are shown in Table I. The solid curve in Fig. 2 shows the theoretical \(\tau_2^{-1}\) for these values.

As a further check on these rate constants, we can use them to calculate the expected amplitudes of the signals for comparison with the experimental data. To do this we must know the changes in absorption for each step of the monomer → dimer → tetramer pathway. While our earlier spectral studies determined the overall tetramer-monomer difference, there is 1

\[^{1}\text{In the temperature jump experiments there is an unresolved kinetic phase within the heating time due to the temperature dependence of the absorption spectra. Also, even though the oxygen saturation is \(\approx 99.8\%\), at some wavelengths a very fast (50 µs) phase can be seen due to an \(O_2\) binding relaxation. However, at this high \(O_2\) saturation the \(O_2\) binding and self-association reactions are essentially entirely uncoupled, and the self-association kinetics we report are not influenced by the kinetics of \(O_2\) binding and release.}\]
not enough dimer present at equilibrium to determine its spectral properties uniquely. Furthermore, to calculate amplitudes for the temperature jump data, we also must know the $\Delta H$ for each step, but only the overall monomer $\rightarrow$ tetramer $\Delta H$ is known. Therefore, we cannot make a rigorous comparison, but we can make some reasonable assumptions about the partitioning of the spectral changes and $\Delta H$ between the monomer $\rightarrow$ dimer and dimer $\rightarrow$ tetramer steps, and then see whether the experimental and predicted amplitudes are in reasonable agreement and to what extent the predicted amplitudes are dependent on the assumptions we have made. (Note that it is entirely unnecessary to make any such assumptions in the above analysis of the relaxation rate data.) For the spectral changes, the simplest assumption is that there is only one spectral change that must occur at either the monomer $\rightarrow$ dimer or the dimer $\rightarrow$ tetramer step, and we have calculated the amplitudes for both of these extreme cases. It is unlikely that the $\Delta H$ would be zero or negative for either step, and it seems most reasonable to assume that the $\Delta H$ for the monomer $\rightarrow$ dimer step is the same as that for the monomer $\rightarrow$ dimer association of hemoglobin $\alpha$ subunits (Valdes and Ackers, 1978a). This choice also makes the $\Delta H$ values nearly equal for each intersubunit contact formed.

The experimental relaxation amplitudes are shown in Fig. 3 along with the theoretical amplitudes for both the fast and slow relaxations for two different assumptions about the spectral changes. In order to include the stopped-flow data with the temperature jump data, the observed stopped-flow amplitude was divided by the theoretical stopped-flow amplitude calculated from the pre- and postmixing species distributions predicted by the equilibrium constants from Table I. This ratio was then multiplied by the slow phase amplitude predicted by the temperature jump simulation at the postmixing concentration for the dimer = tetramer model. That is, the stopped-flow data points are positioned to show the correct ratio of experimental to theoretical amplitude, relative to the upper solid line in Fig. 3. If the spectrum of the dimer is, in fact, intermediate between that of monomer and tetramer, the theoretical curves for both temperature jump and stopped-flow data will lie between the two extreme cases shown.

Increasing the proportion of the overall $\Delta H$ which occurs at the monomer $\rightarrow$ dimer step will increase the amplitude of the fast phase and lower that of the slow phase while keeping the total constant.

The calculations show why we cannot detect the fast phase: its amplitude is always less than 1%. A, which is too small for our instrument to detect at the higher bandwidth needed to follow it. The temperature jump data appear to agree better with the calculations for the dimer = tetramer model (spectral change at the monomer $\rightarrow$ dimer step), but given the uncertainty about the $\Delta H$ values, we would not say that these amplitude data rule out the dimer = monomer case or a dimer spectrum intermediate between that of monomer and tetramer. However, we note that good agreement with the dimer $\rightarrow$ monomer case would require the monomer $\rightarrow$ dimer $\Delta H$ to be less than half that for the monomer $\rightarrow$ dimer association of hemoglobin $\alpha$ subunits (i.e. only about 2 kcal/mol). Therefore, we favor the dimer = tetramer spectral model and consider the agreement of the experimental amplitudes with this model for both the temperature jump and stopped-flow data to be quite good considering the uncertainties involved in the calculations and in scaling the raw experimental data.

**Ligand Binding Kinetics**—The association equilibrium data of Valdes and Ackers (1978a, 1978b) imply the oxygen affinity of $\beta$ is 4 times greater than that of $\beta$ monomers, while the corresponding CO affinities differ by a factor of 3. These differences in affinity imply that the association and/or dissociation kinetics for these ligands must also differ with association state. However, such differences were apparently not recognized in earlier kinetic studies of isolated $\beta$ subunits (Antonini et al., 1965; Brunori et al., 1966; Brunori and Schuster, 1969; Geraci et al., 1969; Noble et al., 1968; Nakamura et al., 1974), although in some cases heterogeneous kinetics and/or inconsistencies with equilibrium data were noted (Antonini et al., 1965; Noble et al., 1969). We, therefore, wanted to look more carefully for such kinetic differences and wanted to determine whether the changes in affinity are due primarily to differences in ligand association or dissociation rates. We have measured the association rate constants for oxygen and CO using laser photolysis. Using the known affinity differences, we can also infer the difference in dissociation rates between species.

**Oxygen Association Kinetics**—Laser photolysis experiments on air-saturated $\beta_4$O$_2$ solutions at 21.5 °C show only a single reaction phase. As shown in Fig. 4, samples at 1 and 30 $\mu$m hem showed essentially identical recombination kinetics, despite a change from about 84% monomer to about 13% monomer. At this high $O_2$ concentration the $O_2$ dissociation reaction contributes negligibly to the observed kinetics. When, as in this case, the amount of $O_2$ released by photolysis is much less than that in solution, the recombination should be ex-
Ligand Binding and Assembly Kinetics of Hb β Subunits

**Fig. 4. Oxygen binding kinetics.** Data are shown for protein concentrations of 1.0 μM (solid trace, 100% photolysis) and 30 μM (dotted trace, 48% photolysis). Oxygen rebinding after photolysis of air-saturated samples was monitored at 436 nm, and the data are plotted as a fraction of the initial absorbance change. For the sake of clarity, less than 20% of the data points are plotted. Sample conditions are the same as in Fig. 2 except that the temperature is 21.5 °C.

Potential and will give a straight line in a semi-log plot such as Fig. 4. Under such pseudo-first order conditions, the maximum deviation of these kinetic data from a single exponential is no more than 0.8% of the total amplitude. We, therefore, conclude that the oxygen association rate constant, $k'$, is essentially independent of association state, with a difference of at most 10% between monomers and tetramers. If the association rates are indeed independent of association state, these data give a value for $k'$ of $6.8 \times 10^4$ M$^{-1}$ s$^{-1}$. Using the oxygen association constant for tetramers measured under these conditions, $2.24 \times 10^4$ M$^{-1}$ (Mills and Ackers, 1979), we infer a dissociation rate, $k$, for tetramers of $30 \times 10^3$ s$^{-1}$. These association and dissociation rates are in reasonable agreement with earlier data for βHb (Brunori et al., 1968, Brunori and Schuster, 1969; Nakamura et al., 1974). For monomers the $O_2$ affinity is 4.2 (3.3, 5.5) times lower (Valdes and Ackers, 1978a), so we infer a dissociation rate of $130 \times 10^3$ s$^{-1}$. This high dissociation rate would perhaps have been seen in earlier temperature jump studies except that these were done at concentrations where the protein samples were probably mostly tetramer (Brunori and Schuster, 1969; Nakamura et al., 1974).

**CO Association Kinetics**—In contrast to the behavior of βHb, photolysis of βCO solutions gives rebinding kinetics which are slightly biphasic. Moreover, the overall rate of rebinding clearly increases with increasing protein concentration. Under conditions where the concentration of CO is greatly in excess over that of protein, CO rebinding after photolysis should be exponential and again should give a straight line in a semi-log plot. However, as can be seen in Fig. 5, the actual plots are clearly curved, and the overall rebinding is significantly faster at the higher protein concentration. The deviation of the data from a single exponential is never large (less than 4% of the total amplitude) but is well outside the noise level. In every case two exponentials are required to fit the kinetic data without systematic deviations, and in no case is a third exponential clearly required. When we use two exponentials, the fits consistently show two phases differing in rate by a factor of about 2.5, with the relative proportion of the amplitude of the faster phase increasing at higher protein concentrations.

There are several possible mechanisms which would produce multiple phase kinetics in these experiments. The first would be that, as in the oxygen temperature jump data of Fig. 1, we are seeing extra phases due to the association-dissociation kinetics and the coupling between assembly and CO affinity. We do not believe this to be true. Because CO rebinding is rapid, there should be little change in association state driven by the photolysis, and both reaction phases are at least 2 orders of magnitude faster than the assembly kinetics observed for βHb. Also, assembly rates would be strongly dependent on protein concentration. We observe no significant concentration dependence of the rates but a strong variation of the proportions of the two phases. A second possibility might be that we are observing a conformational transition. However, as we vary the partial pressure of CO, the rates of both phases seem to be linear with [CO],. We observe no wavelength dependence of the proportions of the two phases and sharp kinetic isosbestic points, both of which imply that the absorption changes associated with both reaction phases are identical. We also see little, if any, change in the proportions of the two phases as we vary the photolysis level from 10 to 100%. Taken together, these facts strongly imply that we are observing two independent CO binding reactions, uncoupled from any association-dissociation process or conformational changes. Since the CO dissociation rate is negligible, we conclude that there must be at least two species present whose CO association rate constants, $l'$, differ by about 2.5 and that the proportion of these species is dependent on protein concentration. We have measured the samples at both 21.5 and 10 °C, and the proportion of the faster species is higher at the higher temperature, as expected if the formation of the faster binding species is driven by self-association. The rate constants we derive from these data are $l_{fast} = 1.31 \pm .02 \times 10^4$ M$^{-1}$ s$^{-1}$ and $l_{slow} = 5.33 \pm .06 \times 10^6$ M$^{-1}$ s$^{-1}$ at 21.5 °C. It is interesting that this faster rate agrees very well with earlier flash photolysis studies at high protein concentrations (Geraci et al., 1969), while the slower one is close to that from stepped-flow data at fairly low concentrations (and where the subunits are deoxy before mixing and, therefore, more dissociated) (Antonini et al., 1965; Brunori et al., 1966). Because the solvent and gas phase are probably not in equilibrium when we cool the cuvette to 10 °C, we are uncertain of the CO concentration at the lower temperature.

---

2 Values in parentheses are 65% confidence limits.
Therefore, we cannot give rate constants at 10 °C or derive activation energies. However, the data suggest that there is little, if any, difference in activation energy for CO binding between the fast and slow binding forms. Also, it is important to note that in order for us to observe these two distinct binding rates, interconversion between the fast and slow conformers must be slow compared to CO binding. This implies that the interconversion rate must be less than 100 s⁻¹.

While the increase in the proportion of the faster binding phase with protein concentration implies a linkage to the association process, a direct assignment of the slower rate constant to the monomer and the faster to tetramer presents several difficulties. First, we noted that even at the lowest and highest protein concentrations we can use (about 0.5 and 100 μM), we still see both components, even though with such a large concentration range we should be going almost completely from monomer to tetramer. In view of our data for the association of β₃₂, we thought that perhaps the failure to see pure species at extremes of concentration was due to the presence of a significant amount of β dimer. The presence of dimers would tend to spread the overall monomer → tetramer association over a broader range of concentration and would also add a third component with an unknown rate constant. Since we saw no evidence for a third binding rate, we tried models where the dimer has the same rate constant as either monomer or tetramer. However, we found no set of association equilibrium constants that could reproduce the observed protein concentration dependence of the proportions of fast and slow CO binding material. That is, the quantitative modeling shows that even with dimer included, with nearly a 200-fold range in concentration, any reasonable values of association constants will give nearly pure fast or slow binding at one of the extremes of concentration. As shown in Fig. 6, we find the proportion of material binding at the slower rate is about 90% at the lowest concentrations and 20% at the highest.

Since we know that association of β₃CO produces a change in the absorption spectrum (Philo et al., 1981), we decided to measure the concentration dependence of this spectral change in order to try to better define the association equilibrium. For the spectral data we have recorded the ratio of the absorbance at 579 nm (a peak in the difference spectrum) to that at 567 nm (an isosbestic point). The concentration dependence of this spectral ratio is also shown in Fig. 6. We then attempted to simultaneously least squares fit the concentration dependence of both data sets (weighted by their relative uncertainties) to a common set of association constants for monomer ↔ dimer ↔ tetramer assembly. As was the case for oxy β, there is uncertainty in assigning the spectral properties of the dimer. We tried models where the dimer has both spectral and CO binding properties either the same as monomer or the same as tetramer. With either assumption we can get only poor fits to the data, again because the strong concentration dependence of a monomer ↔ tetramer equilibrium tends to produce a larger variation in the proportion of fast and slow binding material than that which we observe. In attempting to match the data, the fits yield values for Kᵣ, either 1000 times greater or smaller than those from the gel permeation data. That is, these fits essentially reduce the system to a dimer ↔ tetramer or a monomer ↔ dimer equilibrium (which have a lower concentration dependence), but this is clearly inconsistent with molecular weight data² (Valdes and Ackers, 1975b).

We do not believe that the discrepancy between the data and this simple assembly model can be due to any impurities or degradation of the samples. These data include material derived from two different β chain preparations, and there is no significant difference between them. Oxidation to the ferric form can also be excluded by the spectra and by the inclusion of dithionite in some of the samples. Contamination with the p-mercuribenzoate form of β₃CO is ruled out both by the titration of free—SH groups and by control experiments which show that even the slower CO binding rate is much faster than that for β₃PM. Instead, we believe the reason that these models fail to fit the data is that the assumption that each assembly state has only a single CO binding rate (either fast or slow) is incorrect. That is, we think the data indicate that β subunits have both a fast binding and a slow binding conformation and that both conformations might occur in all association states. However, the equilibrium between the two conformers is association-dependent, with the faster binding form more favored in tetramers. With this assumption, we again simultaneously fitted both the spectral data³ and the fast/slow proportion to a monomer-dimer-tetramer equilibrium. In this fitting process, the fast/slow proportions and spectral ratio for pure monomer and pure tetramer (i.e., the asymptotic values for each type of data at extremely low and high concentrations) are varied freely and independently, and the two types of data are linked only by the common association constants. We find that with this model we get much

³The fitted association constants can easily predict erroneous molecular weights because the properties we are measuring are only sensitive to the proportions of different assembly states and because the models treat the dimer properties as identical to either monomer or tetramer. The least squares fits can only attempt to determine the association stoichiometry from the shape of the association-dissociation curves.

⁴If we allow for two conformations within any association state, then the origin of the spectral change becomes ambiguous. The spectral change could be directly linked to association, or it could reflect a difference spectrum between the fast and slow binding conformers, which is indirectly linked to association. However, this distinction is unimportant for fitting the data, since the fitting function for the spectral ratio is the same in either case.

---

**Fig. 6.** Concentration dependence of the fraction of β subunits binding CO at the slow rate, and changes in absorption spectra for β₃CO. The fraction of the slow binding conformation (open circles, left-hand scale) is determined from the relative amplitudes of a biexponential fit to the CO binding kinetics. The spectral change (closed circles, right-hand scale) is monitored by the ratio of the absorbance at 579 nm (a peak in the difference spectrum) to that at 569 nm (an isosbestic point). Sample conditions are the same as in Fig. 5. The solid line represents the simultaneous best fit of both sets of data to the model described in the text. This fit defines two curves, one for each type of data, but we have chosen the scales for plotting the spectral ratio data so that these two curves are superimposed.
better fits of the data and that the best fit occurs when the
dimer is assumed to have the same properties as the
tetramer. This best fit is plotted as the solid line in Fig. 6. These
parameters imply that monomers are 95% (88%, 100%) in the
slow binding form while dimers are 13% (9%, 17%) in the
slow binding form. These data cannot distinguish whether the
fast binding and slow binding conformers co-exist within the
same tetramer or whether there are two classes of tetraromers,
with all four subunits in each type binding at the same rate.

The association constants obtained from this best fit are

$K_1 = 6.5 \times 10^4 \text{M}^{-1}$ and $K_2 = 5.7 \times 10^5 \text{M}^{-1}$.

This value for dimer association is very close to what we
found from the $\beta O_2$ assembly kinetics, and the overall equilib-
rion is reasonably consistent with the gel permeation data
(Valdes and Ackers, 1978b). These values imply a maximum
dimer fraction of about 20%. On the other hand, when we use
a model with dimer properties the same as monomer, the fits
are significantly poorer, and the parameters imply that a very
large fraction of dimer is present, which is inconsistent with
the gel permeation data. Thus, it appears that the dimer has
properties different from the monomer, but these data cer-
tainly cannot rule out the possibility that dimers have a propor-
tion of fast and slow binding forms which is interme-
diate between monomers and tetraromers.

It may be important to note that while the optimum fit
implies that the monomer contains about 5% of the fast
binding conformation, it makes no significant difference if we
restrict the monomers to be entirely slow binding. If this is
true, the results are qualitatively different, since this would
mean that only the associated states show binding heteroge-
nity. This point and a possible interpretation of the origin
of the heterogeneity will be discussed below. With this assump-
tion, the optimum fit implies that the tetramers are 12(±4)%
in the slow binding form and that slightly more dimer is
present.

**DISCUSSION**

**Presence and Properties of $\beta$ Dimers—**There has been little
agreement in the literature about the predominant stoichi-
ometry for $\beta$ self-assembly. The ultracentrifuge studies by
Tainsky and Edelstein (1973) were interpreted as showing a
tetramer-tetramer equilibrium over a concentration range of 2–
11 $\mu$M. In contrast, Valdes and Ackers (1977, 1978a, 1978b)
fitted their gel permeation data to a monomer-tetramer equi-
lbrium over a 0.6–80 $\mu$M concentration range. Based on
attempts to fit their data with dimer included, Valdes and
Ackers estimated that the maximum fraction of dimer is 10%.

The association constants we obtained in this work imply up
to 17 and 20% dimer for $\beta O_2$ and $\beta CO$, respectively, i.e. they
imply that dimers are neither dominant nor negligible. Since
the oxy $\beta$ assembly kinetics are dominated by the association
of dimers, the assembly rates for $\beta O_2$ are very sensitive to the
dimer concentration. Therefore, we feel there is little doubt
that a significant amount of dimer is present. We do not feel
there is really any discrepancy between our findings and the
Valdes and Ackers data, since the inclusion of ≈20% dimer
has a rather small effect on the shape of the association-
dissociation curves. Rather, our ability to detect the presence
of ($\beta O_2$)$_2$ simply reflects the fact that kinetic techniques are
often more sensitive to low levels of reaction intermediates.

While our data have not given any direct evidence regarding
the properties of $\beta$ dimers, we note that the modeling suggests
that both ($\beta O_2$) and ($\beta CO$) have spectra and/or CO binding
properties the same as the tetrameric forms. This seems to
imply that the intersubunit interactions giving rise to quater-
nary enhancement in $\beta$ subunits occur at the dimer level of
assembly. This view is consistent with the observation that
formation of $\beta$ dimers is strongly enhanced in cyanobet $\beta$, which
proves that the dimer intersubunit contacts are sensi-
tive to heme ligation (Valdes and Ackers, 1978b).

**Kinetics and Energetics of Assembly for Oxy $\beta$—**We find
that the assembly rate constants $k_1$ and $k_2$ are equal and in
fact are very close to values reported for the assembly of $\alpha\beta$
dimers to Hb tetraromers (Ip et al., 1976) and for the association
of $\alpha$ and $\beta$ into dimers (McGovern et al., 1976). Thus, it
appears that Hb subunit association rates are rather inde-
pendent of structural details, and the equilibrium constants
are determined almost entirely by variation in the dissociation
rates. These studies have established that the rate-limiting
step in the dissociation of $\beta$ tetraromers is the tetramer → dimer
step, and, therefore, it is this step which is rate-limiting for
in vitro assembly of Hb A (McGovern et al., 1976).

These studies have shown that the energy of assembly of
dimers is only slightly less than that for the dimer-tetramer
step. This contradicts the suggestion by Valdes and Ackers
(1978b) that the energy is approximately the same per inter-
subunit interface formed. Since there are six interfaces/tetra-
mer, this argument predicts that the energy change at the
dimer-tetramer step would be twice that at the monomer-
dimer step.

**Quaternary Enhancement—**The ligand binding kinetic data
establish that the kinetic expression of the quaternary en-
hancement effect is opposite for $O_2$ and $CO$. For $O_2$, the higher
affinity of tetraromers is due exclusively to a lower dissociation
rate, while for $CO$ it is due almost exclusively to an increased
association rate. This pattern is consistent with the manner
in which the R versus T affinity difference alters ligand
binding kinetics in Hb A. For $O_2$, binding to the $\beta$ subunits in
Hb A, the higher affinity of the R state is due to a ≈100-fold
decrease in dissociation rate and only a ≈5-fold increase in
association rate (Sawicki and Gibson, 1977). For CO binding,
the Hb A data have not distinguished $\alpha$ from $\beta$ but suggest
that the higher R state affinity is due primarily to a ≈70-fold
increase in association rate, with only a ≈10-fold decrease in
dissociation rate (Sawicki and Gibson, 1978). Therefore, these
kinetic data do not suggest that the mechanisms altering the
affinity of the heme between $\beta$ tetraromers and monomers is
fundamentally different than the mechanisms altering heme
affinities in Hb A.

**Origin of the CO Binding Heterogeneity—**The CO binding
kinetic data clearly indicate that not all the subunits within
$\beta$, bind CO at the same rate and that the same is probably
true of $\beta$. A binding heterogeneity may also exist in the
monomer, but the data are also consistent with homogeneous
slow binding for the monomer. Can we saw anything about
the origin and structural basis for this heterogeneity? In
discussing the model used to treat these data, we postulated
an equilibrium between a fast binding and a slow binding
conformation for the subunits, and that association to dimers
and tetraromers tips this equilibrium strongly toward the faster
binding conformer. It is not surprising that formation of
intersubunit contacts might produce a conformation with
different ligand binding properties, since hemoglobin subunits
are clearly designed to make the heme sensitive to such
contacts. We were surprised to find, however, that the for-
mation of $\beta$, does not seem to switch all of the hemes to the
to faster binding form.

We believe a possible explanation of the binding heteroge-
nity is that it is related to rotational disorder of the heme. It
was recently shown that about 10% of the $\beta$ subunits in native
Hb A have the heme group rotated 180° about the $\alpha,\gamma$-meso
axis from the “normal” orientation (La Mar et al., 1985). For
these “backward” hemes, the normal contacts between the heme vinyl and protein side chains are disrupted, including the one with Val-FG5 which has been suggested to be important for heme-heme interaction (Gelin and Karplus, 1977). There is evidence that the subunits with reversed hemes have different ligand affinity (Yamamoto and La Mar, 1986) and that heme reversal can affect the equilibrium between quaternary structures.5

Since 10 ± 3% of the isolated β subunits also have reversed hemes,6 we would like to suggest that the subunits within β4 which bind CO at the slower rate are those which have the reversed heme orientation. That is, our preferred model for these data is that as monomers all the subunits bind CO at the same slow rate. Upon association, the intersubunit contacts cause a conformational change which raises the binding rate only in those subunits with the normal heme orientation. We propose that the reversed heme prevents the subunit from “feeling” the association, so it retains monomer-like properties. This model provides both a structural basis for the heterogeneity and a quantitative explanation for its extent. We should also note that the true equilibrium distribution between the normal and reversed heme orientations in β subunits could itself vary with association state. However, since it is known that heme reorientation in Hb A can require months (La Mar et al., 1985; Yamamoto and La Mar, 1986), it is unlikely that any significant change in orientation occurs during the few hours these samples are at low concentrations (they are stored under conditions where they are tetrameric).

If this model is correct, it predicts that increasing the fraction of disordered heme would increase the fraction of slow binding material at high concentrations. Unfortunately, attempts to prepare heme-disordered β subunits by heme reconstitution have been unsuccessful. Surprisingly, NMR indicates that heme-reconstituted β subunits do not have significantly more reversed heme.7 Therefore, more definitive proof of this hypothesis will have to await the possible development of an alternative method for preparing heme-disordered β subunits.

Finally, we should also note that the association-dissociation reactions of oxy β could also be similarly heterogeneous, but our methods are not sufficiently sensitive to have detected such heterogeneity.

β Subunits as Models for R State Hb A—As noted in the introduction, in many studies of Hb the isolated subunits are used as models for the properties of the Hb R state. Given the 3-4 fold differences between β and βδ in both kinetic (this work) and equilibrium (Valdes and Ackers, 1978a, 1978b) ligand binding properties, such modeling must be approached with great caution. Our earlier spectral studies (Philo et al., 1981) suggested that the β subunits in HbO2 are more similar to β02 than to (β02)δ. Is this supported by the ligand binding kinetics?

Unfortunately, a numerical comparison with literature values for O2 and CO binding kinetics for R state Hb is difficult due to variations in sample conditions. Our new O2 association rate constants for β and βδ are somewhat higher than the average α and β values reported for R state Hb A (Sawicki and Gibson, 1977; Gibson and Edelstein, 1987). The O2 dissociation rate for the β subunits in HbO2 under these solution conditions was recently reported to be 28 s−1 (Gibson and Edelstein, 1987). This is clearly much faster than our derived rate for β monomer but essentially identical to what we derive for β tetramers.

For CO association, a comparison to Hb A is more difficult since the literature data do not distinguish α from β and because in many of the older studies it was not recognized that even low levels of photolysis lead to a partial switch to T. An average α and R state rate of 1.2 × 10−3 M−1 s−1 under similar conditions has been reported (Campbell et al., 1984). Some preliminary studies of α(cyanomet)β(CO) symmetric valence hybrids in our laboratory under these buffer conditions indicate a β R state rate of about 1 × 10−3 M−1 s−1. Therefore, for CO binding it also appears that βδ is more similar to R state Hb A than is β.

Thus, both O2 and CO binding kinetics suggest that βδ is a better (but not perfect) model of the β subunit in R state Hb A, in contradiction to the spectral similarities. These results are further evidence that absorption spectra are a poor indicator of affinity differences.

Acknowledgments—We thank Martin Potschka for help with the relaxation simulation software, Mary L. Adams and Florian Safrer for technical assistance, Ulrich Dreyer for some preliminary CO kinetic measurements, and William Windsor for helpful discussions.

REFERENCES

Antonini, E., Bucci, E., Fronticelli, C., Wyman, J. & Rossi-Fanelli, A. (1985) J. Mol. Biol. 181, 375–384
Antonini, E., Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J. & Rossi-Fanelli, A. (1986) J. Mol. Biol. 181, 735–739
Brunori, M. & Schuster, T. M. (1986) J. Biol. Chem. 241, 4906–4953
Brunori, M., Noble, R. W., Antonini, E. & Wyman, J. (1986) J. Biol. Chem. 241, 5228–5243
Campbell, B. P., Magle, D. & Sharma, V. S. (1984) J. Mol. Biol. 179, 149–150
Chu, A. H., Turner, B. W. & Ackers, G. K. (1984) Biochemistry 23, 604–617
Gelin, B. R. & Karplus, M. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 801–805
Geraci, C., Parkhurst, L. J. & Gibson, Q. H. (1969) J. Biol. Chem. 244, 4646–4667
Gibson, Q. H. & Edelstein, S. J. (1987) J. Biol. Chem. 262, 516–519
Ilgenfritz, G. (1977) in Chemical Relaxation in Molecular Biology (Peicht, I. & Rigler, R., eds) pp. 1–42, Springer-Verlag, Inc., New York
Ip, S. H. C., Johnson, M. L. & Ackers, G. K. (1976) Biochemistry 15, 654–660
La Mar, G. N., Yamamoto, Y., Yue, T., Smith, K. M. & Pandey, R. K. (1965) Biochemistry 4, 3826–3831
McGovern, P., Reisberg, P. & Olson, J. S. (1976) J. Biol. Chem. 251, 7871–7879
Mills, F. C. & Ackers, G. K. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 273–277
Nakamura, T., Sugita, Y. & Yoneyama, Y. (1974) J. Biochem. (Tokyo) 75, 1181–1182
Noble, R. W., Gibson, Q. H., Brunori, M., Antonini, E. & Wyman, J. (1989) J. Biol. Chem. 264, 3905–3908
Pennathur-Das, R. & Schuster, T. M. (1982) in Hemoglobin and Oxygen Binding (Ho, C., ed) pp. 387–391, Elsevier Scientific Publishing Co., Inc., New York
Philo, J. S., Adams, M. L. & Schuster, T. M. (1981) J. Biol. Chem. 256, 7917–7924
Sawicki, C. A. & Gibson, Q. H. (1977) J. Biol. Chem. 252, 7538–7547
Sawicki, C. A. & Gibson, Q. H. (1978) Biophys. J. 24, 21–33
Sheaffer, J. R. (1980) J. Biol. Chem. 255, 2322–2324
Tainsky, M. & Edelstein, S. J. (1975) J. Mol. Biol. 12, 735–739
Tentori, L. & Salvati, A. M. (1981) Methods Enzymol. 76, 707–715
Valdes, R., Jr. & Ackers, G. K. (1977) J. Biol. Chem. 252, 74–81
Valdes, R. L. & Ackers, G. K. (1978a) Proc. Natl. Acad. Sci. U. S. A. 75, 311–314
Valdes, R. L. & Ackers, G. K. (1978b) in Biochemical and Clinical Aspects of Hemoglobin Abnormalities (Caughey, W. S., ed) pp. 527–553, Academic Press, Orlando, FL
Yamamoto, Y. & La Mar, G. N. (1986) Biochemistry 25, 5288–5297

5 G. N. La Mar, private communication.

6 J. S. Philo, unpublished observations.