Carbon Monoxide Binding Properties of Hemoglobin M Iwate†

J. M. Salhany,* C. L. Castillo, and S. Ogawa

ABSTRACT: The kinetic and equilibrium CO binding properties of hemoglobin (Hb) M Iwate (α_2 87 His → Tyr β_2) have been investigated. The results show that the α_2(Met)β_2(CO) *tetramer* of this protein has a low affinity for CO, as indicated by the stopped-flow and flash-photolysis kinetic, as well as the CO binding equilibrium, measurements. However, it has been found that the phosphate-free α_2(Met)β_2(CO) tetramer does tend to dimerize extensively ($K_{4,2}$ = 55 μM). The high-affinity forms seen in earlier kinetic measurements may be explained by this fact. When dimers are accounted for in the functional studies, the results show that the tetramer binds CO noncooperatively either with or without the allosteric cofactor, inositol hexaphosphate (IHP). IHP appears to influence the functional properties of a solution of Hb M Iwate by stabilizing the tetrameric state of aggregation, thereby greatly reducing the population of high-affinity dimers. When the CO "off" rate with IHP present (0.23 s^{-1}) and the CO "on" rate to the tetramer either with or without IHP (1.9×10^5 M^{-1} s^{-1}) at 25 °C are used to calculate the equilibrium constant, the value obtained (8.3×10^5 M^{-1}) is similar to that in equilibrium binding measurements on the phosphate-free tetramer (9.5×10^5 M^{-1}) estimated from the observed $P_{1/2}$ value at 0.48 mM total heme concentration. By showing that dimers account for the high-affinity component seen in earlier kinetic experiments with Hb M Iwate, we can now more strongly suggest that cooperative CO binding to this tetramer is minimal or absent, with both of the active β-hemes presenting a very low affinity.

Hemoglobin (Hb[1]) M Iwate is a mutant where the histidine residue at F8(87) of the α chain is substituted by tyrosine (Konigsberg and Lehmann, 1965; Shimizu et al., 1965; Jones et al., 1966). One consequence of this mutation is the oxidation of the iron atom of the α-chain, while the β chain of the tetramer remains in the ferrous state, capable of binding O_2, CO, or NO. The structure of this mutant hemoglobin has been studied by Greer (1971) using x-ray crystallography. He found that the quaternary structure, with the β-hemes reduced, was the same as normal deoxyhemoglobin and that, when the β-hemes were oxidized in the crystal, the quaternary structure remained unchanged.

The structure of Hb M Iwate in solution has been investigated by Mayer et al. (1973) with high-resolution ^{1}H NMR. Using several NMR spectral features (Shulman et al., 1973; Ogawa and Shulman, 1971, 1972; Ogawa et al., 1972; Lindstrom et al., 1972; Patel et al., 1970; Fung and Ho, 1975), Mayer et al. suggested that Hb M Iwate, without phosphates, is in the deoxy-quaternary structure even when the β-hemes have low-spin ligands, such as CO and O_2 (for ferrous hemes) or CN^- (for ferric hemes).

The functional properties of Hb M Iwate have been studied in various laboratories with conflicting results. Hayashi et al. (1966) measured the O_2 and CO equilibrium curves and found this protein to have a low affinity toward both ligands, n = 1, and essentially no Bohr effect. Interestingly, they also found that the sedimentation coefficients for HbO_2 A and M Iwate were about the same. Recently, Gersonde et al. (1973) have also measured the O_2 and CO equilibrium curves for Hb M Iwate, stripped of organic phosphates and in the presence of 2,3-DPG. When the molecule had two active β chains (i.e., no β-Met present), they observed a large Bohr effect for carbon monoxide and an n value of about 1.5 at pH 7. The magnitude of the cooperative interactions was significantly larger than that observed by Hayashi et al. (1966). Based on these results and the NMR results of Mayer et al. (1973), Gersonde et al. (1973) suggested that cooperative interactions were present within a single quaternary structure. Lastly, Nishikura et al. (1975) recently performed CO binding equilibrium studies and their results support those of Hayashi et al. (1966) and disagree with those of Gersonde et al. (1973).

The kinetics of carbon monoxide binding to Hb M Iwate have been studied by Gibson et al. (1966) and they would appear, on the surface, to confirm the equilibrium measurements of Gersonde et al. (1973). Gibson et al. (1966) found homogeneous, slow CO binding in forward-flow experiments but heterogeneous CO binding in flash-photolysis experiments. Although dithionite was present, the rate of reduction of the abnormal α chain by dithionite was too slow (half-time ~15 h) to have accounted for the presence of fast component in the flash results. The forward-flow experiments are most sensitive to the first CO binding constant and this rate constant for Hb M Iwate was the same as normal adult hemoglobin (~2×10^5 M^{-1} s^{-1}). The flash results could be accounted for by two rates, one being the same as in the forward-flow experiments and the other about an order of magnitude, or more, faster.

One possible interpretation of the flash-photolysis results would be that the fast and slow CO binding rates were a consequence of cooperativity. The observation of no cooperativity in the forward-flow experiments would not be in conflict with this interpretation, since it would be most difficult, if not impossible, to detect cooperative CO binding in forward flow with a two-site system where the second step in CO binding differed by an order of magnitude from the first, functionally limiting,

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; CD, circular dichroism; EPR, electron paramagnetic resonance; IHP, inositol hexaphosphate; Hb, hemoglobin; HbO_2, oxyhemoglobin; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; 2,3-DPG, 2,3-diphosphoglycerate; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

step. This interpretation of the kinetic results of Gibson et al. (1966) would be consistent with the $P_{1/2}$ values and the magnitude of the cooperativity seen in equilibrium measurements by Gersonde et al. (1973). However, if we accept this interpretation, it would mean that the stripped protein with two CO molecules bound was in some high-affinity state,[2] not the low-affinity state as concluded from the previous NMR (Mayer et al., 1973) and x-ray crystallographic measurements (Greer, 1971).

Before we were prepared to abandon the previous interpretations of the NMR and x-ray results, there was one alternative possibility which could explain some of the previous functional measurements on Hb M Iwate. This alternative explanation would require rather extensive dimerization of the protein with two CO molecules bound, as compared with the α_2(Met)β_2(deoxy) form. Under these circumstances, the fast component in the flash-photolysis experiments of Gibson et al. (1966) would be attributable to the α(Met)β(deoxy) dimer in the high ligand affinity state, with the slow component being CO binding to unliganded or partially liganded tetramers in a single low-affinity state. In order to explore this possibility, we reinvestigated the kinetics and equilibrium of CO binding to Hb M Iwate. The present results show that dimerization can be a significant factor in reactions involving this protein. The tetramer shows no detectable kinetic evidence for cooperative CO binding or release, in agreement with the present equilibrium measurements and the results of Hayashi et al. (1966) and also the recent report by Nishikura et al. (1975).

Materials and Methods

Hb M Iwate was isolated as described previously (Gersonde et al., 1973). Kinetic and equilibrium studies were performed anaerobically in the presence of 1 mM ascorbate to keep the β chains in the ferrous state. Ascorbate does not reduce the abnormal ferric α chains. The gases and reagents used in this study have been described elsewhere (Salhany et al., 1975).

Stopped-flow measurements were performed exactly as described elsewhere (Salhany et al., 1974, 1975). Flash-photolysis experiments were performed using the system described by Applebury et al. (1974). The intensity and wavelength of the flash were controlled using a neutral-density filter, and a cutoff filter. The latter filter allowed only light of greater than 480 nm through the hemoglobin sample. The protein was placed in a 1-cm path length cuvette with a rubber septum inserted in the opening. All samples were flushed with pure CO gas at 1 atm. Flash photolysis on CO-bound horse myoglobin was used to test the system. The results show completely pseudo-first-order kinetic behavior for more than 95% of the time course. The rate obtained was the same as that in the literature.

Equilibrium CO binding measurements were carried out by the spectrophotometric method described by Castillo et al. (submitted for publication). A Cary 14 spectrophotometer was employed.

Results

Flash Photolysis Measurements. Gibson's (1959) classic flash-photolysis experiments on concentrated hemoglobin showed that the fraction of fast component in the recombination of CO after photolysis was dependent on the degree of photolysis (see also Antonini et al., 1967). When stripped Hb M Iwate was similarly investigated, the results shown in Figure 1 were obtained. Varying the fraction of CO flashed off from about 4% (curve D) to about 40% (curve A) did not measurably alter the fraction of fast and slow components present. In each case, there was about 73% slow and 27% fast present. Significantly, the fraction of slow component for a 4% flash is quite considerably larger in stripped Hb M Iwate than in stripped adult Hb (Gibson, 1959). The invariance in the fraction of slow and fast components with the degree of photolysis could be explained if the fast component were due to CO binding to high-affinity dimers, with the slow phase involving tetramers in the low-affinity state.

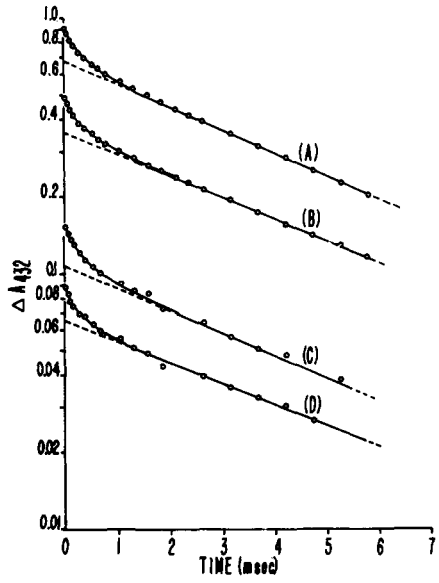

FIGURE 1: Flash photolysis on stripped Hb M Iwate in 0.2 M Bistris, pH 7.0. The concentration of total heme was 50 μM. A 1-cm path length was used. The concentration of CO was 1 mM with T = 25 °C. The sample contained 1 mM ascorbate. The reactions can be represented by two rates: $l'_{slow} = 1.9 \times 10^5$ M^{-1} s^{-1} and $l'_{fast} = 5 \times 10^6$ M^{-1} s^{-1}. The fraction of fast = 0.27 and the fraction of slow = 0.73 for all curves presented.

To test this, the sample of Hb M Iwate was diluted by a factor of 3 with oxygen-free, CO-flushed 0.2 M Bistris buffer at pH 7.0. When a 42% flash was made on the diluted sample, almost all of the material recombining with CO showed rapid binding (Figure 2, curve B) in contrast to the threefold more concentrated solution where, for about the same flash, far more slow phase was present (Figure 2, curve A). Partial flash-photolysis experiments at a very high protein concentration (1 mM in heme) were also performed using a thin sample cell (0.2 mm), which was placed at 45° with respect to both the flash and observing light. There was no detectable fraction of rapid reacting component for a 10% flash. These results, and the fact that the fraction of fast and slow phase was essentially independent of the degree of photolysis, seem to suggest that the fast component comes from the α(Met)β(deoxy) dimer, with the slow phase coming from tetramers in the low-affinity state. The rate constants extracted for the fast and slow phases were 5×10^6 M^{-1} s^{-1} and 1.9×10^5 M^{-1} s^{-1}, respectively.

When IHP (in 0.2 M Bistris buffer, pH 7.0, flushed with pure CO) was added to the same diluted sample of Hb M Iwate used above and the flash-photolysis experiments were repeated, no rapid component was detectable (Figure 3, curve B). The time course shown in Figure 3, curve b, was the same as that observed for stripped Hb M Iwate at much higher protein concentrations. The fraction of CO photodissociated was 69% in this case. Precisely the same result was obtained on Hb M

[2] The Hill constant n = 1.5 reported by Gersonde et al. (1973) corresponds to a factor of ten increase in the affinity from the first to the second CO binding.

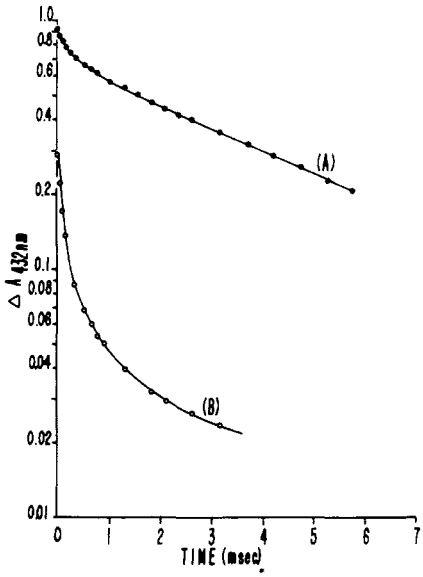

FIGURE 2: Flash photolysis on stripped Hb M Iwate. Conditions are the same as in Figure 1, except that the protein concentration for curve B was 16.2 μM, whereas that for curve A was 50 μM. The percent CO flashed off in both cases was 42%. The fraction of fast component in curve A is 0.27 and in curve B is $\geq$0.85.

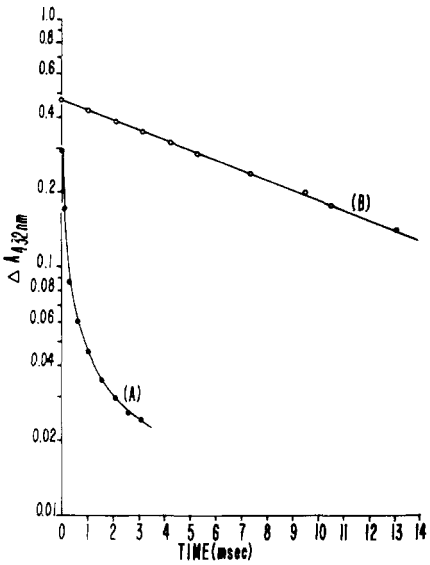

FIGURE 3: Flash photolysis on Hb M Iwate at about 16 μM (A) stripped and (B) in the presence of 1 mM IHP. Otherwise, the conditions are the same as in Figure 1.

Iwate plus IHP for an 11% flash. In both cases, no fast component was observable and the CO "on" rate was the same as the slow component in stripped Hb M Iwate (1.9×10^5 M^{-1} s^{-1}). The elimination of the fast phase by addition of IHP is consistent with the reduction in dimer formation seen in other experiments upon addition of organic phosphates to hemoglobin solutions (Gray, 1974).

Stopped-Flow CO Binding Measurements. When stripped α_2(Met)β_2(deoxy) Hb M Iwate was reacted with CO in the stopped-flow apparatus in the absence of dithionite, the time courses shown in Figure 4A were observed. This reaction is homogeneous and pseudo-first-order (when plotted semilogarithmically), and wavelength independent, presenting a good kinetic isosbestic at 426.1 $\pm$ 0.45 nm. This is the isosbestic for the deoxy and CO forms of the β chain and is about 1-nm red shifted as compared with stripped adult hemoglobin whose

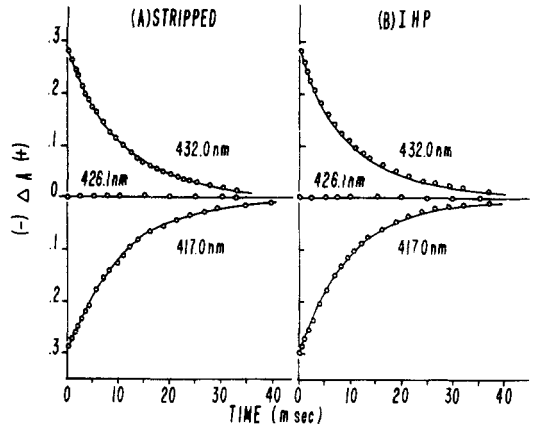

FIGURE 4: Stopped-flow CO binding to α_2(Met)β_2(deoxy) Hb M Iwate (A) stripped and (B) in the presence of IHP, as a function of wavelength. Concentration of heme (before the mix) was about 77 μM in both cases. In reactions (B), the concentration of IHP on the hemoglobin side (before the mix) was 0.1 mM. The initial concentration of CO was 1 mM before the mix. The samples were buffered with 0.2 M Bistris, pH 7.0. The temperature was 25 °C.

isosbestic in this region is the average of the α and β chains (Gray and Gibson, 1971). The homogeneous nature of this reaction is the same as that seen by Gibson et al. (1966). When IHP was added to the protein and the kinetics of CO binding was investigated, exactly the same results were obtained (Figure 4B). There was no effect of IHP on CO binding to α_2(Met)β_2(deoxy) and the rate constant was 1.9×10^5 M^{-1} s^{-1}, in excellent agreement with the flash-photolysis results discussed above.

Stopped-Flow CO Replacement by NO Measurements. The CO replacement reaction by NO has been extensively studied by Salhany et al. (1974, 1975). It was shown that the kinetics of this replacement reaction depended on the quaternary structure of the protein, as determined by NMR and CD spectroscopic measurements. Within the low-affinity quaternary structure of nitrosylhemoglobin there occurred a slow spectral change not related to ligand binding per se (Salhany et al., 1974, 1975; Cassoly, 1974). Salhany et al. (1974, 1975) presented evidence which indicated that this spectral change was due to some tertiary event within this state. In a recent paper, Sugita (1975) has shown that this spectral change occurs almost exclusively within the α chains. Perutz et al. (1976) have recently suggested that this spectral change may come from the tension exerted on the α-NO heme within the T (or deoxy) quaternary structure. As has been stressed elsewhere (Salhany et al., 1974, 1975), it is usually necessary to study kinetic reactions involving NO at wavelengths isosbestic for that spectral change. However, when hemoglobin M Iwate was initially investigated using this reaction, it was observed that the extra spectral change was absent. Recent EPR measurements on Hb M Iwate by Dr. Mordechae Chevion at Bell Laboratories (personal communication) show why this is so. When the α_2(Met)β_2(CO) form of this protein was flushed with NO and the EPR spectrum was measured, he observed two EPR resonances, one with a g value of about 2 for the ferrous NO complex and one with a g value which is characteristic of the high-spin Met heme of the α chain. This result clearly shows that NO does not react with the abnormal Met heme of the α chain in Hb M Iwate in any reasonable time period. Normal Met hemoglobin, however, does apparently form a complex with NO and NO will reduce it to the ferrous state (Chien, 1969; Henry and Banerjee, 1973).

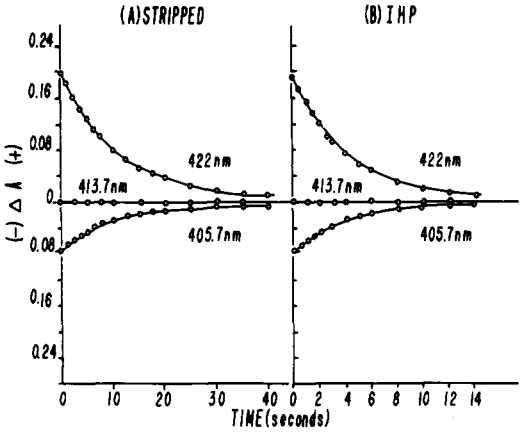

FIGURE 5: The CO replacement reaction by NO for Hb M Iwate stripped (A) and in the presence of IHP (B). The concentration of heme before the mix was 55.5 μM. The concentration of ascorbate on the hemoglobin side was 1 mM (before the mix) with the concentration of NO at 2 mM. The reactions with IHP added to the hemoglobin had 1 mM IHP present before the mix. The system was in 0.2 M Bistris, pH 7.0, at 25 °C.

When stripped Hb M Iwate in the CO form is reacted with NO in the stopped-flow, relatively fast and wavelength-independent time courses were observed (Figure 5A). The CO "off" rate was apparently first order (when plotted semilogarithmically) with a rate constant that is about an order of magnitude faster (0.09 s^{-1}) than stripped adult hemoglobin in the R state (0.008 s^{-1}) (Salhany et al., 1974, 1975). Addition of IHP to this system causes the reaction to remain first order and wavelength independent with the rate constant increasing to 0.23 s^{-1} (Figure 5B).

Carbon Monoxide Binding at Equilibrium. CO binding at equilibrium was performed on stripped Hb M Iwate at pH 7.0, with the protein concentration at 0.48 mM (total heme). The binding curves showed low CO affinity ($P_{1/2}$ = 0.48 mmHg) and essentially no cooperativity (n = 1.05). This result is in agreement with the results of Hayashi et al. (1966) and of Nishikura et al. (1975), but disagrees with the measurements by Gersonde et al. (1973).

Discussion

Identification of the Fast CO Binding Component in the Flash Photolysis Experiments. The observation that stripped Hb M Iwate showed no significant dependence of the fraction of fast and slow phases on the degree of photolysis can be most directly interpreted to mean that the species responsible for these two rates are not rapidly interconvertible on a time scale comparable with CO binding (3–4 ms at the CO concentration used). This result is in marked contrast with normal cooperative hemoglobin (Gibson, 1959). When normal hemoglobin undergoes a so-called "tickle" flash, almost no slow phase is usually detectable. The rate constant observed at pH 7 is about 6×10^6 M^{-1} s^{-1}. This value is also about the same as the rate constant observed for CO binding to noncooperative deoxy dimers of adult hemoglobin (Andersen et al., 1971). Full flash photolysis of normal hemoglobin at pH 7 produces a nearly homogeneous recombination reaction with a rate constant of about 2×10^5 M^{-1} s^{-1} (Gibson, 1959). Since the two phases in CO binding to stripped Hb M Iwate after photolysis are independent of the degree of photolysis (Figure 1), strongly dependent on protein corcentration (Figure 2), and show a rate constant for the fast phase which is almost identical to that for the dimer of adult hemoglobin and a rate constant for the slow phase which is almost identical to the tetramer of adult hemoglobin, we suggest that these two phases come from dimers and tetramers present under the conditions of the experiments.

The suggestion that the fast component in flash experiments with Hb M Iwate comes from dimers implies that the tetramer-dimer equilibrium constant ($K_{4,2}$) is relatively large. Edelstein and Ogawa have tested this directly by performing ultracentrifugation measurements on Hb M Iwate in 2 mM ascorbate and 0.2 M Bistris, pH 7, fully saturated with CO (unpublished results, method of Crepeau et al., 1974). The value they obtained was 55 μM, as compared with a value of 1 μM for the CO form of adult hemoglobin (Crepeau et al., 1974). This value of $K_{4,2}$ would qualitatively confirm the unusual dependence of the fraction of the fast and slow phases on protein concentration. However, there is an apparent quantitative inconsistency when one calculates the fraction of fast and slow phases expected from the measured $K_{4,2}$ value and compares it to the fraction observed.[3] For example, under the conditions of Figure 2, curve A, we should expect to see about 64% fast phase and 36% slow phase in the recombination reaction after photolysis. Instead, we observed about 27% fast and about 73% slow. Furthermore, in curve B of the same figure, where the protein concentration is about 16 μM, we expect about 81% fast and about 19% slow. This expectation does appear to be realized by the data of Figure 2, curve b, but we must have less confidence in this estimate owing to the relatively small number of data points near the end of the reaction which are available for extrapolation to zero time.

Addition of IHP to a 16 μM solution of stripped Hb M Iwate not only caused the reaction to become completely homogeneous, slow, and identical to CO binding to stripped Hb M Iwate at high protein concentration, but also caused the fraction of CO flashed off to be reproducibly greater by about 40% as compared with stripped Hb M Iwate under the same instrumental conditions. Addition of this quantity of IHP to Hb M Iwate would be expected to strongly favor the association of dimers to tetramers (Gray, 1974; Hensley et al., 1975). The absence of fast component in the recombination reaction would confirm this expectation.

Kinetic Evidence Concerning Cooperative CO Binding to the Tetramer of Hb M Iwate. Having identified the fast component in the flash experiments as CO binding to dimers of Hb M Iwate, we next consider the functional properties of the tetramer. Is there any evidence from the kinetic studies that CO binds to the tetramer cooperatively? It would appear that the answer to this question is no. Forward-flow experiments (Figure 4) would be expected to predominantly yield information about the first CO "on" constant in this two-site system. The value of this rate constant was found to be 1.9×10^5 M^{-1} s^{-1}, and IHP had no measurable effect. The last CO binding rate constant would be obtained from the "tickle" flash experiments. At 50 μM heme, the slow component for a 4% flash had the same rate constant as in forward-flow experiments. Furthermore, a 10% flash at high-protein concentration (1 mM, heme) also gave the same rate constant and no fast phase was detectable. Addition of IHP to solutions of Hb M Iwate only appears to reduce the population of fast reacting, dimeric, species without measurably altering the value of the rate constant when compared with the slow phase seen in the stripped protein. Since the first (forward flow) and the last ("tickle" flash) CO molecules bind to the tetramer with the same rate constant and addition of IHP does not alter the value

[3] The calculation assumes that the spectra of the β-chain in the deoxy dimer and tetramer are the same, as suggested by Sugita (1975).

of that constant, it would appear safe to conclude that both homotropic and heterotropic phenomena, characteristic of cooperative hemoglobins, do not appear in the "on" constant of CO binding to the tetramer of Hb M Iwate.

We would suggest that the results of the kinetic experiments, where CO is replaced by NO (Figure 5), also would be consistent with the conclusion that cooperative effects are absent in the tetramer of this mutant hemoglobin. As was shown elsewhere (Salhany, 1974; Salhany et al., 1974; 1975), this replacement reaction can have acceleration in the CO "off" rate when the protein is cooperative in equilibrium binding measurements. No acceleration was observed for stripped Hb M Iwate. The apparent rate constant was fully an order of magnitude larger than stripped adult hemoglobin, where the molecule remains in the high-affinity state throughout the reaction (Salhany, 1974; Salhany et al., 1974; 1975). The effect of IHP on this reaction for Hb M Iwate was to increase the rate constant by a factor of about two. However, from the flash-photolysis results discussed above, it would appear that an increase in the initial population of tetramers accounts for the observed increase in the CO "off" rate. Therefore, we can probably accept 0.23 s^{-1} as the value of the CO "off" rate constant for the tetramer of Hb M Iwate at 25 °C, pH 7. Although we cannot absolutely rule out a minor effect of IHP on the CO "off" rate from the tetramer, it seems simpler, owing to the flash and forward flow results with and without IHP, to ascribe the increase in the CO "off" rate to an increase in the initial tetramer population.

Equilibrium CO Binding to the Tetramer of Hb M Iwate and Comparison with the Kinetic Measurements. The effect of dimerization on the value of $P_{1/2}$ and Hill's constant, n, can be estimated using Wyman's (1964) formulation. The degree of ligation, Y, is given by

$$Y = \alpha Y_d + (1 - \alpha)Y_t \quad (1)$$

where Y_d and Y_t are the degrees of ligation of the dimer and tetramer, respectively, and α is the fraction of total dimer in hemes. Hill's constant for this system at half-saturation is given by

$$n = 2n_tY_t + 4(1 - Y_t)(1 - 2Y_t)/(3 - 2Y_t) \quad (2)$$

where n_t is Hill's constant for the tetramer only. The derivation of this equation is accomplished by setting Y_d equal to unity. This is justified, since the affinity of the dimer is far higher than the tetramer and also because α is relatively small for the conditions under which the equilibrium studies reported here were performed. From these considerations, Y_t can be approximated by Hill's equation

$$Y_t = (P/P_t)^n t/(1 + (P/P_t)^n t) \quad (3)$$

with P_t being the $P_{1/2}$ value of the tetramer. Using this approximation, the values of $P_{1/2}$ for the entire system can be given by

$$(P_{1/2}/P_t)^n t = (1 - 2\alpha) \quad (4)$$

$$\alpha^2/(1 - \alpha) = K/4C_0 \quad (5)$$

where K is the dimerization constant of the liganded tetramer and C_0 is the total ferrous heme concentration. In the present study, C_0 = 240 μM and K = 55 μM. The value of α at half-saturation is estimated to be 0.2. From the observed value of $P_{1/2}$ (0.48 mmHg) and n (1.05), the same values for the *stripped tetramer* are: P_t = 0.80 mmHg (1.05 × 10^{-6} M) and n = 1.03. Thus, Hill's constant is apparently unity for the *tetramer,* in agreement with the kinetic results which show no evidence for cooperativity. The CO binding equilibrium constant ($1/P_t = 0.95 \times 10^6$ M^{-1}) for the *stripped tetramer* is quite close to the value of the same constant calculated from the kinetic measurements *with IHP present* (0.83×10^6 M^{-1}), where dimer contributions would be minimal.

Concluding Remarks. The original purpose of this study was to investigate the kinetics of CO binding to Hb M Iwate in the hope of resolving the disagreement about the presence or absence of cooperativity for this mutant. Our kinetic results basically confirm those of Gibson et al. (1966) and extend them to show that the fast component they observed in flash-photolysis experiments is not due to cooperativity but rather is due to the presence of significant populations of high-affinity dimers. Thus, kinetic measurements from two laboratories and equilibrium measurements from three (Hayashi et al., 1966; Nishikura et al., 1975, and the present results) all agree that the *tetramer* of this mutant hemoglobin binds CO noncooperatively and with a very low affinity. There is a disagreement between all of these results and the results of Gersonde et al. (1973) who reported relatively high cooperativity (n = 1.5 at pH 7.0) in spite of the high protein concentration used. No clear explanation for this disagreement can be given at present.

We might ask what the structural basis is for the absence of cooperativity and the very low affinity observed for the tetramer of this mutant. Although we currently have no detailed answer to this question, one reliable piece of evidence on the solution structure is the observation of the −14 ppm line (from DSS) in the proton NMR spectrum (Mayer et al., 1973). It has been observed in adult deoxyhemoglobin and noncooperative, low affinity, liganded hemoglobins, but not in liganded (O_2 or CO) adult hemoglobin or in unliganded noncooperative, high-affinity hemoglobins (Patel et al., 1970; Shulman et al., 1973, 1975; Fung and Ho, 1975; Salhany et al., 1975). Of course, any single ^1H NMR line only gives structural information about the local environment of that proton. In the case of the −14 ppm line, Fung and Ho (1975) have suggested that this resonance appears when the intersubunit hydrogen bond is formed between Tyr-42 (C7) of the α_1 chain and Asp-99 (G1) of the β_2 chain. X-ray diffraction studies on hemoglobin (see Baldwin, 1975) have strongly suggested that this bond can only form when a hemoglobin tetramer has the deoxy quaternary structure. It was the observation of this NMR line in deoxyhemoglobin which was the original reason for suggesting that it could be used as one marker for the low-affinity state (Patel et al., 1970; Shulman et al., 1975). If we accept the detailed assignment of this line to the bond mentioned above, then the observation of Mayer et al. (1973), showing the presence of the −14 ppm line in *both* liganded and unliganded Hb M Iwate, suggests that this bond does *not* break with CO binding, as it does in normal, cooperative hemoglobin. If this bond only breaks when there is a major change in quaternary structure (i.e., spatial arrangement of the four subunits), the observations of Mayer et al. (1973) may suggest that CO binding to Hb M Iwate does not cause such a change. *It should be emphasized that this argument does not constitute a proof.* It is only presented to allow the reader to recall what may be a significant correlation between the presence of an important NMR line in both liganded and unliganded Hb M Iwate, and the absence of cooperative CO binding associated with a very low CO affinity.

Acknowledgments

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