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Oxygen Equilibrium Studies of Cross-Linked Asymmetrical Cyanomet Valency Hybrid Hemoglobins: Models for Partially Oxygenated Species†

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ABSTRACT: Oxygen equilibrium curves have been measured to determine the binding constant at each oxygenation step (K_i) for various cross-linked hemoglobins, $(\alpha\beta)_A(\alpha\beta)_C$XL, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, where the subscripts A and C denote that the $\alpha\beta$ dimer is derived from human normal adult hemoglobin and mutant hemoglobin C (β6Glu → Lys), respectively, and XL denotes cross-linking between the lysyl residues at position 82 in the two β chains by bis(3,5-dibromosalicyl) fumarate as described by Miura and Ho [Miura, S., & Ho, C. (1982) *Biochemistry 21*, 6280–6287]. The oxygen equilibrium data indicate that the oxygen affinity increases with the number of cyanomet hemes carried by the cross-linked mixed valency hybrid hemoglobins. The oxygen binding property depends not only on the number of the subunits carrying cyanomet hemes but also on the distribution of cyanomet hemes among the four subunits. A striking effect is observed in singly cyanomet valency hybrid hemoglobins; namely, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL exhibits lower oxygen affinity and higher cooperativity than $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL. The magnitude of the Adair constants and their pH dependency of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL ($K_i$, i = 1–3) are analogous to those of the Adair constants of $(\alpha\beta)_A(\alpha\beta)_C$XL ($K_i$, i = 2–4), whereas such an analogy is not observed between $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL and $(\alpha\beta)_A(\alpha\beta)_C$XL. The doubly cyanomet mixed valency hybrid cross-linked hemoglobins exhibit high oxygen affinity and reduced cooperativity, and their Adair constants are not analogous to K_3 and K_4 of $(\alpha\beta)_A(\alpha\beta)_C$XL. The present results on the oxygen binding properties of cross-linked mixed valency hybrid hemoglobins provide additional support to the conclusion based on a previous proton nuclear magnetic resonance investigation of these hybrid hemoglobins that there are at least three functional and energetically important structures of hemoglobin in going from the deoxy to the ligated state. Thus, the cooperative oxygenation process of human normal adult hemoglobin cannot be simply described by two-structure allosteric models.

In spite of the large amount of research during the last several decades, the molecular mechanism of cooperative oxygen binding by hemoglobin (Hb)[1] is not yet fully understood. The cooperativity arises from the reversible transition between fully ligated and fully deoxy forms of human normal adult hemoglobin (Hb A), whose structures have been determined by X-ray crystallography [for example, see Baldwin (1980), Shaanan (1983), and Fermi et al. (1984)]. One of the most challenging tasks in current Hb research is to describe the structural change induced at each oxygenation step by characterizing the properties of the intermediate ligated species, namely, Hb A with one, two, and three ligand molecules bound within a tetramer, and comparing them with the properties of the fully deoxy and ligated species, and thus to be able to interpret alterations in functional properties in terms of molecular structural changes. Because at any given fractional saturation between the fully deoxy and fully oxy states, various molecular species with binding of zero to four ligand molecules coexist as a statistical mixture, it is not feasible to isolate a stable intermediate ligation state by using Hb A. (This difficulty in studying the intermediate ligation states is not due to the Hb A itself but rather to the use of O_2 as the ligand.) The cooperative nature of oxygen binding further reduces the relative populations of intermediate ligated states to a small fraction in comparison with those of fully deoxy and ligated forms. Thus, the majority of the information available about the properties of the Hb molecule has been unfortunately limited to either the fully ligated or the fully deoxy form of

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; met-Hb, methemoglobin; α^{+CN} or β^{+CN}, α or β subunit containing heme iron that is in the ferric state and is combined with cyanide; $(\alpha\beta)_A(\alpha\beta)_C$XL, cross-linked Hb, where the subscripts A and C denote that the $\alpha\beta$ dimer comes from Hb A and Hb C, respectively, and XL denotes cross-linked Hb; $\alpha(Fe)_2\beta(Co)_2$, hybrid Hb containing iron protoporphyrin IX in the α subunits and cobaltous protoporphyrin IX in the β subunits; $\alpha(Co)_2\beta(Fe)_2$, hybrid Hb containing cobaltous protoporphyrin IX in the α subunits and iron protoporphyrin IX in the β subunits; NMR, nuclear magnetic resonance; n, Hill coefficient; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; RMS, root mean square.

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Hb A, and the functional properties of Hb have been interpreted on the basis of the information on deoxy and oxy structures.

Some efforts have been made to study the intermediate states of ligation, mainly focused on symmetrically ligated hybrid intermediate Hb molecules. Three types of symmetric hybrid Hbs have been extensively studied: (i) cyanomet valency hybrid Hbs, which contain two ferric hemes with cyanide at the α or β subunits (Brunori et al., 1970; Maeda et al., 1972; Bannerjee &Cassoly, 1969; Ogawa & Shulman, 1972; Cassoly & Gibson, 1972); (ii) the M type of hemoglobins, such as Hb M Milwaukee (Fung et al., 1976, 1977); and (iii) iron–cobalt hybrid Hbs in the presence of carbon monoxide, which binds only to the iron-containing subunits (Ikeda-Saito et al., 1977; Ikeda-Saito & Yonetani, 1980; Inubushi et al., 1983; Hofrichter et al., 1985). Because of rapid dimer exchange, only the symmetrical intermediates, such as either $(\alpha^{+CN}\beta)_2$ and $(\alpha\beta^{+CN})_2$ or $\alpha(Fe–CO)_2\beta(Co)_2$ and $\alpha(Co)_2\beta(Fe–CO)_2$, two out of eight intermediate forms, have been isolated as stable tetramers. Consequently, so far, most of the studies on model intermediates have been limited to symmetrical intermediate Hb preparations. The results obtained on symmetric hybrid Hbs have made important contributions to the recent advance in Hb research. However, the characterization of other intermediate species is required for fully understanding the mechanism of cooperative oxygen binding by Hb.

One of the major breakthroughs has been recently accomplished by Miura and Ho (1982), who demonstrated that the exchange of two dissociative dimers can be prevented by introduction of a cross-linking reagent, i.e., bis(3,5-dibromosalicyl) fumarate, which cross-links between the lysine-82 residues of the two β subunits (Walder et al., 1980). This has enabled them to prepare various asymmetrical cyanomet mixed valency hybrid Hbs, i.e., with one cyanomet heme in either an α or a β subunit, or with two cyanomet hemes, one in an α and one in a β subunit within a tetramer Hb molecule. Since it has been recognized that, as a first approximation, cyanomet heme is a good model for ligated heme, it is now possible to prepare stable Hb molecules with various degrees of ligation at any specific subunit. Miura and Ho (1982) have further demonstrated the inadequacy of two-state allosteric models in interpreting their proton nuclear magnetic resonance (NMR) spectra of deoxy singly cyanomet valency hybrid Hbs, namely, $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ and $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$.

Measurements of the precise O_2 equilibrium curves of these asymmetric cyanomet valency hybrid Hb preparations will not only characterize the O_2 affinity of these hybrids but will also allow us to determine the oxygen equilibrium constants at each oxygenation step (Adair constants) of the ferrous subunits. Comparison of the Adair constants of these cross-linked hybrid Hbs with those of cross-linked Hb is expected to provide clues to our investigation of the effect of introducing cyanomet hemes at specific subunits in the Hb tetramer upon the O_2 affinity of the remaining ferrous subunits. To this end, we have measured precise O_2 equilibrium curves of the following cross-linked Hbs: $(\alpha\beta)_A(\alpha\beta)_CXL$, $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$, $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_CXL$, and $(\alpha\beta^{+CN})_A$-$(\alpha^{+CN}\beta)_CXL$, where the subscripts A and C denote that the $\alpha\beta$ dimer in parentheses is from Hb A and Hb C (β6Glu → Lys), respectively, and XL denotes cross-linked Hb. We also report a pH dependence of the Adair constants of these cross-linked Hbs.

Experimental Procedures

Preparation of Asymmetrical Cyanomet Hybrid Hbs. Cross-linked Hbs are prepared as described by Walder et al. (1980). The preparation of various cross-linked mixed valency asymmetric cyanomet hybrid Hbs followed the procedures of Miura and Ho (1982). In order to achieve better separation, preparative isoelectric focusing on polyacrylamide gel (Righetti & Drysdale, 1971) was used as the final step of the preparative procedure in place of CM-cellulose column chromatography. The band containing the desired cross-linked Hb sample was cut out, and the Hb sample was passed through a Sephadex G-25 column to remove ampholites.

Measurement of the O_2 Equilibrium Curves. The O_2 equilibrium curves were recorded by the automatic recording system of Imai et al. (1970), which is interfaced to a PDP 11/40 computer system for on-line data acquisition, storage, and analysis (Imai & Yonetani, 1977). All the measurements were carried out at 25 °C in 0.1 M phosphate buffer. For cyanomet valency hybrid Hbs, the experiments were performed in the presence of 2 mM KCN to ensure that ferric hemes were saturated with cyanide. The concentration of Hb samples was 60 μM in terms of heme, and the absorbance change during the successive deoxygenation and reoxygenation was monitored by a Cary 118C spectrophotometer at 560 nm. Because of the cross-linking between the two β subunits, a partial dissociation into $\alpha\beta$ dimers, which has been observed at this Hb concentration, does not take place. In order to calculate the amount of methemoglobin (met-Hb) formed during the equilibrium measurements, the absorption spectra in the visible region were measured before and after each measurement. The extrapolation of the bottom and top portions of the O_2 equilibrium curve was carried out as described by Imai and Yonetani (1977).

The oxygen equilibrium curves of Hb A and cross-linked Hb prepared from Hb A and Hb C were analyzed according to the four-step oxygenation scheme (Adair, 1925), and those of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ and $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$ were analyzed according to the three-step oxygenation scheme. In both cases, the fractional saturation of oxygen Y is expressed as

$$Y = \{\sum_{i=1}^{j}[(i/j)A_iP^i]\}/[1 + \sum_{i=1}^{j}(A_iP^i)] \quad (1)$$

where $j = 3$ and 4, respectively, for the three-step and four-step oxygenation schemes.

In eq 1, P is the partial pressure of oxygen and A_i (i = 1, 2, 3, or 4) is a constant composed of several equilibrium constants of the intermediate reactions. In the four-step oxygenation scheme, K_i, the intrinsic oxygen binding constant of the ith oxygenation step, is related to A_i as follows: $A_1 = 4K_1$, $A_2 = 6K_1K_2$, $A_3 = 4K_1K_2K_3$, and $A_4 = K_1K_2K_3K_4$. In the three-step oxygenation scheme, the relationships between A_i and K_i are as follows: $A_1 = 3K_1$, $A_2 = 3K_1K_2$, and $A_3 = K_1K_2K_3$. The binding constants were estimated by fitting eq 1 to each oxygen binding curve with the least-squares curve fitting for nonlinear functions developed by Imai (1973, 1981). Since the details of the least-squares curve fitting procedures for estimation of the Adair constants have been given by Imai (1982), we only briefly describe the procedure here. The initial set of constants, which was subjected to successive least-squares iteration, was obtained by solving the four (or three for the three-step oxygenation) simultaneous equations for A_i with a set of four (or three for the three-step oxygen binding) experimental points (P_j, Y_j) substituted in eq 1. The remaining points were taken around 20% and 80% saturation for the four-step oxygenation and around 50% saturation for the three-step oxygenation. This set of A_i was subjected to successive iteration until the third significant figure of each A_i was not altered by further iteration. When the oxygen

Table I: Oxygen Binding Constants for Cross-Linked Hemoglobins in 0.1 M Phosphate Buffer at 25 °C[a]

sample	pH	K_1	K_2	K_3	K_4	RMS[b]
$(\alpha\beta)_A(\alpha\beta)_C$XL	7.41	0.3497 (0.0085)	0.1409 (0.0098)	0.2686 (0.0198)	2.802 (0.121)	8.1×10^{-5}
$(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL	7.47	0.680 (0.0623)	0.623 (0.0946)	2.77 (0.3022)		5.6×10^{-5}
$(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL	7.48	0.938 (0.0982)	0.974 (0.1600)	2.04 (0.235)		6.9×10^{-5}

[a] K_i's are in $Torr^{-1}$ and their calculated errors are given inside parentheses. [b] The root mean square of the residual of Y.

Table II: Calculated Cyanomet Subunit Contents of Each Sample before and after Oxygen Equilibrium Curve Measurements at pH 7.1

	before		after	
samples	optical density ratio OD_{577}/OD_{540}	cyanomet content (%)	optical density ratio OD_{577}/OD_{540}	cyanomet content (%)
$(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL	0.960	28	0.930	36
$(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL	0.980	23	0.940	33
$(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL	0.875	50	0.850	55
$(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL	0.873	50	0.824	61
$(\alpha^{+CN}\beta_2)_A$XL	0.864	53	0.820	62
$(\alpha_2\beta^{+CN})_A$XL	0.885	47	0.845	58

equilibrium curve was determined by Imai's method, the dependence of the standard error of Y was roughly simulated by a parabolic curve of $0.08Y(1 - Y)$ (Imai, 1973). Each ith data point was proportionally weighted to the inverse square of this standard error equation, as described by Imai (1973). The iteration procedure gave identical values of A_i's irrespective of the way of choosing the initial set of four (or three) experimental points. The values of the root mean square (RMS) of the residual of Y, a measure of the goodness of the curve fitting, were between 10^{-4} and 10^{-5}. K_i, P_m, P_{50}, n_{max}, and n_{50} were evaluated from the A_i. Table I gives the estimated O_2 binding constants for $(\alpha\beta)_A(\alpha\beta)_C$XL, $(\alpha^{+CN}\beta)_A$-$(\alpha\beta)_C$XL, and $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL at pH ~7.4 together with the calculated errors and the RMS of the residual of Y. It is difficult to estimate the standard errors from all sources in our measurements. However, repeated experiments under identical conditions and with different preparations of the samples suggest an estimate of approximately ±20% for the first and last K's and ±20 to ±30% for K's at the other oxygenation steps.

Table II summarizes the contents of cyanomet subunits calculated from the ratio of absorbance at 577 and 540 nm recorded before and after the O_2 equilibrium measurements. The proportion of cyanomet subunits is increased during the equilibrium measurements, which covered both the deoxygenation and reoxygenation processes, by about 10%. The total absorbance changes at 560 nm recorded during the deoxygenation process were always close to the expected values, while those obtained during the reoxygenation process were considerably larger than the calculated values, possibly due to extra oxidation during the measurements. Thus, we have used the data obtained during the deoxygenation process for our data analysis.

Results

Oxygen Equilibrium Curves of Cross-Linked Hbs. Figure 1A shows the Hill plot of the O_2 equilibrium curves at various pH values of the cross-linked Hb, $(\alpha\beta)_A(\alpha\beta)_C$XL, in which one of the two $\alpha\beta$ dimers comes from Hb A and the other from Hb C. The lines are calculated from the estimated K_i values by using eq 1. The curves in Figure 1A approach closely positioned asymptotes at the high-saturation range and diverge at the low-saturation range as seen in Hb A (Imai & Yonetani, 1975). The cross-linking between two $\alpha\beta$ dimers causes a reduction in cooperativity as measured by the Hill coefficient ($n = 2.3$ for the cross-linked Hb as compared to $n \approx 2.9$ for Hb A) and increased O_2 affinity as compared to that of Hb A. It should be mentioned that essentially the same Hill

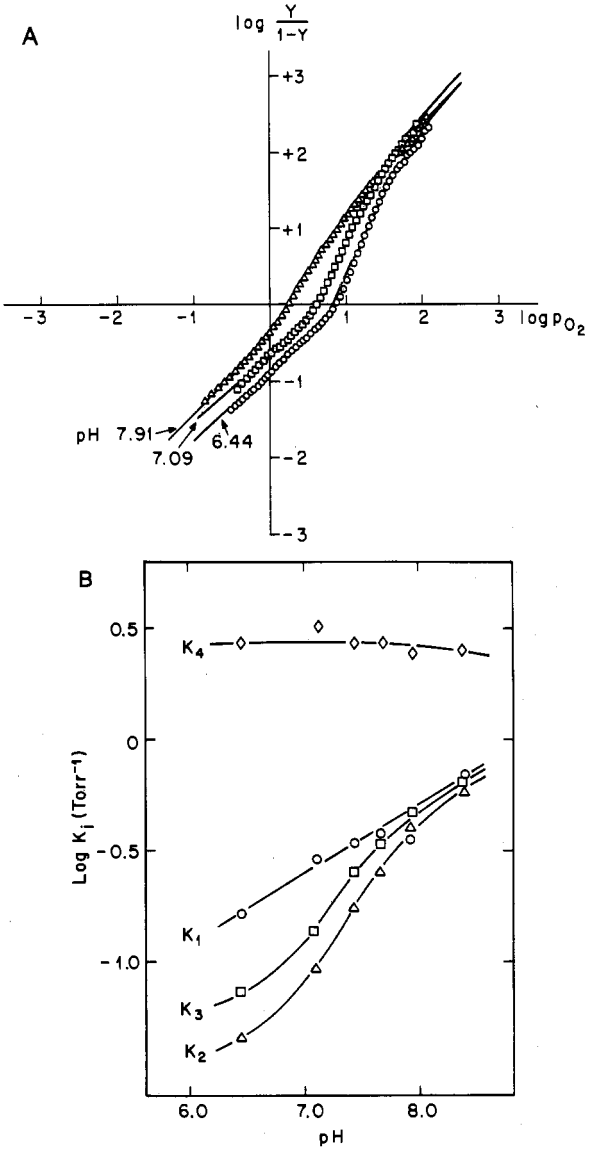

FIGURE 1: Oxygen equilibrium curves of cross-linked hemoglobin $[(\alpha\beta)_A(\alpha\beta)_C$XL], in 0.1 M phosphate buffer at various pH values: (A) Hill plots; (B) O_2 binding constants.

coefficient is obtained whether or not the two $\alpha\beta$ dimers are derived from Hb A or one $\alpha\beta$ is derived from Hb A and the other from Hb C (results not shown). The P_{50} value and Hill coefficient are not significantly affected by organic phosphate (results not shown), in agreement with the fact that one of the

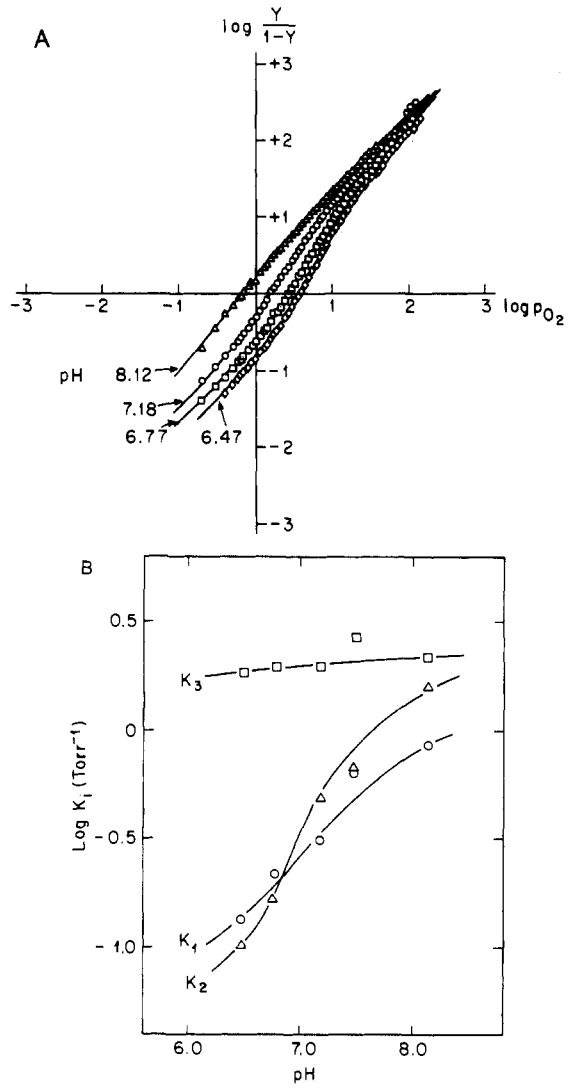

FIGURE 2: Oxygen equilibrium curves of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ in 0.1 M phosphate buffer at various pH values in the presence of 2 mM KCN: (A) Hill plots; (B) O_2 binding constants.

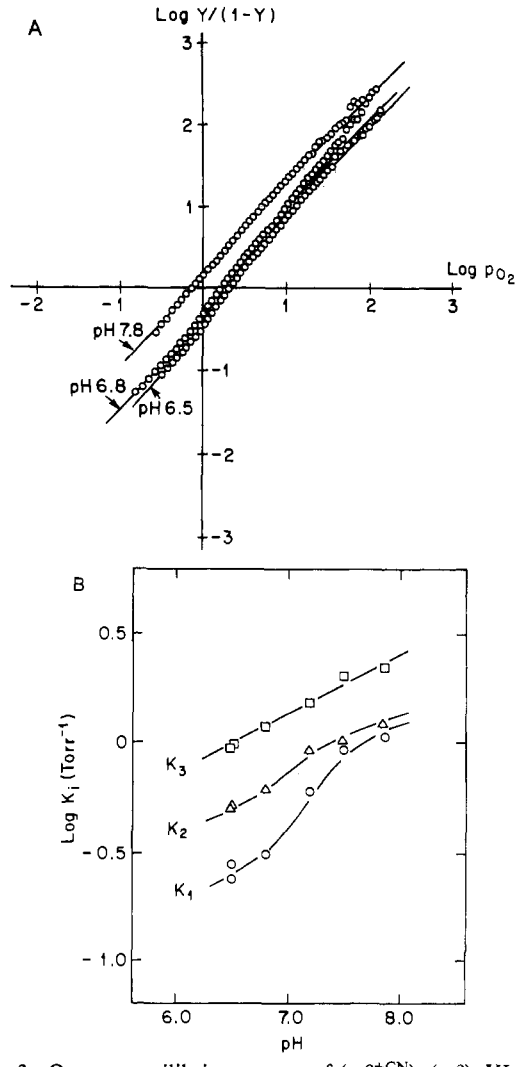

FIGURE 3: Oxygen equilibrium curves of $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$ in 0.1 M phosphate buffer at various pH values in the presence of 2 mM KCN: (A) Hill plots; (B) O_2 binding constants.

2,3-diphosphoglycerate (DPG) binding sites is occupied by the cross-linking reagent. Estimated K_i values are plotted against pH as shown in Figure 1B. A comparison of each estimated K_i value with that of Hb A shows the effects of the cross-linking on the intrinsic binding constants. The K_4 is essentially unaffected by the reaction with bis(3,5-dibromosalicyl) fumarate. The increase in O_2 affinity must be attributed to an increase of K_i's other than K_4. The pH dependence of K_1 is smaller than that of K_2 and K_3 as is seen in Hb A (Imai & Yonetani, 1975). However, the relative magnitudes of K_1, K_2, and K_3 are altered compared to those of Hb A. The relative magnitudes of K_i of cross-linked Hb are in the order of $K_2 \leq K_3 < K_1 \ll K_4$, while those of Hb A are in the order of $K_1 \leq K_2 < K_3 \ll K_4$. The number of protons released during oxygenation from cross-linked Hb (Bohr protons), which was derived from the pH dependence of P_{50} (Figure 4), is about 0.5 per heme, which is equal to that of Hb A.

Asymmetrical Hybrid Hbs with One Cyanomet Heme. Figure 2A illustrates the Hill plot of the O_2 equilibrium curves of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ in 0.1 M phosphate buffer at various pH values. The O_2 equilibrium curves converge at a high-saturation range as seen in $(\alpha\beta)_A(\alpha\beta)_CXL$ (Figure 1A), while the lower asymptotes diverge more than those of $(\alpha\beta)_A(\alpha\beta)_CXL$. The O_2 binding curve shows high cooperativity at low pH with a Hill coefficient value of 1.8. The estimated K_i (i = 1, 2, or 3) values are plotted against pH as shown in Figure 2B. K_3 of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ is observed to be less pH dependent than K_1 and K_2. Its value is very similar to those of K_4 of $(\alpha\beta)_A(\alpha\beta)_CXL$ and Hb A, which are essentially pH independent. The K_1 value of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ shows a pH dependence similar to that of K_2 or K_3 of $(\alpha\beta)_A(\alpha\beta)_CXL$ rather than that of K_1 of $(\alpha\beta)_A(\alpha\beta)_CXL$. P_{50} of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ is plotted as a function of pH as shown in Figure 4. The slope of the curve is about 0.6 at pH 7.1, which is steeper than that of $(\alpha\beta)_A(\alpha\beta)_CXL$, indicating that about 0.6 proton per ferrous heme and a total of 1.8 protons per tetramer of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ are released upon oxygenation. Since the Bohr effect on K_1 of $(\alpha\beta)_A(\alpha\beta)_CXL$ is about 0.2 (Figure 1), the number of 1.8 is comparable to that released by $(\alpha\beta)_A(\alpha\beta)_CXL$ after the initial ligation, suggesting that the same structural change as that of $(\alpha\beta)_A(\alpha\beta)_CXL$ would occur upon full oxygenation of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$.

The O_2 equilibrium curves of $(\alpha\beta^{+CN})_A(\alpha\beta)_CXL$ at various pH values are shown in Figure 3A. Introducing a cyanomet heme at the β subunit causes a drastic change in the O_2 equilibrium curve. The affinity for O_2 of this cross-linked hybrid Hb is considerably higher than that of $(\alpha^{+CN}\beta)_A(\alpha\beta)_CXL$ but significantly lower than that of isolated α or β subunits. The curve shows less cooperativity and gives an n value of 1.3 at pH 6.6. The pH dependence of the estimated O_2 binding constants is markedly different from that of

$(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL. K_1 and K_2 of $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL are much larger than those of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL at low pH. Each O_2 equilibrium constant increases upon raising the pH. Consequently, the Hill coefficient remains constant over the pH range examined (Figures 3B and 4). A large Bohr effect is still present in this hybrid Hb which has, in alkaline pH, a similar slope of P_{50} against pH to that of $(\alpha\beta)_A(\alpha\beta)_C$XL (Figure 4). Thus, the same number of protons per ferrous subunit as that from $(\alpha\beta)_A(\alpha\beta)_C$XL are released from $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL during oxygenation.

Asymmetrical Hybrid Hbs with Two Cyanomet Hemes. The O_2 equilibrium curves of asymmetrical cyanomet hybrid Hbs with two cyanomet subunits, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, have also been measured. However, it is not feasible to estimate reliable values of K_1 and K_2 by curve fitting. We have found that it is difficult to fit the O_2 binding scheme with two binding constants over the entire saturation range of the O_2 equilibrium curve. The difficulty comes possibly from the instability of the ferrous heme, which is readily oxidized to the met form. Thus, only the pH dependence of P_{50} and n are plotted in Figure 4, together with those of the other samples. Both of these asymmetrical hybrid Hbs with two cyanomet subunits show a low cooperativity, although the value of the Hill constant (n = 1.2) of $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL is slightly higher than that of $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL ($n$ = 1.1). The O_2 affinity of these two hybrids is much lower than that of the isolated α or β subunits of Hb A. No essential difference in affinity for O_2 is found between these two samples, so that only the data for $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL are plotted in Figure 4.

We have evaluated K_1 and K_2 of these asymmetrical hybrid Hbs with two cyanomet subunits by means of the equations:[2]

$$K_1 = \frac{(2/n_{max}) - 1}{P_{50}} \quad (2)$$

$$K_2 = \frac{1}{[(2/n_{max}) - 1]P_{50}} \quad (3)$$

Unfortunately, K_1 and K_2 of doubly ligated species, $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL, fail to represent the characteristics of K_3 and K_4 of $(\alpha\beta)_A(\alpha\beta)_C$XL. The values of log P_{50} of these models of the doubly ligated species are much closer to an average of log K_3 and log K_4 of $(\alpha\beta)_A(\alpha\beta)_C$XL than to the log P_{50} of isolated α or β subunits over the pH range examined. It is inferred that the affinity state of these doubly ligated species is different from that of fully ligated Hb but perhaps an intermediate affinity state. We do not know why the cooperativity of these intermediate species is greatly reduced. The symmetrical cyanomet valency hybrid Hbs, $(\alpha^{+CN}\beta)_2$ and $(\alpha\beta^{+CN})_2$, have also been shown to have reduced cooperativity (n = 1.1–1.2) and increased affinity for O_2 compared to Hb A (Cassoly & Gibson, 1972; Maeda et al., 1972; Hofrichter et al., 1985). Thus, none of the four models for doubly ligated species can represent the features of K_3 and K_4 of cross-linked Hb or Hb A.

[2] Derivation of eq 2 and 3:

$$n = \frac{\text{d log } [Y/(1 - Y)]}{\text{d log } P}$$

where $Y = (K_1P + K_1K_2P^2)/(1 + 2K_1P + K_1K_2P^2)$. When $K_1 = mK_2$, m is an enhancement factor, and

$$P_{50} = (K_1K_2)^{-1/2} = 1/(m^{1/2}K_2)$$

$n_{50} = 2/(1 + m^{1/2})$ and $P_{50} = [(2/n_{50}) - 1]^{-1}K_2^{-1}$. Since asymmetry of the oxygen binding curve reduces n_{50}, n_{max} is a better estimate than n_{50}.

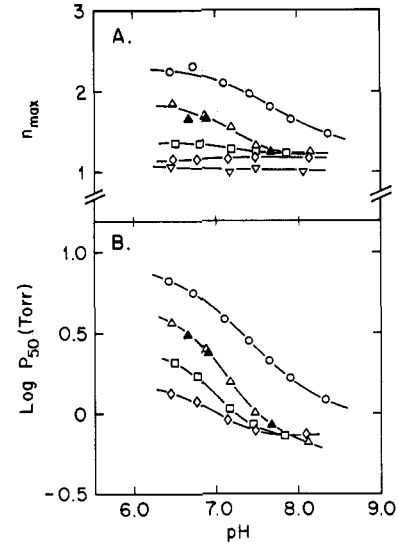

FIGURE 4: Effects of pH on parameters of oxygenation of cross-linked hybrid Hbs in 0.1 M phosphate buffer: (A) the Hill coefficient (n); (B) P_{50}. (○) $(\alpha\beta)_A(\alpha\beta)_C$XL, (△) $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, (□) $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, (▽) $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL, and (◇) $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL. Filled symbols denote samples with 2 mM IHP.

DISCUSSION

This study indicates that the overall O_2 binding property as measured by P_{50} is in the order of $(\alpha\beta)_A(\alpha\beta)_C$XL > $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL > $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL > $(\alpha^{+CN}\beta^{+CN})_A(\alpha\beta)_C$XL ≈ $(\alpha\beta^{+CN})_A(\alpha^{+CN}\beta)_C$XL. The results are consistent with the assumption that the subunit carrying cyanomet heme behaves like a model of an oxygenated subunit. The O_2 equilibrium properties of the cross-linked Hb are characterized by its high affinity for the first O_2 molecule. The perturbation due to the cross-linking between two $\alpha\beta$ dimers on the oxygen binding affinity appears to be not in the oxy quaternary structure but in the deoxy quaternary structure of Hb. The present results are consistent with the results of X-ray crystallography showing that there are small shifts toward the central cavity in E and F helices of cross-linked β subunits, because the cross-linking reagent is too short to span between two lysines at β82 in the deoxy structure (Walder et al., 1980).

The functional differences between the models of singly ligated Hbs, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL and $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, are prominent. That is to say, $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL has lower affinity for O_2 and is more cooperative than $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL. K_1 and K_2 of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL are similar in pH dependence to K_2 and K_3 of $(\alpha\beta)_A(\alpha\beta)_C$XL, respectively, while the three binding constants of $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL behave quite differently upon changing pH. In Figure 5, we have summarized the intrinsic O_2 binding constants of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL, and $(\alpha\beta)_A(\alpha\beta)_C$XL at pH 6.5. Clearly, there exists a good correlation between K_1, K_2, and K_3 of $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL and K_2, K_3, and K_4 of $(\alpha\beta)_A(\alpha\beta)_C$XL. On the contrary, the binding constants of $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL increase with each oxygenation step and no longer have any correlation with the O_2 binding constants of $(\alpha\beta)_A(\alpha\beta)_C$XL. The present results strongly suggest that only $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL would work as a model for the singly oxygenated cross-linked Hb. This implication is consistent with our earlier ^{1}H NMR investigations of the binding of O_2 to Hb A (Lindstrom & Ho, 1972; Johnson & Ho, 1974; Viggiano & Ho, 1979; Viggiano et al., 1979). The intensity of the exchangeable proton resonance at 9.3 ppm downfield from H_2O in deoxy $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL, due to the intersubunit hydrogen bond between the phenolic proton of tyrosine at α42 and aspartate at β99 in the $\alpha_1\beta_2$ interface (Fung & Ho, 1975), is

Table III: Hill Coefficients and P_{50} of Doubly Ligated Cyanomet Hybrid Hemoglobins and Equivalent Intermediates of Partially Oxygenated Hemoglobin

Hill coefficient n and P_{50} (mmHg)[a]					
$(\alpha^{+CN}\beta)_2$	$(\alpha\beta^{+CN})_2$	$(\alpha\beta^{+CN})(\alpha^{+CN}\beta)$	$(\alpha^{+CN}\beta^{+CN})(\alpha\beta)$	conditions	ref
		1.2 (0.81)[b]	1.1 (1.04)[b]	0.1 M phosphate, pH 7.1, at 25 °C	this work
1.17 (0.40)	1.10 (0.47)			0.1 M NaCl, pH 7.4, at 25 °C	Maeda et al. (1972)
			1.7 (1.75)	0.1 M phosphate, pH 7.4, at 25 °C	Imai (1982)
1.0[c]	1.0[c]	1.8[c]	1.8[c]	0.1 M NaCl, pH 7.4, at 21.5 °C	Smith and Ackers (1985)

Hill coefficient n and P_{50} (mmHg)[a]				
$(\alpha^{oxy}\beta)_2$	$(\alpha\beta^{oxy})_2$	$(\alpha\beta^{oxy})(\alpha^{oxy}\beta)$ and $(\alpha^{oxy}\beta^{oxy})(\alpha\beta)$	conditions	ref
1.8 (0.54)[d]	1.5 (2.53)[d]	1.3 (0.77)[d,e]	0.1 M phosphate, pH 7.0, at 15 °C	Imai et al. (1980)

[a] The P_{50} value is given inside the parentheses. [b] Samples are cross-linked between two dimers. [c] From the free energies for dimer–tetramer assembly reported by Smith and Ackers (1985), one can calculate n_{50} from the equations given in footnote 2 of this paper. [d] Calculated from the microscopic equilibrium binding constants of Hb A, which were estimated from the oxygen equilibrium curves of iron–cobalt hybrid Hbs. [e] The values are calculated as an average of two asymmetrical intermediates.

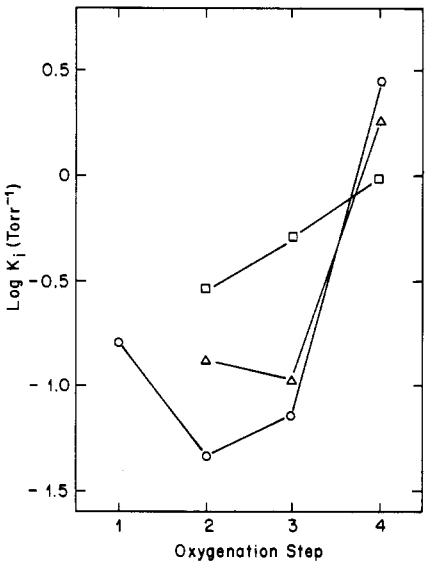

FIGURE 5: O_2 binding constants of cross-linked Hbs in 0.1 M phosphate buffer at pH 6.5 as a function of oxygenation step: (○) cross-linked Hb, $(\alpha\beta)_A(\alpha\beta)_C$XL; (△) $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL; and (□) $(\alpha\beta^{+CN})_A(\alpha\beta)_C$XL.

reduced by about half (Miura & Ho, 1982). Thus, the quaternary structure of deoxy $(\alpha^{+CN}\beta)_A(\alpha\beta)_C$XL manifested by the exchangeable proton resonance at 9.3 ppm should be considered to be in neither the deoxy nor the oxy quaternary structure (Miura & Ho, 1982).

Recently, Smith and Ackers (1985) have carried out an experimental determination of the cooperative free energies for the ten ligation states of Hb from measurements on the dissociation into dimers of tetramers in which each subunit is either in the unligated deoxy form or in the ligated cyanomet form. Their results suggest that each Hb tetramer acts as a three-level molecular switch. Their results further suggest that the following hybrid Hbs should be cooperative in their ligation process: $(\alpha^{+CN}\beta)(\alpha\beta)$, $(\alpha\beta^{+CN})(\alpha\beta)$, $(\alpha^{+CN}\beta^{+CN})(\alpha\beta)$, and $(\alpha^{+CN}\beta)(\alpha\beta^{+CN})$. The Hill coefficients for our analogous singly ligated cross-linked hybrid Hbs are in good qualitative agreement with their free energy determinations, but there are considerable discrepancies among the published Hill coefficients of various doubly ligated hybrid Hbs. Table III summarizes the published Hill coefficients and P_{50} values of various doubly ligated Hbs. The reasons for their discrepancies are not understood.

In conclusion, our present data on the O_2 equilibrium measurements of various cross-linked mixed valency hybrid Hbs have provided us with a deeper insight into the functional properties of partially oxygenated species of Hb A during the cooperative oxygenation process. In addition, they also provide a strong indication that various structural changes associated with the oxygenation of Hb A observed in ^{1}H NMR studies by Ho and co-workers (Viggiano & Ho, 1979; Viggiano et al., 1979) as well as ^{1}H NMR studies of cross-linked mixed valency asymmetrical hybrid Hbs by Miura and Ho (1982) are both energetically and functionally important. Thus, the present studies on O_2 binding properties and our previous ^{1}H NMR results with cross-linked mixed valency hybrid Hbs and Hb A together with the recent determination of the cooperative free energies of the ligation states of Hb reported by Smith and Ackers (1985) strongly suggest that there are at least three functional and energetically important structures of Hb A in going from the deoxy to the oxy state. However, it should be emphasized that much work is needed to clarify various published data on the oxygenation of hybrid Hbs and to understand the structural and functional properties of intermediate ligated species formed during the oxygenation of hemoglobin.

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Limitations of *N*-Hydroxysuccinimide Esters in Affinity Chromatography and Protein Immobilization†

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ABSTRACT: The carbodiimide-mediated reaction of *N*-hydroxysuccinimide with carboxyl groups immobilized to hydroxyl-containing polymers (such as Sepharose or Trisacryl) leads to an undesirable side reaction in high yields. The product of this reaction interferes with the application of such columns for further affinity-based purification. In addition to the desired *N*-hydroxysuccinimide ester, a bis(*N*-hydroxysuccinimide) derivative of β-alanine [namely, *N*-[(succinimidooxy)carbonyl]-β-alanine *N*-hydroxysuccinimide ester] is probably produced that reacts subsequently with the hydroxyl group of the polymer via ester and carbamate bonds. These β-alanine derivatives are formed upon interaction of dicyclohexylcarbodiimide with 3 equiv of *N*-hydroxysuccinimide followed by a Lossen rearrangement. The amount of β-alanine thus coupled is very high compared to the number of carboxyl groups present on the resin. The β-alanine bound through the ester bond comprises about 90% of the β-alanine bound. Alkaline treatment of the ester-bonded β-alanine-containing polymers (prior to coupling of amino-containing ligands) causes a rearrangement yielding β-alanine with a free carboxyl group coupled through a stable carbamate linkage. After coupling of amino-containing ligands, the above-described rearrangement cannot occur, and the β-alanine-linked ligand leaks from the polymer via hydrolysis of the ester bond. The newly formed carboxyl groups (derived from the rearrangement) can be used to prepare active esters. In view of the above, we developed methods for the preparation of nitrophenyl esters as well as *N*-hydroxysuccinimide esters free of unstable β-alanine derivatives on polymers containing hydroxyl groups. Upon coupling with amino-containing ligands, these esters yield resins bearing chemically stable bonds.

N-Hydroxysuccinimide (NHS)[1] esters were introduced by Anderson et al. (1964) and are widely used as coupling agents in peptide synthesis. These esters were later introduced by us for the modification of lysine residues on proteins (Becker et al., 1971) and cells (Becker & Wilchek, 1972). NHS esters are also widely used to activate carboxyl groups on spacer arms bound to agarose (Cuatrecasas & Parikh, 1972). Affinity gels containing NHS ester are commercially available and commonly used (Wilchek et al., 1984).

Recently, during an affinity study for the isolation of receptors and lymphokinases, we needed activated supports containing spacer arms that give stable products. We therefore applied both commercially available and "homemade" NHS derivatives of agarose and Trisacryl (Miron & Wilchek, 1985). To our surprise, upon reaction with ligands containing an amino group, all the gels yielded columns that were unstable to alkali. All of these columns were plagued by constant leakage during use. Furthermore, upon total hydrolysis of the activated gels or their coupled derivatives, a new amino acid appeared in large quantities. Leakage of ligands from affinity columns is a serious problem in affinity chromatography (Wilchek et al., 1975). This is particularly true when minute amounts of different biologically active factors are being pu-

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[1] Abbreviations: NHS, *N*-hydroxysuccinimide; ε-cap, ε-aminocaproic (or 6-aminocaproic) acid; DCC, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenyl.

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