THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 24, Issue of December 25, pp. 12390-12398, 1979
Printed in U.S.A.

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The Linkage between Oxygenation and Subunit Association in Human Hemoglobin Kansas

CONCENTRATION DEPENDENCE OF THE OXYGEN BINDING EQUILIBRIA*

(Received for publication, June 19, 1979)

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The oxygen binding equilibria of human hemoglobin Kansas (a variant with a substitution at the $\alpha_1\beta_2$ interface) has been measured as a function of protein concentration with an automated Imai apparatus and a Gill cell. Measurements were made at 20°C in 0.05 M Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM EDTA. The overall oxygen affinity of the hemoglobin decreases greatly when the concentration is increased from 0.36 μM to 6.2 mM heme; the pressure of half-saturation increases from 4.7 to 32 mm Hg. The Hill coefficient at half-saturation is near 1.5 at all concentrations. These data were combined with an earlier determination of the dimer-tetramer association constant (Atha, D. H., and Riggs, A. (1976) *J. Biol. Chem.* 251, 5537-5543) and analyzed with the linkage relations (Ackers, G. K., and Halvorson, H. R. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 4312-4316) to yield changes in the free energies of the formation of the intersubunit contacts and of ligand binding for the dimer and tetramer at successive oxygenation steps. The results show that only a small degree of cooperativity is manifested in the differences between the successive steps of oxygen binding by hemoglobin Kansas. The β subunit, known to have an intrinsically low affinity for oxygen (Riggs, A., and Gibson, Q. H. (1973) *Proc. Natl. Acad. Sci. U. S. A.* 70, 1718-1720), appears to contribute pseudonegative cooperativity to the oxygen binding by both the dimers and the tetramers. A small positive contribution to the cooperativity occurs in the association equilibrium between the high affinity dimeric species and the low affinity tetrameric species.

The $\alpha_1\beta_2$ and $\alpha_2\beta_1$ intersubunit contact regions are believed to play a major role in the mechanism of hemoglobin cooperativity (1-3). The variant human hemoglobin Kansas is of particular interest because its substitution (β_{102}, asparagine → threonine) (4) is in this contact region. Previous studies have shown that hemoglobin Kansas has an unusually low affinity for oxygen, a low Hill coefficient, and a high degree of subunit dissociation of the ligated form (4-6). In human hemoglobin A, precise measurements of the dimer-tetramer association (7) and of the concentration dependence of the oxygen binding curves (8, 9) have yielded values for the changes in the free energies for the formation of the intersubunit contacts and for the successive steps of binding oxygen to dimer and tetramer. These studies are independent of mechanistic models and thus provide thermodynamic constraints for possible models of the mechanism of action of hemoglobin. We report in this paper similar measurements of hemoglobin Kansas designed to determine what effect the β_{102} substitution has on these constraints and to what extent we can explain the differences between hemoglobins A and Kansas.

* This work was supported by Grants PCM 76-06719 from the National Science Foundation and GM 21314 from the National Institutes of Health, and a Robert A. Welch Foundation Grant F-213. A preliminary account of this work has appeared in abstract form (Atha, D. H., Riggs, A., and Johnson, M. L. (1979) *Biophys. J.* **25,** 37a. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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MATERIALS AND METHODS

Hemoglobin Kansas was prepared as described previously (4) except that the blood sample came from the mother.

Oxygen binding equilibria were measured by two techniques. Hemoglobin solutions in the range 3×10^{-7} to 4×10^{-4} M heme were measured with an Imai apparatus as described (10) except that the inside surface of the stainless steel cell was coated with Pyre M. L. enamel (Dupont No. RC5019) which greatly reduced methemoglobin production. A Yellow Springs Instruments oxygen electrode (No. 5331) was used instead of the Beckman No. 39065 electrode. The electrode was calibrated and checked for linearity by equilibrating with standard O_2/N_2 gas mixtures (Matheson, Inc.) (11). Hemoglobin solutions in the range 6×10^{-5} to 6×10^{-3} M were measured with a Gill apparatus as described (12) except that the cell holder was mounted to the bottom of the cell compartment of the Cary 118C so that it could be interchanged easily with the Imai cell assembly. All measurements were done at 20°C in 0.05 M Tris/HCl buffer of pH 7.5 containing 0.1 M NaCl and 1 mM EDTA. The hemoglobin solutions (approximately 10 ml) in the Imai cell and (approximately 2.6 μl) in the Gill cell contained the components of the methemoglobin reductase system (13) at concentrations specified previously (11). Solutions of carbonmonoxy hemoglobin to be used in the Imai apparatus were converted to the oxygenated form by equilibrating them with purified oxygen at pH 7.5, 0°C, in a glass rotary evaporator under a high intensity sun lamp (Sylvania No. SG-50 with a General Electric DWY bulb). Solutions to be used in the Gill apparatus were converted to the oxygenated form within the cell by equilibration at 20°C with pure oxygen and illuminating the cell with the sun lamp. This was accomplished by placing a metal mirror within the cell compartment of the spectrophotometer so that light from the sun lamp placed above would be reflected horizontally through the cell. Spectra were measured after each procedure to ensure complete conversion to the oxygenated form. The barometric pressure was measured during calibration of the electrode and during the oxygen binding experiments. Corrections were made for the vapor pressure of water and

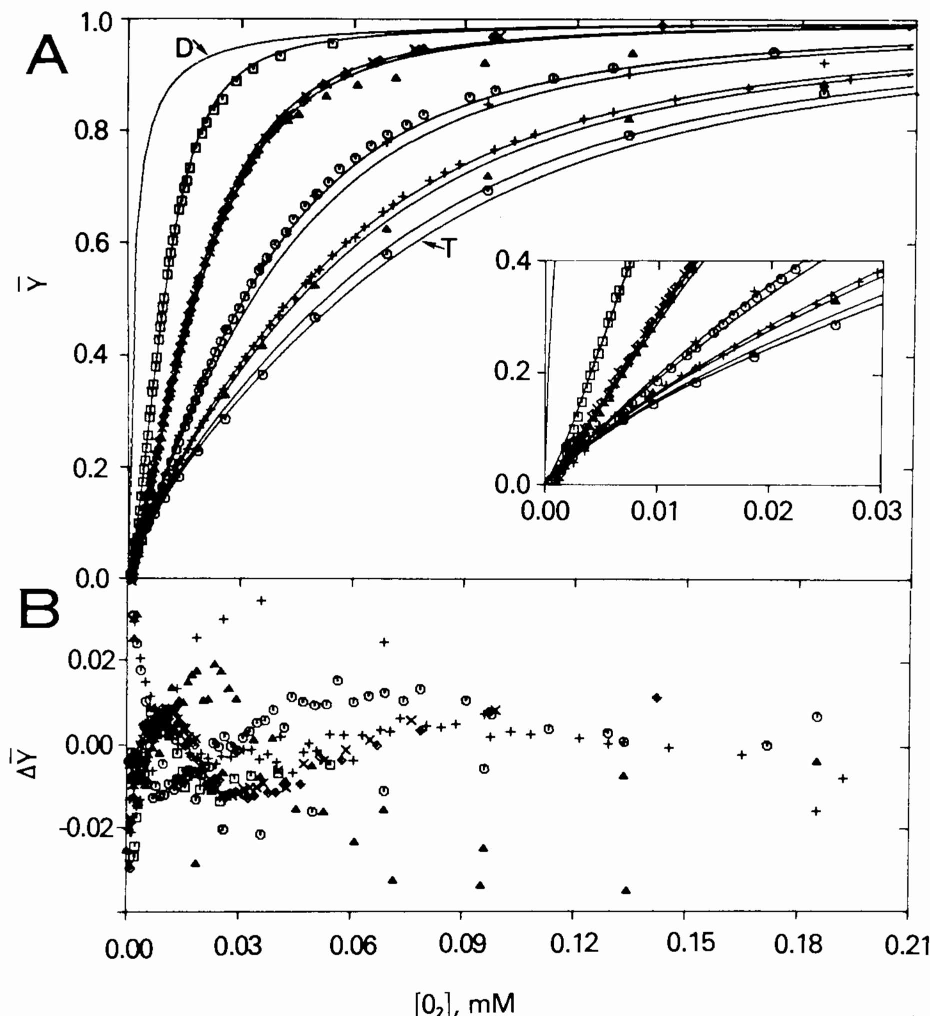

FIG. 1. **Dependence of the oxygen binding curves of hemoglobin Kansas on protein concentration.** *A*, $\bar{Y}$ *versus* $[O_2]$ at various protein concentrations. Symbols show data from oxygen binding curves obtained at a fixed protein concentration and wavelength. The protein concentrations are given in Table I and decrease from *right* to *left*. Symbols used are 6.24×10^{-3} M heme (⊙) [Gill cell]; 6.24×10^{-4} M (▲) [Gill cell]; 3.82×10^{-4} M (+); 6.24×10^{-5} M (+) [Gill cell]; 4.28×10^{-5} M (⊙); 4.28×10^{-6} M (▲); 3.62×10^{-6} M (× and ◆); and 3.62×10^{-7} M (⊡). The *solid lines* are a least squares computer fit of the data (see text and Table II, Case B). *Curves D* and *T* are for dimer and tetramer, respectively. *B*, difference between data calculated from the values in Table II, Case B and the experimental data. The variance of the fit was 1.35×10^{-4}.

oxygen concentrations were calculated using the Henry's law constant at 20°C: 1.82×10^{-6} M O_2/mm Hg.[1]

The data were analyzed by a least squares fitting program described previously (18). This program uses a linkage function which couples oxygen binding and subunit association (19). The fractional saturation is expressed as

$$\bar{Y} = \frac{(Z'_2 + Z'_4\sqrt{Z_2^2 + 4\,{}^0K_2Z_4[P_t]} - Z_2)/4Z_4}{Z_2 + \sqrt{Z_2^2 + 4\,{}^0K_2Z_4[P_t]}} \quad (1)$$

where Z_2 and Z'_2 are functions of the oxygen binding constants for dimers and Z_4 and Z'_4 are the corresponding functions for oxygen binding to tetramers, $[P_t]$ is the protein concentration expressed as molar heme, and ${}^0K_2$ is the dimer to tetramer subunit association constant. (See "Appendix" for exact definitions of constants.)

The confidence regions of both fitted and derived parameters are calculated by searching the variance space for an F statistic corresponding to a 65% probability (18). These regions are expected to be asymmetric and are thus expresed as a range instead of a single ± value.

TABLE I

Log P_{50} and n_{50} for hemoglobin Kansas at various protein concentrations

Measurements were made at 20°C in 0.05 M Tris/HCl, pH 7.5, containing 0.1 M NaCl and 1 mM EDTA.

Hemoglobin concentrations	Wavelength	log P_{50}	Hill coefficient
M Heme	*nm*	*mm Hg*	n_{50}
6.24×10^{-3}	560	1.51[a]	1.4
6.24×10^{-4}	560	1.46[a]	1.3
3.82×10^{-4}	700	1.40	1.5
6.24×10^{-5}	415	1.18[a]	1.5
4.28×10^{-5}	560	1.22	1.5
4.28×10^{-6}	560	0.97	1.7
3.62×10^{-6}	560	1.0	1.6
3.62×10^{-6}	560	0.97	1.4
3.62×10^{-7}	415	0.67	1.6

[a] Measurements were made with a Gill cell.

RESULTS

The oxygen binding equilibria of hemoglobin Kansas at each of eight protein concentrations between 6.24×10^{-3} M heme and 3.62×10^{-7} M heme are shown in Fig. 1*A*. The solid lines were generated from a least squares fit of these data as described in the following section. The limiting oxygen binding curves for the tetramer and dimer, calculated from the least squares fit, are included for comparison.

The oxygen pressure at half-saturation, P_{50}[2] and Hill coefficient at half-saturation, n_{50}, calculated from the data in Fig. 1 are given in Table I. The data show a large increase in

[1] This widely quoted, 50-year-old number is from the International Critical Tables (14). We have used it to be consistent with other workers in the field. More recent values for pure water, summarized by Battino and Clever (15) and Wilhelm *et al.* (16) average 1.77×10^{-6} M O_2/mm Hg at 20°C, which is 2.9% lower. The value under our conditions may be even lower since the value obtained in 0.1 M chloride is about 6% lower (17). However, since all our measurements refer back to the oxygen pressure which reflects the oxygen activity, no correction for differing salt conditions would be appropriate.

[2] The abbreviations used are: P_{50}, the oxygen pressure in mm Hg at half-saturation; n_{50}, the Hill coefficient at half-saturation.

TABLE II
Fitted parameters in the analysis of oxygen binding to hemoglobin Kansas

	Case A	Case B	Case C	Case D
$\ln {}^0K_2$	23.56[a]	23.49 (23.44, 23.55)	23.56[a]	23.56[a]
$\ln K_\alpha$	14.65 (14.42, 14.87)[b]	14.25[c]	14.28 (14.26, 14.31)	14.25[c]
$\ln K_\beta$	12.49 (12.26, 12.76)	12.87[d]	12.87[d]	12.90 (12.88, 12.93)
$\ln {}^4K_2$	9.10 (8.87, 9.29)	9.12 (8.93, 9.27)	9.12 (8.93, 9.25)	9.13 (8.94, 9.27)
${}^0K_2/{}^1K_2$	32.82 (23.48, 41.42)	22.89 (19.65, 25.20)	23.53 (20.66, 26.00)	23.07 (19.81, 25.41)
${}^3K_2/{}^4K_2$	16.13 (9.01, 24.68)	21.50 (11.43, 36.07)	21.57 (11.97, 36.51)	21.81 (11.97, 37.11)
$\ln k_{43}$	9.13 (8.46, 9.93)	9.17 (8.58, 10.02)	9.16 (8.61, 10.02)	9.15 (8.60, 10.00)
Variance	1.30×10^{-4}	1.35×10^{-4}	1.35×10^{-4}	1.36×10^{-4}
Highest cross-correlation	0.9953	0.9574	0.9584	0.9611

[a] Fixed parameter; assumed to be 1.7×10^{10} M^{-1} (dimer) (6).
[b] The asymmetrical confidence region corresponding to a 65% probability is given in parentheses following the parameter.
[c] Fixed parameter; assumed to be the affinity of an isolated α chain; *i.e.* −8.27 kcal/mol of O_2 (20).
[d] Fixed parameter; assumed to be the affinity of an isolated β chain; *i.e.* −7.47 kcal/mol of O_2 (20).

affinity without a large change in Hill coefficient as the protein concentration is lowered from 6.24×10^{-3} to 3.62×10^{-7} M heme.

Analysis of Oxygen Binding Data—Seven equilibrium constants are required to describe the linkage between oxygenation and subunit association, as given in Equation 1. These constants can be expressed in different ways, some of which are more useful than others in describing functional properties of hemoglobin (18). The problem of fitting experimental data to seven parameters can be simplified by independent determination of some of the parameters. Therefore, we have chosen a parameter set which includes those which can be independently measured as well as those which are of greatest interest for a complete thermodynamic description of oxygen binding and subunit association of hemoglobin Kansas. The values of the subunit association constants for the unligated and CO forms of hemoglobin Kansas, ${}^0K_2$ and ${}^4K_2$, respectively, have been previously determined to be 1.7×10^{10} M^{-1} (dimer) and 2×10^4 M^{-1} (dimer), respectively (6). Similarly, the oxygen binding free energy changes of α and β chains isolated from hemoglobin Kansas have been reported to be −8.27 kcal/mol and −7.47 kcal/mol (20). Perutz (3) has shown that very little or no structural change at the $\alpha_1\beta_1$ contact region occurs upon oxygenation of hemoglobin A which implies that the dimer of hemoglobin should be noncooperative. Experimental results on hemoglobin A are consistent with this (11, 21, 22). We can relate the values of K_{21} and K_{22} to the independently measured binding constants of the isolated α and β chains, K_α and K_β by assuming that the hemoglobin Kansas dimer has 2 nonequivalent and noninteracting oxygen binding sites with the same affinity as the isolated chains. The ligand binding isotherm for such a dimer is

$$\bar{Y}_2 = \frac{1}{2}\frac{(K_\alpha + K_\beta)[X] + 2K_\alpha K_\beta[X]^2}{1 + (K_\alpha + K_\beta)[X] + K_\alpha K_\beta [X]^2} \quad (2)$$

where $[X]$ is the free ligand concentration. We note that in this binding isotherm, K_{21} is the sum of the binding constants of the isolated chains and K_{22} is the product of these binding constants. Thus, four equilibrium constants of the seven required to define the linkage system have been previously measured independently. The logarithms of these equilibrium constants were used as four of the fitting parameters; *i.e.* $\ln {}^0K_2$, $\ln {}^4K_2$, $\ln K_\alpha$, and $\ln K_\beta$. The use of the logarithms restricts the parameters to positive values and is more easily related to the free energy changes. Other parameters of primary interest, the ratios ${}^0K_2/{}^1K_2$ and ${}^3K_2/{}^4K_2$, correspond to the changes in the intersubunit contact-free energy upon oxygenation. The only remaining fitting parameter required to complete the energy scheme is the stepwise binding constant of the third oxygen to the tetrameric form; we used $\ln k_{43}$.

Because of the large value of the association constant of the deoxygenated subunit, ${}^0K_2$, only a very small fraction of the hemoglobin will occur as deoxygenated dimer in our oxygen binding experiments. Consequently, it is expected that $\ln {}^0K_2$ should be highly correlated with $\ln K_\alpha$ and $\ln K_\beta$. Such a correlation implies that these values will be very sensitive to small experimental errors and thus unreliable. A complete least squares analysis of all seven parameters was attempted. Although the variance of the fit was 1.32×10^{-4} ($\bar{Y}$ units), the fit was deemed to be poor because of the expected and observed high cross-correlation between the parameters, *i.e.* 0.9999.[3]

The value of the association constant for the unligated subunit, ${}^0K_2$, from the seven parameter fit was 2.3×10^9 M^{-1} (65% confidence range, 2.1×10^9 to 2.4×10^9). Fixing this parameter at the independently measured value of 1.7×10^{10} M^{-1} did not significantly change the variance of the fit; 1.3×10^{-4} (see Table II, Case A). The values of the oxygen binding free energies of the individual chains of the dimer, obtained from the fit with ${}^0K_2$ fixed at 1.7×10^{10} M^{-1}, were −8.50 kcal/mol (−8.37 to −8.63) and −7.25 kcal/mol (−7.12 to −7.39). These values are quite close to the previously published values for the isolated chains; −8.27 kcal/mol and −7.47 kcal/mol (20). The cross-correlation between $\ln K_\alpha$ and $\ln K_\beta$ was still found to be high: 0.9953. Consequently, either $\ln K_\alpha$ or $\ln K_\beta$ must also be independently known in order to resolve the linkage system uniquely.

Three least squares fits were performed by fixing pairs of the independently determined values. A tabulation of the fitted parameters is given in Table II: Case B, fixing $\ln K_\alpha$ and $\ln K_\beta$; Case C, fixing $\ln {}^0K_2$ and $\ln K_\beta$; and Case D, fixing $\ln {}^0K_2$ and $\ln K_\alpha$. This table shows that the results of fitting for each of these three cases are essentially identical, indicating that any choice of two fixed parameters among these three is equally successful. Furthermore, the three cross-correlation coefficients have decreased significantly which suggests that the least squares procedure should give reliable values for the determined parameters.

Table III gives the Adair oxygen binding constants for dimeric and tetrameric hemoglobin Kansas, Case B. Case B has been arbitrarily selected for these constants because it gave a slightly lower variance and cross-correlation coefficient. The *solid lines* in Fig. 1*A* were calculated from the values for Case B. Fig. 1*B* gives the distribution of residuals of Case B, which appear to be random. It should be noted that any one

[3] The cross-correlation coefficient is calculated as previously described (18). The possible range of values is ±1. By simulation studies with synthetic data, we have observed that if the absolute value of the cross-correlation coefficient is greater than approximately 0.96, the least squares analysis may be unreliable.

oxygen binding curve will be expected to show a high correlation from one data point to the next because of the method of data collection. The important measure of randomness is, therefore, the randomness of the entire set of data and not the individual curves.

One test for the accuracy and internal consistency of these experimentally determined values is to compare them, where possible, with independently determined values. The free energy change for the association of unligated dimers is only 0.1 kcal/mol less than the independently measured value (6). The corresponding free energy change for oxygenated hemoglobin Kansas has not been measured independently, but our value of −5.29 kcal/mol is 0.46 kcal/mol less than the CO subunit association free energy (6). This deviation could be due to the difference between CO and O_2. The values of k_{41}, 0.11 mm Hg^{-1}, and k_{44}, 1.66 mm Hg^{-1}, have been independently determined at 10°C by extrapolation of oxygen binding data at low and high oxygen concentrations (23, 24). These would correspond to intrinsic free energy changes of −6.84 and −6.78 kcal/mol, respectively, at 10°C. A direct comparison of these oxygen binding free energy changes cannot be made at one temperature since the corresponding enthalpy changes have not been measured for hemoglobin Kansas. However, in light of this temperature difference, the values of Shulman *et al.* (23) and Kilmartin *et al.* (24) are in agreement with our values and support the accuracy of the numerical values given in Table II.

TABLE III

Constants derived from least squares analysis of hemoglobin Kansas data

Parameter	Equilibrium constant[a]	Free energy change[b]
K_{21} and k_{21}	1.93×10^{6c}	-8.40^{c}
K_{22}	6.00×10^{11c}	-15.74^{c}
K_{41} and k_{41}	8.44×10^4 (7.67, 9.83)[d]	−6.58 (−6.53, −6.67)
K_{42}	1.50×10^9 (0.93, 1.82)	−12.26 (−11.99, −12.38)
K_{43}	1.43×10^{13} (0.84, 2.09)	−17.58 (−17.27, −17.80)
K_{44}	2.07×10^{17} (1.76, 2.39)	−23.14 (−23.05, −23.23)
k_{22}	3.10×10^{5c}	-7.34^{c}
k_{42}	1.77×10^4 (0.95, 2.16)	−5.68 (−5.32, −5.79)
k_{43}	9.56×10^3 (5.31, 22.43)	−5.32 (−4.98, −5.82)
k_{44}	1.44×10^4 (0.86, 2.71)	−5.56 (−5.26, −5.93)

[a] Units are expressed as molar oxygen and molar dimer; see text for details.

[b] Units are expressed as kilocalories/mol of dimer or O_2; see text for details.

[c] Taken to be the best available experimental values; see text for details.

[d] The asymmetrical confidence region corresponding to a 65% probability is given in parentheses following the parameter.

The same data and least squares fit which appear in Fig. 1 are presented as Hill plots in Fig. 2. The *solid lines* in Fig. 2 were calculated from Case B. In view of the fact that the upper and lower ends of the plot are expanded relative to the curves in Fig. 1, the overall fit of the data to the linkage model is quite good. It should also be noted that the data obtained using the Gill cell and the Imai cell give very similar results where their ranges of protein concentrations overlap. This provides a check on the accuracy of the two independent techniques.

The data obtained at high protein concentration indicate that the slope of the Hill plot, n, is less than unity at low oxygen concentrations and is greater than unity at higher oxygen concentrations (see Figs. 2 and 3). The Hill plot for the calculated dimer equilibrium shows that $n < 1$, an apparent negative cooperativity (see Fig. 3). This shape results from nonidentical binding affinities for the α and β chains of the dimer, as has been observed for the isolated α and β chains of hemoglobin Kansas (20).

Fig. 4 shows the weight fraction of dimer plotted as a

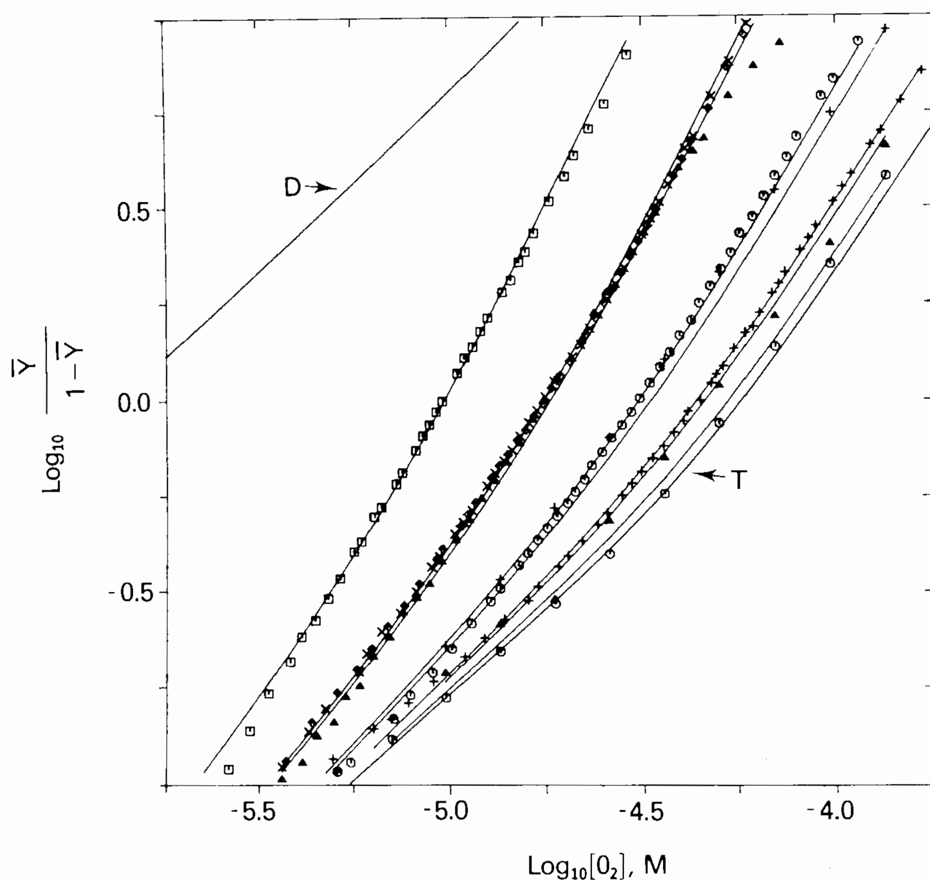

FIG. 2. **Hill plots of oxygenation data of hemoglobin Kansas.** The data and symbols are identical with those shown in Fig. 1.

function of fractional saturation at 60 μM heme. These calculations were based on the values of Case B and are compared with those found previously for hemoglobin A (11). Hemoglobins A and Kansas have essentially the same low degree of dissociation at less than 20% saturation, but hemoglobin Kansas is progressively more dissociated than hemoglobin A above this level.

Fig. 5 shows that the fraction of dimer in totally oxygenated solutions of hemoglobins Kansas and A is substantial even at relatively high protein concentrations. It has previously been shown that neglecting the few per cent of dimer present in hemoglobin A at 1 mM heme is sufficient to cause as much as 0.4 kcal/mol error in the Adair constants (25). Consequently, Fig. 5 demonstrates that it is impossible in an Imai apparatus or a Gill cell to measure oxygen binding data at a sufficiently high concentration of hemoglobin Kansas to avoid the necessity of including the dimeric species in the data analysis. Even in the intact red blood cell, oxygenated hemoglobin Kansas would be about 7% dissociated if present to the extent of 50% of the total hemoglobin.

The weight fraction of the various oxygenated species of hemoglobin Kansas at the lowest protein concentration employed, 0.36 μM, is given in Fig. 6. At low fractional saturation, the hemoglobin Kansas is predominately unligated tetramer. As the fractional saturation increases, the unligated tetramer is replaced predominately by singly ligated tetramer although

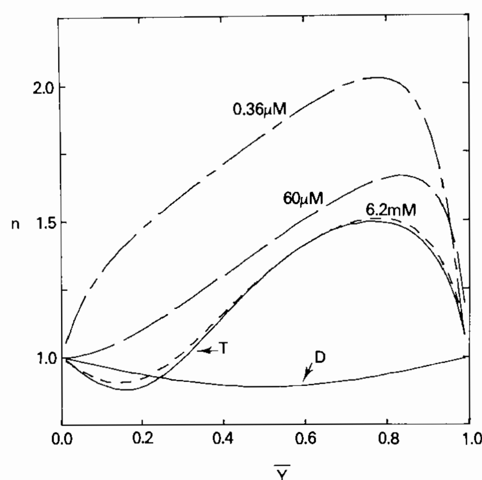

FIG. 3. **Slope of the Hill plots as a function of fractional saturation estimated from the equilibrium constants tabulated in Table II, Case B for various concentrations of hemoglobin Kansas.** Estimates for dimeric and tetrameric species are shown as *solid lines* (*D* and *T*, respectively). Protein concentration is expressed as molar heme.

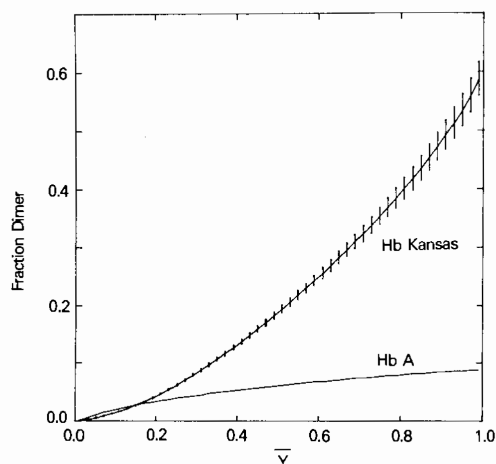

FIG. 4. **A comparison of the weight fraction of dimer present in solutions of hemoglobins *A* and *Kansas* as a function of fractional saturation of oxygen.** The hemoglobin concentration is fixed at 60 μM heme and the equilibrium constants tabulated in Tables IV and V. *Vertical bars* correspond to propagated standard errors.

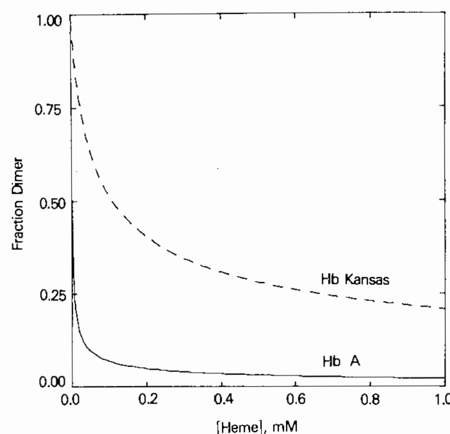

FIG. 5. **A comparison of the weight fraction of dimer present in totally oxygenated solutions of hemoglobins *Kansas* and *A* as a function of protein concentration.**

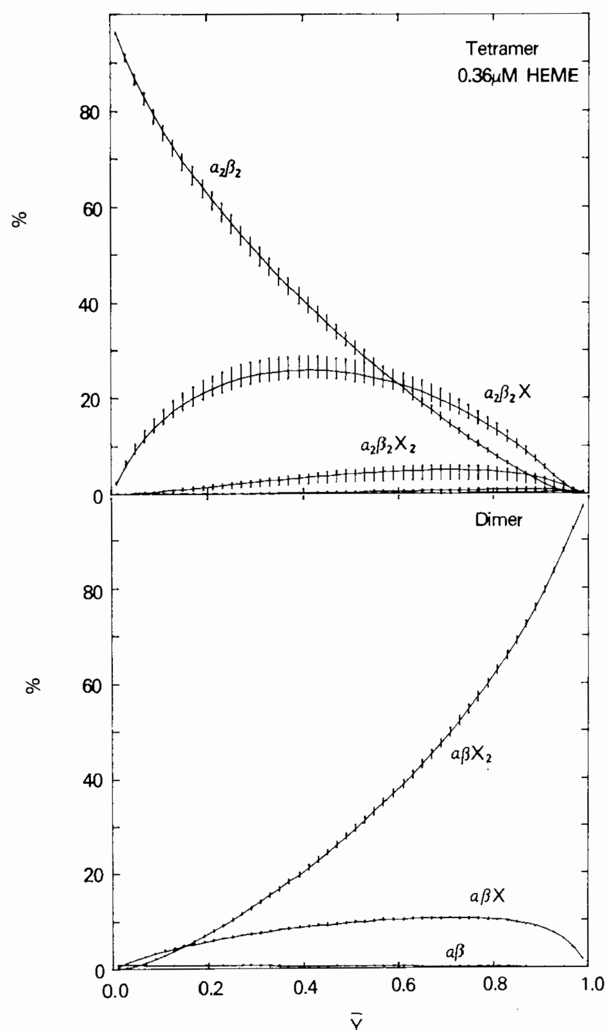

FIG. 6. **Estimated distribution of tetrameric and dimeric species of hemoglobin Kansas as a function of fractional saturation at 0.36 μM heme.** Calculated from the equilibrium constants in Table II, Case B. Triply and quadruply liganded hemoglobin does not occur in significant amounts. *Vertical bars* correspond to propagated standard errors.

doubly ligated dimer is also increasing. Above a fractional saturation of 0.4 all of the tetrameric species are decreasing and the doubly ligated dimer continues to increase almost to 100%. Doubly, triply, and fully ligated tetramer and unligated dimer do not occur in significant amounts. Singly ligated dimer is never more than approximately 10%. As the hemoglobin concentration is increased to 60 μM heme, the same species predominate (Fig. 7). However, the amounts of the dimeric species are decreased and the amounts of the higher ligated tetrameric species are increased. The unligated dimer never occurs in a significant amount. At the highest hemoglobin Kansas concentration studied, 6.2 mM, the dimeric species never exceeded 10% (Fig. 8).

Since unligated and singly ligated dimers do not occur in significant amounts (Figs. 6 to 8), the equilibria involving them should be very difficult to resolve. Consequently, one would expect the observed high cross-correlation coefficients encountered while curve fitting for ln K_α, ln K_β, and ln $^0K_2$.

The calculated *solid lines* in Fig. 2 are based upon considerations of the entire body of data (327 data points), not upon extrapolation from individual curves. Although the accurate determination of the asymptotes has been widely believed to be essential for the determination of the first and last Adair constants, the asymptotes are subject to substantial systematic errors such as the presence of traces of carbonmonoxy hemoglobin (26) and uncertainties in the determination of the end points.

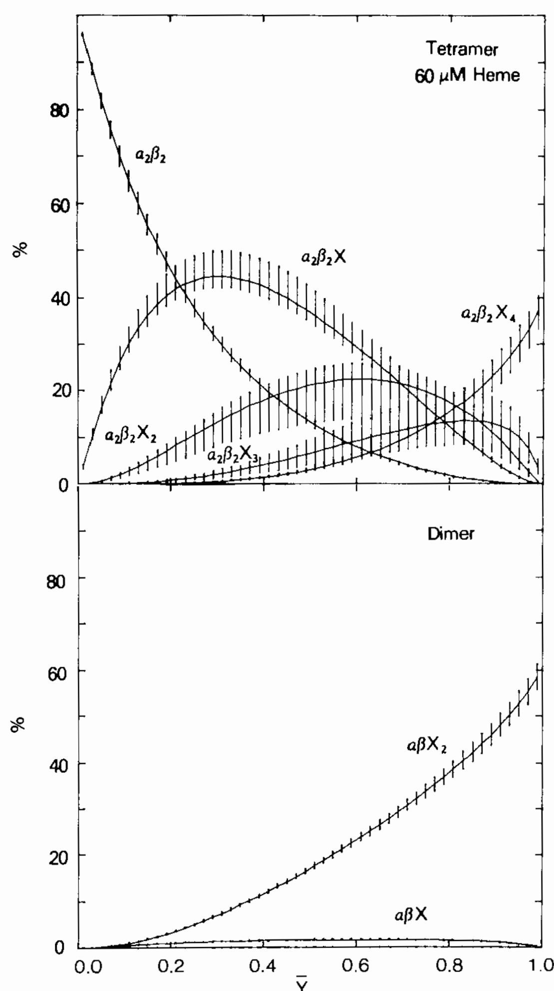

FIG. 7. **Estimated distribution of tetrameric and dimeric species of hemoglobin Kansas as a function of fractional saturation at 60 μM heme.** Calculated from the equilibrium constants in Table II, Case B. *Vertical bars* correspond to propagated standard errors.

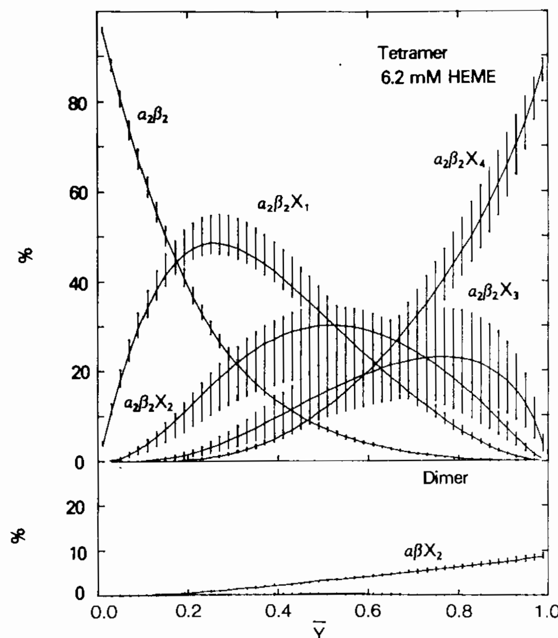

FIG. 8. **Estimated distribution of tetrameric and dimeric species of hemoglobin Kansas as a function of fractional saturation at 6.2 mM heme.** Calculated from the equilibrium constants in Table II, Case B. *Vertical bars* correspond to propagated standard errors.

DISCUSSION

The x-ray analyses of oxygenated and deoxygenated forms of hemoglobin A show a significant quaternary structural change in the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) contact region (3). This structural change appears to play a major role in the mechanism of cooperative oxygen binding to hemoglobin (1, 2). It is this contact region which is broken upon dissociation of the tetramer to $\alpha\beta$ dimers. Consequently, direct measurements of the free energy of association of $\alpha\beta$ dimers to form tetramers, as a function of degree of oxygenation, can be used as a measure of cooperative structural changes which take place in tetrameric hemoglobin (19). It should be noted that a thermodynamic analysis of this type is independent of mechanistic models for the function of hemoglobin and can thus be used to test various mechanistic models.

Oxygenated hemoglobin Kansas has previously been shown to have a lower degree of association than hemoglobin A (4, 5). The subunit association constants of deoxyhemoglobins A and Kansas are essentially identical (6). The free energies of subunit association of fully oxygenated hemoglobins A and Kansas differ by about 3 kcal/mol. In contrast, the corresponding difference for the deoxygenated hemoglobins is either small or zero. The difference, $^0\Delta G_2 - {}^4\Delta G_2$, for hemoglobin Kansas is −8.34 kcal/mol compared with −6.34 kcal/mol for hemoglobin A. These findings are consistent with structural observations which show that the hydrogen bond at the $\alpha_1\beta_2$ interface in human oxyhemoglobin A between α aspartyl 94 and β asparaginyl 102 residues (27) cannot form in oxyhemoglobin Kansas (28) because the β_{102} residue is threonine (4). Since this hydrogen bond does not exist in unligated hemoglobins A or Kansas, this substitution would not be expected to have a large effect on the dimer-tetramer equilibrium and little or none was found. X-ray analysis of deoxygenated hemoglobin Kansas has shown slight movements relative to hemoglobin A throughout the region between the α and β

heme pockets (29). X-ray analyses of the deoxygenated α and β chains within the tetramer do not yield evidence of the abnormally low oxygen affinity of the hemoglobin Kansas tetramer (29).

The subunit association free energies for hemoglobin Kansas (Table IV) indicate that the structural changes, upon oxygenation, at the $\alpha_1\beta_2$ contact region are quite similar to those of hemoglobin A for the early stages of ligation ($^0\Delta G_2$, $^1\Delta G_2$). The small difference ($^0\Delta G_2 - {}^1\Delta G_2$) between −2.97 and −2.22 kcal/mol is in agreement with the findings of Kilmartin *et al.* (24). In marked contrast, statistically significant differences in these structural changes occur during the last stage of ligation ($^4\Delta G_2$). This is in agreement with the x-ray crystallographic data of Anderson (29) which show major structural differences between hemoglobins Kansas and A for the carbonmonoxy but not for the unligated form.

The free energy change for subunit association for hemoglobin Kansas appears to increase approximately linearly with each oxygenation step (Fig. 9). Thus, the 8.34 kcal of oxygenation-linked subunit interaction energy appears to be approximately evenly partitioned among the four binding steps. In hemoglobin A, 6.34 kcal of oxygenation-linked subunit interaction energy is partitioned so that approximately 3 kcal is released upon binding the first oxygen and approximately 4.2 kcal during the second and third binding steps. It should be kept in mind that this analysis cannot resolve the interaction energies of the second and third binding steps and instead yields their sum. Recent findings with hemoglobin A have demonstrated a "quaternary enhancement" effect at the last step of oxygen binding (8, 9); the difference between the intrinsic values, $^3\Delta G_2$ and $^4\Delta G_2$, is significantly positive. The results obtained with hemoglobin Kansas (Table IV) do not exhibit such an effect.

TABLE IV

Comparison of the intrinsic free energy changes for subunit association of hemoglobins A and Kansas at various states of ligation

Units are kilocalories/mol (dimer). Values are corrected for statistical factors.

	Hb A[a]	Hb Kansas (Case B)
$^0\Delta G_2$	−14.36 ± 0.2	−13.63 (−13.60, −13.67)[b]
$^1\Delta G_2$	−11.39 ± 0.2	−11.41 (−11.35, −11.47)
$^3\Delta G_2$	−7.21 ± 0.3	−7.48 (−7.19, −7.66)
$^4\Delta G_2$	−8.01 ± 0.1	−5.29 (−5.18, −5.38)[c]
$^0\Delta G_2 - {}^1\Delta G_2$	−2.97 ± 0.3	−2.22 (−2.13, −2.28)
$^1\Delta G_2 - {}^3\Delta G_2$	−4.18 ± 0.4	−3.94 (−3.81, −4.12)
$^3\Delta G_2 - {}^4\Delta G_2$	+0.80 ± 0.3	−2.18 (−1.82, −2.48)

[a] Values are from Mills and Ackers (9).

[b] Independent measurement of the association constant of deoxyhemoglobin Kansas yields $^0\Delta G_2$ = −13.67 kcal/mol (dimer) (6).

[c] Independent measurement of the association constant of carbonmonoxy hemoglobin Kansas yields $^4\Delta G_2$ = −5.75 kcal/mol (dimer) (6).

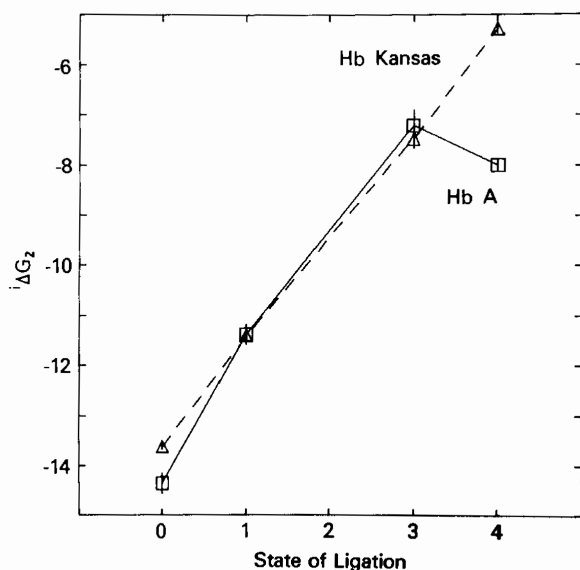

FIG. 9. **Intrinsic free energy of association as a function of ligation.** *Triangles* and *dashed line* correspond to hemoglobin Kansas. *Squares* and *solid lines* correspond to hemoglobin A. *Vertical bars* correspond to standard errors.

TABLE V

Comparison of the macroscopic free energy changes for oxygen binding to the tetramer of hemoglobins A and Kansas

Units are kilocalories/mol of O_2. Intrinsic values are corrected for statistical factors.

	Observed intrinsic	Observed macroscopic	Expected macroscopic	Difference
		Hb Kansas[a]		
ΔG_{41}	−5.81	−6.58 (−6.53, −6.67)	−8.80	2.22 (2.13, 2.27)
ΔG_{42}	−5.41	−5.68 (−5.31, −5.79)	−8.16	2.48 (2.37, 2.85)
ΔG_{43}	−5.59	−5.32 (−4.98, −5.81)	−7.62	2.30 (1.81, 2.64)
ΔG_{44}	−6.36	−5.56 (−5.26, −5.93)	−6.94	1.38 (1.01, 1.68)
		Hb A[b]		
ΔG_{41}	−5.39	−6.19 ± 0.21	−9.19	2.30 ± 0.21
ΔG_{42}	−5.71	−5.44 ± 0.90	−8.59	3.15 ± 0.90
ΔG_{43}	−6.67	−6.94 ± 0.50	−8.09	1.15 ± 0.50
ΔG_{44}	−9.15	−8.35 ± 0.35	−7.49	−0.86 ± 0.35

[a] The expected free energy change for hemoglobin Kansas was calculated assuming two chains with an affinity corresponding to −8.27 kcal/mol of O_2 and two chains with an affinity corresponding to −7.47 kcal/mol of O_2 (20).

[b] The expected free energy change for hemoglobin A was calculated assuming two chains with an affinity corresponding to −8.11 kcal/mol of O_2 and two chains with an affinity corresponding to −8.57 kcal/mol of O_2 (30).

The oxygen binding properties of tetrameric hemoglobins A and Kansas are compared in Table V. The expected macroscopic oxygen binding free energies to tetrameric hemoglobins Kansas and A in the absence of cooperativity have been calculated by taking into account the differences in the intrinsic binding energies of the isolated chains. For a molecule with two pairs of noninteracting, noncooperative binding sites, the macroscopic product Adair constants can be shown to be related to the intrinsic affinities, K_α and K_β:

$$K_{41} = 2K_\alpha + 2K_\beta \tag{3}$$

$$K_{42} = K_\alpha^2 + K_\beta^2 + 4K_\alpha K_\beta \tag{4}$$

$$K_{43} = 2K_\alpha^2 K_\beta + 2K_\beta^2 K_\alpha \tag{5}$$

$$K_{44} = K_\alpha^2 K_\beta^2 \tag{6}$$

For hemoglobin A, the values of K_α and K_β correspond to free energy changes of −8.11 kcal/mol and −8.57 kcal/mol, respectively (30). The values for hemoglobin Kansas correspond to free energy changes of −8.27 kcal/mol and −7.47 kcal/mol, respectively (20). Subtraction of the predicted free energies of binding (due to differences in intrinsic binding energies of the chains in the absence of cooperativity) from the observed binding energies, yields a set of differences which are a measure of the cooperative changes in the oxygen binding of tetrameric hemoglobins A and Kansas. A positive difference denotes a "quaternary constraint"; a negative one indicates a quaternary enhancement relative to the isolated chains (8, 9). In both hemoglobins A and Kansas, the largest difference from the expected value for the constituent chains is in the

second state of ligation and is approximately 3.2 kcal/mol and 2.5 kcal/mol for hemoglobins A and Kansas, respectively. The sign of these constraints in hemoglobin A is actually reversed by the final stages of ligation (the quaternary enhancement effect), but in hemoglobin Kansas they remain significantly positive, indicating that the structures of oxygenated hemoglobins A and Kansas are dramatically different. It should be noted that the total change in these constraints is 4.01 kcal/mol (3.15 to (−0.86)) for hemoglobin A and 1.10 kcal/mol (2.48 to 1.38) for hemoglobin Kansas. This indicates that even at very high concentration, where essentially no subunit dissociation occurs, hemoglobin Kansas should be less cooperative than hemoglobin A. This difference is also responsible, in part, for the marked difference in affinity of the dimeric and tetrameric forms.

The observed low affinity and low Hill coefficient of hemoglobin Kansas are a reflection of several of the observed properties. 1) The differences in the isolated chain affinities would, in the absence of cooperative interactions, yield a pseudonegative cooperativity to both the dimeric and tetrameric forms as is shown in Fig. 3 and Table V. 2) The positive differences in Table V demonstrate that hemoglobin Kansas tetramers exhibit quaternary constraints. However, these are not fully released upon oxygenation since the ΔG_{44} difference is significantly positive. Another way of viewing this effect is to note that the binding free energy, ΔG_{44}, is still greatly reduced relative to the values for the isolated chains. Consequently, the tetramer of hemoglobin Kansas has a low Hill coefficient and a low overall affinity. The Hill coefficient of hemoglobin Kansas at high protein concentration has previously been reported as approximately 1.5 (5, 23). This value is in agreement with our results (Table I and Fig. 3). 3) As the total protein concentration is lowered, the resulting increased dissociation would increase the cooperativity by increasing the proportion of the higher affinity dimeric form (31). This can be seen in Table I and Fig. 3 where the calculated slope of the Hill plot increases as the protein concentration is decreased to 0.36 μM heme. Such an increase in Hill coefficient of hemoglobin Kansas has also been observed above neutral pH where the subunit dissociation is increased (5, 6). The increase in Hill coefficient is counterbalanced by the pseudonegative cooperativity of the dimer, thus giving a Hill coefficient which is essentially constant over a wide range of protein concentration.

The Wyman interaction energy for tetrameric hemoglobin is the difference in the free energies of the last and first steps of oxygen binding, corrected for statistical factors (11, 32, 33). The calculated value of the Wyman interaction energy of tetrameric hemoglobin Kansas from our data is 0.55 kcal/mol (from data in Table V). The value calculated from the data of Shulman *et al.* (23) is 0.05 kcal/mol.

The cause of the low affinity of the hemoglobin Kansas β chain is not known. Under similar conditions, the binding free energy change of hemoglobin A β chains has been determined as −7.72 kcal/mol for β monomers (8) and −8.56 kcal/mol for β tetramers (30). In tetrameric hemoglobin A, the α and β chains have been shown to have nearly equal binding free energies (34, 35). Under the conditions of this study, the affinity is approximately −7.5 kcal/mol (11). An intriguing possibility is that the low value measured for the affinity of the hemoglobin Kansas β chain is due to the β_{102} substitution inhibiting the quaternary enhancement which the β chain of hemoglobin A undergoes upon association.

One hypothesis for the cause of the cooperativity in hemoglobins is that the heme ring tilts (36). The x-ray structure indicates that the β_{102} residue is in contact with the edge of the heme ring. It is, therefore, logical to assume that a substitution of the β_{102} residue could effect the ability of the heme ring to tilt and that this is related both to the low cooperativity of hemoglobin Kansas and to its low affinity for oxygen.

Acknowledgments—We thank Dr. Gary K. Ackers for many helpful suggestions and access to unpublished data. We also thank Dr. Stanley J. Gill for arranging for the construction of the special oxygenation cell at the University of Colorado. We thank Dr. Dennis A. Diederich for supplying us with the blood sample with hemoglobin Kansas.

APPENDIX

The subunit association-saturation function, Equation 1, is defined in terms of the following polynomials:

$$Z_2 = 1 + K_{21}[X] + K_{22}[X]^2 \quad (7)$$

$$Z'_2 = K_{21}[X] + 2K_{22}[X]^2 \quad (8)$$

$$Z_4 = 1 + K_{41}[X] + K_{42}[X]^2 + K_{43}[X]^3 + K_{44}[X]^4 \quad (9)$$

$$Z'_4 = K_{41}[X] + 2K_{42}[X]^2 + 3K_{43}[X]^3 + 4K_{44}[X]^4 \quad (10)$$

where

$$K_{2i} = \frac{[(\alpha\beta)X_i]}{[\alpha\beta][X]^i} \qquad i = 1, 2, 3, 4 \quad (11)$$

and

$$K_{4i} = \frac{[(\alpha_2\beta_2)X_i]}{[\alpha_2\beta_2][X]^i} \qquad i = 1, 2, 3, 4 \quad (12)$$

The sequential bindings are defined as:

$$k_{2i} = \frac{[(\alpha\beta)X_i]}{[(\alpha\beta)X_{i-1}][X]} = \frac{K_{2i}}{K_{2(i-1)}} \qquad (K_{20} = 1) \quad (13)$$

$$k_{4i} = \frac{[(\alpha_2\beta_2)X_i]}{[(\alpha_2\beta_2)X_{i-1}][X]} = \frac{K_{4i}}{K_{4(i-1)}} \qquad (K_{40} = 1) \quad (14)$$

The sequential binding free energy changes are defined in terms of the sequential binding constants as:

$$\Delta G_{ni} = -RT \ln k_{ni}$$

The subunit association constants ${}^iK_2$ are defined as the dimer-tetramer association constants, per mol of dimer, to form a tetramer with i oxygens bound.

$${}^iK_2 = \frac{[(\alpha_2\beta_2)X_i]}{[(\alpha\beta)X_j][(\alpha\beta)X_k]} \qquad \begin{matrix} i = j + k \\ j, k = 0, 1, 2 \end{matrix} \quad (15)$$

The corresponding subunit association free energy changes are defined as:

$${}^i\Delta G_2 = -RT \ln {}^iK_2 \quad (16)$$

For a more complete definition of terms, see the previously published discussions (11, 18, 19).

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