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ENERGETICS OF OXYGENATION-LINKED SUBUNIT INTERACTIONS IN HUMAN HEMOGLOBIN

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SUMMARY: The intersubunit contact energy changes associated with successive oxygenation of human hemoglobin have been determined by measurements on the linkage between oxygenation and dissociation of tetramers into dimeric species. Experimental conditions were: 0.1M Tris HCl, 0.1M NaCl, 1mM Na_2 EDTA, pH 7.4, 21.5°C. The results establish that (1) there are at least three major energetic states assumed by the tetramer upon oxygenation, (2) dimers bind oxygen noncooperatively under these conditions and have the same affinity as isolated chains, and (3) successive changes in ligand binding energy are compensated by equal and opposite changes in the intersubunit contact energy during the oxygenation-deoxygenation cycle.

In human hemoglobin the intersubunit contact region between $\alpha^1\beta^1$ and $\alpha^2\beta^2$ dimer pairs contains specific chemical linkages (salt bridges, hydrogen bonds) and other interactions believed responsible for stabilizing the constrained quaternary structure of the molecule in its unliganded state (1,2). This form of the molecule has a large positive free energy of subunit dissociation (3,4) in contrast to the liganded form (5,6) where the "constraining interactions" are absent. Determination of the energy changes occurring within this contact region upon successive oxygen binding steps is therefore of paramount importance in (a) defining the major energetic states assumed by the molecule during the course of oxygenation, and (b) correlating those energy changes with structural features in order to identify molecular events which accompany cooperative ligand binding.

We wish to report a determination of the intersubunit contact energy changes between $\alpha\beta$ dimer pairs in tetrameric human hemoglobin which accompany binding of the first, middle two, and last oxygen molecules.

Our approach has been to study the linkage between oxygen binding and dissociation of hemoglobin tetramers into dimeric species (7-9). Dissociation

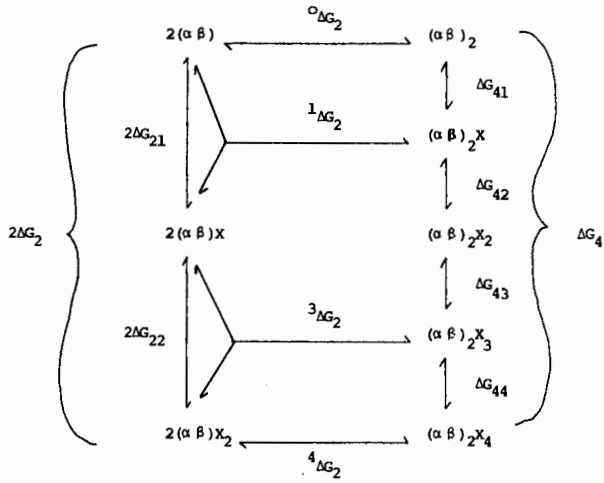

Figure 1.: Linkage diagram for subunit dissociation and oxygenation of human hemoglobin. The energetic quantities represented have been defined previously (7). $^{o}\Delta G_2$ and $^{4}\Delta G_2$ are free energies for tetramer formation (from dimers) in the unliganded and fully liganded states, respectively. $^{1}\Delta G_2$ and $^{3}\Delta G_2$ are respective association energies for formation of singly liganded tetramer (from combination of singly-liganded and unligand dimers) and triply-liganded tetramer (from combination of singly and doubly-liganded dimers). Energies for the successive binding steps to tetramer are ΔG_{41}, ΔG_{42}, ΔG_{43}, ΔG_{44}. For dimer, the successive free energies of oxygenation are ΔG_{21} and ΔG_{22}. The free energies for saturation of tetramer and dimer, respectively, are ΔG_4 and ΔG_2. The successive differences ($^{1}\Delta G_2$-$^{o}\Delta G_2$), ($^{3}\Delta G_2$-$^{1}\Delta G_2$), ($^{4}\Delta G_2$-$^{3}\Delta G_2$) are measures of the intersubunit contact energy changes accompanying the first, middle two, and last binding steps, respectively.

into $\alpha\beta$ dimers in dilute solution destroys the contacts within the region where the major structural changes occur in the tetramer upon oxygenation (10,11). Since the oxygenation-linked energy differences within this contact region are large (3,4,9), measurements of subunit dissociation vs state of oxygenation and of oxygenation vs hemoglobin concentration provide powerful means to probe the contact energy changes associated with ligand binding (7).

We have now succeeded in resolving the free energies depicted in Fig. 1 under one set of experimental conditions: (0.1M Tris HCl, 0.1M NaCl, 1mM Na_2 EDTA, pH 7.4, 21.5°C). Our primary goal was to determine the energies "across" the scheme: $^{o}\Delta G_2$, $^{1}\Delta G_2$, $^{3}\Delta G_2$, and $^{4}\Delta G_2$. To this end, a set of experimentally

derivable energy terms depicted in Fig. 1 serve to define the quantities of interest; seven of these are independent (7). The strategy we have used is based upon obtaining essentially independent determinations of $^{o}\Delta G_2$, $^{4}\Delta G_2$, and ΔG_4, and subsequently analyzing oxygenation curves measured over a wide range of protein concentration to estimate the remaining four parameters. The key to solution of the problem was obtaining a reliable independent estimate of $^{o}\Delta G_2$, the free energy for association of unliganded dimers into tetramers. This is the most difficult quantity to determine because of its high value (the equilibrium constant $^{o}K_2$ is $> 10^{10}$)(4,9) and because of its high statistical correlation with several of the other energies to which it is linked (12).

EXPERIMENTAL METHODS AND RESULTS

Human hemoglobin was prepared from freshly drawn blood by the method of Williams and Tsay (13), and isolated chains were made according to Tyuma et al (22). Haptoglobin 1-1 was prepared by the method of Connell and Shaw (14). The buffer used in all experiments was 0.1M Tris HCl, 0.1M NaCl, 1mM Na_2 EDTA, pH 7.4. Determination of the various energetic quantities at 21.5°C are described below.

The Energy For Formation of Unliganded Tetramers From Dimers, $^{o}\Delta G_2$: The equilibrium constant $^{o}K_2$ for dimer-tetramer association of unliganded hemoglobin was estimated from independent determinations of the rate constants for forward and reverse reactions. The dissociation rate constant was measured by a haptoglobin binding technique described in detail elsewhere (15,9, see Fig. 2), and was found to be $2.24 \pm 0.1 \times 10^{-5} sec^{-1}$. The reverse rate constant was determined from stopped flow measurements similar to those described by Kellett and Gutfreund (16) and was found to be $1.14 \pm 0.1 \times 10^{6} M^{-1} sec^{-1}$ (dimer). From the ratio of these rate constants the equilibrium constant $^{o}K_2$ was determined to be $5.11 \pm 1.3 \times 10^{10} M^{-1}$ (dimer). This leads to a value of -14.38 ± 0.2 Kcal/mole dimer for $^{o}\Delta G_2$. Confidence limits in this paper correspond to approximately one standard deviation.

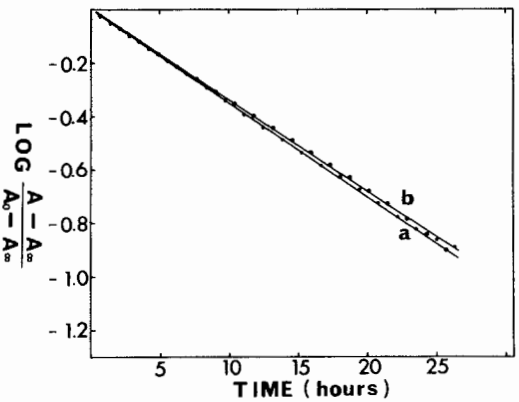

Figure 2.: First order plots of kinetic data for dissociation of deoxyhemoglobin tetramers into dimers in 0.1M Tris HCl, 0.1M NaCl, 1mM Na_2 EDTA pH 7.4, 21.5°C. Deoxygenated hemoglobin was reacted under anaerobic conditions with an excess of human haptoglobin, type 1-1. The haptoglobin binds rapidly and irreversibly to the $\alpha\beta$ dimers formed upon dissociation of the tetramers (15). The reaction is followed by measuring the decrease in absorbance at the Soret maximum, 430 nm, in a Cary 118C spectrophotometer (9). Concentrations of hemoglobin were (a) 1.4×10^{-5}M heme, (b) 6.0×10^{-6}M heme. The rate constant, corresponding to the rate-limiting dissociation of tetramers, was determined as $2.24 \pm 0.1 \times 10^{-5}sec^{-1}$. This result was combined with the (second order) rate constant for association of dimers obtained by stopped-flow experiments, to yield the equilibrium constant $^{o}K_2 = 5.11 \pm 1.3 \times 10^{10}M^{-1}$. $^{o}\Delta G_2 = -RT\ln\ ^{o}K_2 = -14.38 \pm 0.2$ Kcal/mole dimer.

The Energy For Formation of Liganded Tetramers From Dimers, $^{4}\Delta G_2$: From large-zone gel chromatography experiments (5,6) carried out as a function of hemoglobin concentration (using a Sephadex G-100 column, 0.9 x 38.5 cm) the association constant for formation of oxygenated tetramers from oxygenated dimers was determined to be $9.81 \pm 2.5 \times 10^{5}M^{-1}$ (dimer) so that $^{4}\Delta G_2 = -8.05 \pm 0.2$ Kcal/mole (dimer).

The Energy of Tetramer Binding Four Oxygens, ΔG_4: Measurements of oxygenation curves were made as a function of hemoglobin concentration using the automatic oxygenation technique of Imai (17) with the ferredoxin reductase system of Hayashi et al (18). Results are shown in Fig. 3. From the oxygenation curves (Fig. 3), median ligand concentrations X_m (19) were determined, and are shown

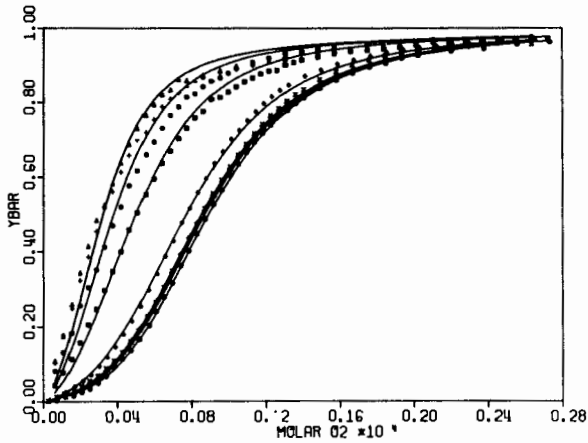

Figure 3.: Dependence of oxygenation curves upon hemoglobin concentration. Experiments were carried out using an Imai type automatic oxygenation apparatus (17) with a Cary 118C spectrophotometer and ferredoxin reductase system (18). The curves correspond to concentrations shown in Table I. The five leftmost data sets were measured at 415 nm. The next two (nearly overlapping) were measured at 480 nm and 610 nm. The two on the right (highest hemoglobin concentrations) were determined at 610 nm. The oxygen electrode was calibrated against standard O_2-N_2 gas mixtures. Oxygenation data were found to be independent of wavelengths used. Solid curves are calculated saturation functions for the concentrations used (Table I) based upon the energies derived from these data and the independently determined energies.

in Table I. For each of the curves a value of ΔG_4 is uniquely determined by X_m and values of $^{o}\Delta G_2$ and $^{4}\Delta G_2$ (12). The value determined from all the curves was -27.16 ± 0.05 Kcal/4 moles O_2. This quantity is not highly correlated with the sequential binding energies.

The Energy of Dimer Binding Two Oxygens, ΔG_2: The value of ΔG_2 was calculated as $\Delta G_2 = (\Delta G_4 + {}^{o}\Delta G_2 - {}^{4}\Delta G_2)/2 = -16.75 \pm 0.25$ Kcal/2 moles O_2. We have experimentally determined corresponding values for the isolated chains which sum to -16.5 ± 0.2 Kcal/mole, in agreement with previous determinations (21), and with ΔG_2 as determined in this study.

Determination of Remaining Parameters: By fixing the independently determined values of $^{o}\Delta G_2$, ΔG_4, ΔG_2, and $^{4}\Delta G_2$, the oxygenation curves were analyzed by non-linear least-squares minimization (12,20) to yield the intersubunit

Table I: Median Oxygen Concentration and Total Binding Energy

Hemoglobin Concentration (P_t) Molar Heme x 10^6	Median, X_m Molar O_2 x 10^6	Calculated* K_{44} x $10^{20} M^{-4}$
382.	8.67	1.60
76.5	8.21	1.76
76.5	8.16	1.80
38.2	8.01	1.78
5.4	7.32	1.60
0.27	4.78	1.53
0.08	3.67	1.53
0.04	3.05	1.68
0.04	3.17	1.44
Mean Adair constant for four oxygens		1.64 ± 0.04
ΔG_4 = -27.16 ± 0.05 Kcal/four moles O_2		

*Calculated from the formula (12):

$$K_{44} = (X_m)^{-4} \left[\frac{1 - {}^4f_2}{1 - {}^of_2}\right] \exp({}^of_2 - {}^4f_2)$$

where ${}^4f_2 = \dfrac{[1 + 4\,{}^4K_2(P_t)]^{\frac{1}{2}} - 1}{2\,{}^4K_2(P_t)}$, ${}^of_2 = \dfrac{[1 + 4\,{}^oK_2(P_t)]^{\frac{1}{2}} - 1}{2\,{}^oK_2(P_t)}$

and using the independently determined values:
${}^oK_2 = 5.11 \times 10^{10} M^{-1}$ (dimer), ${}^4K_2 = 9.81 \times 10^{5} M^{-1}$ (dimer)

contact energy changes.

The Intersubunit Contact Energy Changes: The energy changes within the dimer-dimer contact region are calculated as the following differences:

a. First Step: $\delta\Delta G_{01} = {}^1\Delta G_2 - {}^o\Delta G_2 = 2.59 \pm 0.3$ Kcal/mole (dimer)

b. Middle Step: $\delta\Delta G_{13} = {}^3\Delta G_2 - {}^1\Delta G_2 = 3.75 \pm 0.4$ Kcal/mole (dimer)

c. Last Step: $\delta\Delta G_{34} = {}^4\Delta G_2 - {}^3\Delta G_2 = -0.1 \pm 0.25$ Kcal/mole (dimer)

Thus the 6.34 Kcal of oxygenation-linked subunit interaction energy is partitioned so that approximately 2.6 Kcal are released within the contact region upon binding of the first oxygen, approximately 3.7 Kcal during the second and third binding steps, and essentially no energy at the last step.

We have analyzed two independent extensive sets of oxygenation curves such as those shown in Fig. 3, and find very nearly the same energies.

Successive Energies of Oxygen Binding By Dimers: Least-squares analysis of the oxygenation data also provide a resolution of ΔG_2 into terms for the successive binding steps by dimers: $\Delta G_{21} = -8.78 \pm 0.30$ Kcal/mole O_2 $\Delta G_{22} = -7.97 \pm 0.31$ Kcal/mole O_2. The difference between these values very nearly equals the expected statistical factor (-.808 Kcal/mole) for non-cooperative binding. These binding energies also nearly equal the value of ΔG_{44} (corrected for statistical factors) as well as the energies obtained for the isolated chains, thus providing highly consistent estimates of the intrinsic binding energy, ΔG_{int}, for hemoglobin in the absence of cooperative effects. Then for cooperative tetrameric hemoglobin, the energy of binding oxygen at each step(corrected for the appropriate statistical factor)differs from the intrinsic binding energy by an amount equal and opposite to the change in intersubunit contact energy accompanying that step.

CONCLUSIONS

The high degree of consistency found in this study between results from diverse experimental techniques provides strong verification of the basic linkage relationships postulated to be operative in human hemoglobin under these conditions (7,8). Based upon this work there appear to be at least three major energetic states assumed by tetrameric hemoglobin as a function of oxygenation state: (a) unliganded, (b) singly-liganded, (c) triply and quadruply-liganded species. There may be significant energetic partitioning between the second and third binding steps but this cannot be resolved by present methods (7). It is not known whether these energetic states can be assumed by a single molecule, or whether they arise as averages over a distribution of conformational states (23). The results provide unequivocal evidence against a concerted transition at a particular binding step in a system with only two energetic states of tetramer.

Results of this study clearly establish that during the oxygenation-deoxygenation cycle, successive changes in ligand binding energy (i.e. the cooperative energy changes) are compensated by equal and opposite changes in the average intersubunit contact energy:

i.e. $$(\Delta G_{4m} - \Delta G_{41}) = \delta \Delta G_{13} - \delta \Delta G_{01}$$

$$(\Delta G_{44} - \Delta G_{4m}) = \delta \Delta G_{34} - \delta \Delta G_{13}$$

where $\Delta G_{4m} = \Delta G_{42} + \Delta G_{43}$ and represents the binding energy for the middle two steps. Thus all the cooperative energy in oxygen binding by human hemoglobin is reflected in the contact region separating $\alpha^1 \beta^1$ and $\alpha^2 \beta^2$ dimer pairs within the tetramer.

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