ENERGETICS OF SUBUNIT ASSEMBLY AND LIGAND BINDING IN HUMAN HEMOGLOBIN

Gary K. Ackers, *Department of Biology and McCollum Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218 U.S.A.*

ABSTRACT An extensive and self-consistent set of thermodynamic properties has recently been established for the coupled processes of subunit assembly and ligand binding (oxygen and protons) in human hemoglobin. The resulting thermodynamic values permit a consideration of the possible sources of energetic terms accounting for stability of the tetrameric quaternary structures at different stages of ligation, and of the possible sources of cooperative energy. The analysis indicates that: (*a*) The change in buried surface area upon oxygenation (i.e., hydrophobic stablization) does not play a dominant role in stabilizing the unliganded tetramer relative to the liganded tetramer. (*b*) The pattern of enthalpic and entropic contributions to the free energies of dimer-tetramer assembly provides evidence against a dominant role of salt bridges in stabilizing the deoxy tetramer. (*c*) The thermodynamic results are consistent with a dominant role of increased hydrogen bond formation in the deoxy quaternary structure. (*d*) Within tetramers the variation in free energy for successive oxygenation steps arises from both enthalpic and entropic contributions and the enthapic contributions are almost entirely attributable to the heats of Bohr proton release. At pH 7.4 the pattern of thermodynamic values suggests that a large contribution to the free energy of cooperativity may arise from the energetics of Bohr proton release. It is suggested that a combination of proton ionization and hydrogen bonding may account for the main energetic features of cooperativity. Possible contributions from fluctuation behavior cannot presently be evaluated.

INTRODUCTION

Cooperative ligand binding in multisubunit proteins such as human hemoglobin arises from a coupling between the effects of ligand binding at the individual subunits and the interactions between subunits within the quaternary structure. An approach to understanding these coupling processes lies in physically "decoupling" the interactions by dissociation of the oligomeric molecule into smaller combinations of its constituent subunits. Subunit dissociation brings about the elimination of intersubunit contacts and thus provides a useful means of studying their effects. Characterization of ligand binding properties for the subunits in various stages of assembly, and of the assembly reactions themselves, provides an experimental basis for understanding how subunit interaction brings about the observed alterations in affinity and regulatory properties. For normal human hemoglobin a comprehensive set of thermodynamic properties pertaining to these processes has recently been developed (1–16). The resulting energetic picture imposes constraints that must be satisfied by any theory proposed to explain the mechanism of cooperative oxygen binding.

Many of the concepts developed to date for explaining the mechanism of cooperative interactions in human hemoglobin have been based upon interpretations of the elegant structural results derived from x-ray crystallographic analysis (cf. reference 19 for a general review). The structural analyses have provided an admirable wealth of detailed information,

whereas the corresponding development of energetic information has been severely lagging. This discrepancy in quantity and quality between structural and thermodynamic information is particularly serious in view of the fact that cooperativity is an energetic concept developed to recognize the change in free energy which accompanies the successive oxygenation steps. The difference between the energy of binding the first and last oxygens is only ~3–4 kcal. This small difference accounts for most of the observed cooperativity in the binding curves.

Structural information alone is incapable of identifying the sources of cooperative energy, since the net driving force undoubtedly arises as the sum of a number of terms which mostly cancel. Like the thermodynamic information, structural results provide essential constraints against which all hypothetical mechanisms may be tested. The necessary correlation between thermodynamic and structural information that will be required for an accurate theory has not been achieved to date. In this article we consider what can be learned from thermodynamic studies on ligand binding (oxygen and protons) under conditions where the quaternary structure is altered through subunit dissociation.

The first part of this paper is a summary of the model-independent thermodynamic results. Second, we consider possible sources of the dominant energetic terms which may acount for stability of hemoglobin tetramers relative to the dissociated dimers in various states of oxygenation and protonation. A third section deals with the problem of assigning the molecular basis of cooperativity, i.e., the molecular sources of variations in binding free energies for the various oxygenation steps.

THERMODYNAMIC RESOLUTION OF THE LIGAND-LINKED ASSEMBLY FOR HUMAN HEMOGLOBIN

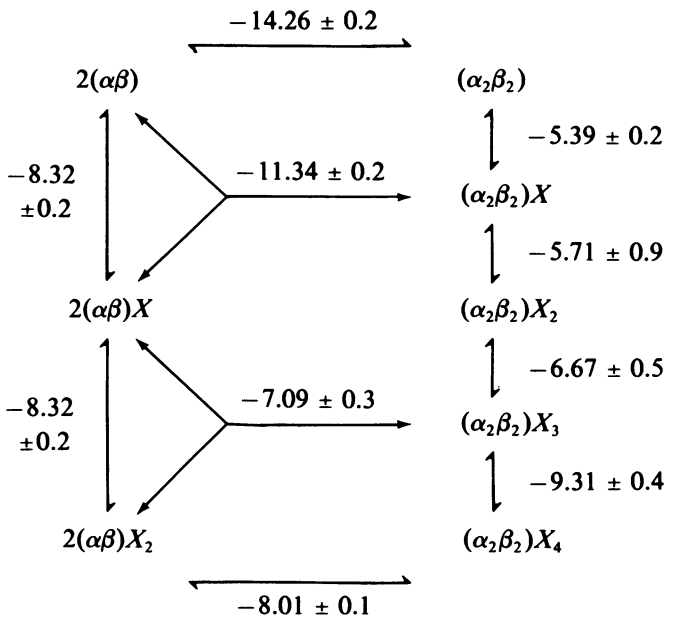
A series of recent experimental studies on human hemoglobin (1–16) has provided an extensive and self-consistent set of thermodynamic properties pertaining to (*a*) the linkage between dimer tetramer association and oxygen binding, (*b*) the effects of pH and chloride on the oxygenation linked dimer-tetramer reactions, (*c*) the properties of isolated chains, including their ligand binding and self-association reactions, and (*d*) the reconstitution of functional hemoglobin tetramers from the constituent subunits. The results summarized here will focus mainly upon the processes of categories *a* and *b*.

Free Energies of Dimer-Tetramer Assembly and Oxygen Binding

Reversible dissociation of hemoglobin tetramers near neutral pH leads to elimination of the $\alpha^1\beta^2$ intersubunit contact region so that the dimers formed are of the $\alpha^1\beta^1$ type (17). The $\alpha^1\beta^2$ contact is known from x-ray studies to undergo major structural change upon conversion from the deoxy quaternary form into the oxy quaternary structure (18, 19). In particular, this contact contains a number of salt bridges and hydrogen bonds which are eliminated or formed during the oxygenation process. It is therefore of great interest to know how the degree of oxygenation by tetramers affects the energy of dissociation along this oxygenation-sensitive contact surface. Experimental studies on the concentration dependence of oxygen binding curves as a function of protein concentration (5, 14, 16) and temperature (16) and corresponding studies of the subunit assembly reactions (10, 13) have permitted a complete

resolution of the free energies, enthalpies and entropies pertaining to the reactions of the linkage scheme:

SCHEME 1



This diagram depicts the coupled reactions for dimer-tetramer assembly and binding of oxygen (denoted X). Values of intrinsic free energies (i.e., corrected for statistical factors) are given on the diagram. The values pertain to experimental conditions of pH 7.4, 0.1 M Tris buffer, 0.1 M NaCl, 1 mM EDTA and 21.5°C, with the reactions taken from left to right and top to bottom. Extensive documentation on the reliability of these results is provided in references 6 and 14. The free energies, given in kilocalories, are physically meaningful and accurate to within the specified error limits.

A necessary consequence of these results is that oxygenation experiments carried out at low concentrations which are analyzed according to tetrameric properties alone may be subject to significant errors due to the presence of dimers (20). Such artifacts, recently verified by Imai (21), may affect only some of the constants appreciably but seriousness of the errors can be known only by a study of concentration effects in each particular case (22).

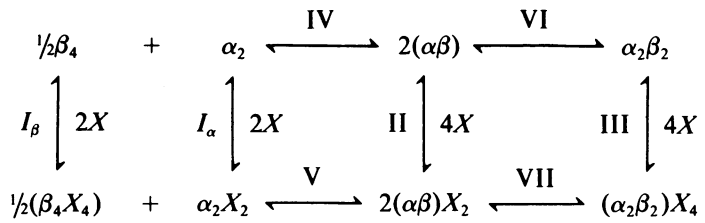
From the experimental results shown in scheme 1 and similar sets obtained for temperatures between 10°–37°C (16), the following conclusions may be drawn on model-independent grounds: (*a*) The dimers bind oxygen noncooperatively and with high affinity (similar to that of the isolated α and β chains). (*b*) Assembly of two unliganded $\alpha^1\beta^1$ dimers leads to a quaternary structure (the deoxy tetramer) of high stability (−14.3 kcal/mol) and substantially reduced oxygen affinity (−5.4 vs. −8.3 kcal/mol), whereas the assembly of fully ligated dimers results in a tetramer of comparatively low stability (−8.0 kcal/mol). The deoxy tetramer is more stable than the oxy tetramer by 6.3 kcal/mol. These results are consistent with the notion, derived largely from x-ray structure analysis, that the deoxy tetramer is subject to constraints arising from pair-wise noncovalent interactions which are localized at

the dimer-dimer interface, and that these constraining interactions are eliminated or reduced upon oxygenation (18, 19). (*c*) The oxygenation-sensitive linkage free energy, 6.3 kcal, is seen (scheme 1) to be partitioned between the first binding step, the middle two steps taken together, and the last step. The partitioning is distributed over these oxygenation states in a sequential manner, i.e., the total transition does not occur at any particular ligation step. Thus models which embody an all-or-none transition between two states of the molecule at a particular binding step are unequivocally excluded by these experimental findings. (*d*) The assembly of triliganded tetramer leads to a quaternary structure with enhanced affinity for binding oxygen at the remaining site (i.e., −9.3 kcal/mol). This phenomenon of quaternary enhancement has been found at all temperatures between 10°C and 37°C (14, 16). Quarternary enhancement is also observed in the assembly of tetramers from monomers—i.e., the tetramers have higher affinity for oxygen (12). The structural and energetic bases of these quaternary enhancement effects are not known (cf. reference 14 for a discussion of mechanistic possibilities).

Enthalpies and Entropies

The reactions of linkage scheme [1] have been studied as a function of temperature (16) and by calorimetry (2–4, 11) to determine the reaction enthalpies and entropies. In addition, values of the thermodynamic quantities for ligation and self-assembly of isolated chains (9, 11, 12, 14) have also been estimated for the overall reaction paths depicted in scheme 2:

SCHEME 2



Certain results of these determinations are summarized in Tables I, II, and III. We will discuss these results in relation to the mechanistic issues which form the subject of the next sections. Worth noting at this point is the fact that both the enthalpies and entropies of dimer-tetramer assembly have large negative values for the deoxy state, but change to distinctly positive values upon oxygenation (Table I). The oxy and deoxy molecules both exhibit enthalpy-entropy compensation (21) in their assembly reactions, but with dramatically opposite signs for the enthalpic and entropic terms. It should also be noted that the deoxy tetramer absorbs protons upon assembly from dimers whereas the oxy tetramer is formed with release of protons (Table I).

SOURCES OF ENERGETIC TERMS ACCOUNTING FOR STABILITY OF TETRAMERS IN DIFFERENT STATES OF LIGATION

The relationships between the thermodynamic quantities summarized in the previous section and in Table I, II, and III permit us to make a more realistic assessment of the possible sources of energetic effects in human hemoglobin than has previously been possible. In considering the known structure changes which accompany ligation of the tetramer (18, 19, 24–26) and its dissociation it is evident that a variety of types of interaction may be

TABLE I

ENTHALPIES AND ENTROPIES OF DIMER-TETRAMER ASSEMBLY FOR HUMAN HEMOGLOBIN AT VARIOUS STATES OF OXYGENATION

Dimer-tetramer assembly (scheme 1)*			n_{H+} mols H^+ released / mols tetramer formed
i	${}^{i}\Delta H_2$ kcal*	${}^{i}\Delta S_2$ cal/deg/mol‡	
0	−28.9 ± 0.5	−49.8 ± 1.7	−0.9§
1	−17.7 ± 0.8	−21.4 ± 2.7	—
3	+3.1 ± 3.8	+34.8 ± 12.4	—
4	+3.9 ± 1.6	+40.4 ± 5.7	+0.8§

*${}^{i}\Delta H_2$ is the enthalpy of formation of a tetramer with i oxygens bound. Values are from reference 16.
‡${}^{i}\Delta S_2$ is the corresponding enthalpy of formation. Values are from reference 16.
§Newly determined values pertaining to 0.1 M NaCl, 0.1 M Tris buffer, pH 7.4, 21.5°C. (A. Chu and G. K. Ackers, unpublished results).

simultaneously involved in contributing to the observed energetics. A detailed accounting of such effects would have to include energies which accompany the alterations in van der Waals contacts, hydrogen bonds, salt bridges, electrostatic and hydrophobic interactions. In addition, we would have to include a consideration of thermodynamic effects arising solely from the motional dynamics of the molecules in their various states of ligation and association. Since the net enthalpies and entropies undoubtedly arise from the sum of many large terms which mostly cancel, it is not possible from the observed energetics to decide whether a given type of noncovalent interaction is contributing significantly. We can, however, test any theories which are based on the idea that a particular type of non-covalent interaction plays a dominant role in contributing to the energetic effects. Inconsistency with the pattern of experimentally determined thermodynamic quantities permits a given theory of this type to be ruled out. Consistency, on the other hand, does not provide positive verification of any such theoretical mechanism. A number of theories have been proposed to account for the enhanced stability of unliganded tetramers as compared with tetramers in the fully liganded quaternary structure

TABLE II

ENTHALPIES OF O_2 BINDING FOR HUMAN HEMOGLOBIN AND ITS SUBUNITS

Path (scheme 2)	n_{H+}	$\Delta H_{O_2}^{corr}$
Mean I_α, I_β	0	−15.8 ± 1.0*
II	0.1‡	(−16.6 ± 0.9)§
III	0.52‖	−14.5 ± 0.7¶
	0.52‖	−14.0 ± 0.6**
	0.52‖	−14.3 ± 0.5‡‡
Mean		−14.3 ± 0.8

Conditions for all values in the table are: pH 7.4 0.1 M Tris-HCl, 0.1 M NaCl. The values from reference 15.
*Average of van't Hoff and calorimetric values for α and β chains corrected to O_2(eq).
‡Chu, A., and G. K. Ackers, unpublished results.
§One-half the enthalpy for oxygenating an $\alpha\beta$ dimer (2 oxygens) derived from van't Hoff values for Reactions III, VI, and VIII. (scheme 2).
‖From reference 40.
¶Calorimetric values measured for CO(g) binding corrected for differential heat of replacement of CO (g) by O_2(g) and by the heat of solution of O_2 (15).
**Calorimetric value for O_2 (aq) binding (2).
‡‡Van't Hoff value (−8.6 ± 0.3 kcal) corrected for heat for Bohr proton release (15).

TABLE III
THERMODYNAMICS OF OXYGENATING HEMOGLOBIN TETRAMERS

Oxygenation steps	ΔG	ΔH	$T\Delta S$
$0 \rightarrow 1$	-5.39 ± 0.2	-6.3 ± 0.7	-0.91 ± 0.7
$1 \rightarrow 3$	-12.38 ± 0.5	-11.4 ± 3.9	$+1.02 \pm 3.9$
$3 \rightarrow 4$	-9.31 ± 0.4	-16.0 ± 3.8	-6.69 ± 3.8
$0 \rightarrow 4$	-27.1 ± 0.1	-33.7 ± 9.6	-6.6 ± 0.6

Conditions: 0.1 M Tris, 0.1 M NaCl, pH 7.4, 21.5°C. Values are from reference 16.

in terms of dominance by a single energetic factor. We consider here several of the possibilities and proposals of current interest.

Solvent Acessibility (Hydrophobic Stabilization)

Chothia et al., (27) have proposed changes in solvent accessibility as the dominant factor in accounting for higher stability of the deoxy quaternary structure as compared with the oxy quaternary structure. This theory is based on the assumption that the free energy ${}^{i}\Delta G_2$ for assembly of tetramers from dimers can be described as the sum of two terms:

$$ {}^{i}\Delta G_2 = \Delta G_a + \Delta G_s, \tag{1} $$

where ΔG_a is the contribution arising from surface area buried at the dimer-dimer interface upon association, and ΔG_2 is the free energy due to loss of translational and rotational entropy upon assembly of the tetramers. The value of i in Eq. (1) denotes either the deoxy tetramer ($i = 0$) or the oxy tetramer ($i = 4$). The value of ΔG_a is estimated as the product of the surface area which is buried at the protein:protein interface and a hydrophobic energy constant $k_a = -25$ cal mol^{-1} Å^2. The buried surface area is evaluated from crystallographic data (28). The constant k_a is estimated from (*a*) the mean free energy of transfer from ethanol to water of the amino acid side chains (29), and (*b*) the corresponding average accessible surface area for the amino acids, *M*, as calculated for the tripeptide Ala-*M*-Ala (30). From the x-ray structures of deoxyhemoglobin and methemoglobin (representing the oxy quaternary structure) Chothia et al. (27) calculated the buried surface areas to be 3,100 Å^2 and 2,300 Å^2, respectively. Calculations of ${}^{0}\Delta G_2$ and ${}^{4}\Delta G_2$ by means of Eq. (1) and the independently determined value of k_a leads to an estimated increase in free energy of 20 kcal at 25°C for the deoxy structure as compared with the oxy structure. This difference between ${}^{0}\Delta G_2$ and ${}^{4}\Delta G_2$ arises from the surface area terms alone since the ΔG_s terms cancel in taking the difference according to Eq. (1). By contrast the experimentally determined free energy difference ${}^{0}\Delta G_2 - {}^{4}\Delta G_2$ at 25°C is 5.9 kcal (10). Using the experimental values for methemoglobin (31) rather than oxyhemoglobin leads to a value of 5.0 kcal for the difference ${}^{0}\Delta G_2 - {}^{4}\Delta G_2$. The discrepancy is not minor: to bring the theoretical and experimental values into coincidence either the difference in buried surface area must have been overestimated by a factor of 4 (i.e., requiring a decrease from 800 Å to 200 Å) or the ratio of the experimentally determined equilibrium constants corresponding to ${}^{0}\Delta G_2$ and ${}^{4}\Delta G_2$ would have to be in error by a factor of ~5,000. Neither of these seems likely. The discrepancy is probably even larger since the $\alpha_1\alpha_2$ and $\beta_1\beta_2$ contributions estimated for the methemoglobin interface are probably spurious. Thus a change of 1,200 Å^2 rather than 800 Å^2 would result for the difference in buried surface area.

The hydrophobic stabilization idea can be tested further by considering the signs and magnitudes of the enthalpic and entropic contributions to the free energy of tetramer

stabilization. Since hydrophobic "bonds" generally have positive enthalpies and entropies of formation (32, 33) one would expect, if the theory were correct, to observe increasingly positive values for both the enthalpy and entropy of dimer-tetramer assembly in going from the oxy to deoxy forms. The experimental values, however, for the enthalpies and entropies of dimer-tetramer association become strongly more negative upon deoxygenation, as shown in Table I. These experimental results argue strongly against a dominant role of increased hydrophobic interaction upon deoxygenation.

The Contribution of Salt Bridges

Perutz has identified a number of salt bridges which are present in the deoxy quaternary structure of hemoglobin but are abolished upon conversion to the oxy form of the molecule. The most recent crystallographic analyses (24–26) indicate a total of six interchain salt bridges involving amino acid side chains within the $\alpha^1\beta^2$ contact of the deoxy molecule as well as two intrachain salt bridges. In addition there are two sets of electrostatic interactions in which a chloride ion "bridges" two positively charged groups, associated with the carboxy terminal Arg-141α and amino terminal group of Val-1α. This makes a total of ten salt bridges, all of which are oxygenation sensitive.

Perutz (19) has proposed that these salt bridges are the dominant factor in stabilizing the deoxy quaternary structure relative to the oxy structure and that a major fraction of the cooperative energy is stored in these bonds. In addition, four of the groups involved in these bridges are believed to be a source of Bohr protons (i.e., the Val-1α amino groups and the His-146β imidazole nitrogens). Formation of the salt bridges involving these groups result in pK_a shifts such that protons are absorbed.

In considering the relationship of these salt bridges to energetics of the dimer-tetramer assembly reactions we note that at least the interchain bridges will be broken upon dissociation of the deoxy tetramer into dimers. The first six interchain salt bridges mentioned above (excluding the chloride-mediated ones) consist both of hydrogen bonding and ion pair interaction and involve groups with pK_a's so remote from pH 7.4 that no protonation reactions will accompany their formation. The energetics of such salt bridges, all located either on the surface or at clefts accessible to water, will most likely be dominated by the energetics of the ion pair interactions with solvent, leading to significantly positive entropies and small enthalpies of either sign (32, 34–36). The hydrogen bonding characteristics of such salt bridges will differ little energetically from those of the surrounding water, whereas closer proximity of the charge groups reduces the electrostricted water, yielding an increase in entropy upon salt bridge formation.

The same considerations apply to the two intrachain salt bridges except that their formation may also be accompanied by an absorption of protons. All protonation reactions of amino acid ionizable groups occur with negative $\Delta H°$ and positive $\Delta S°$ values. Thus the formation of these salt bridges will contribute a distinctly positive $\Delta S°$ as a result of both processes. The two chloride-mediated salt bridges will exhibit the same properties as regards the sign of their entropic contribution. Thus a dominant role of the ten salt bridges would be expected to result in a distinctly more positive entropy of tetrameric formation in going from the oxy to deoxy states. By contrast, the experimentally determined entropy of assembly is more negative for the deoxy form of the tetramer by ~90 cal/mol/deg. Since the enthalpies of salt bridge formation are generally quite small, the large enthalpic linkage ${}^4\Delta H_2 - {}^0\Delta H_2$, amounting to 33 kcal, also casts doubt on a dominant role of salt bridges as the energy source for stabilizing the deoxy tetramer relative to the oxy molecule.

It should be emphasized that these results do not imply that the energies of the salt bridges make no contribution to the overall free energy difference between quaternary structures. Nor do they imply that the salt bridges are unimportant. There is considerable evidence that the salt bridges play a crucial mediating role in the interconversion between quaternary states of the molecule. The above considerations strongly suggest that the salt bridges are not the dominant source of the stabilization energies.

Assembly-linked Proton Reactions

Since dimer-tetramer formation is found to be accompanied by the absorption of protons in the deoxy state and by a release of protons in the oxy state (Table I) it is of interest to ask whether we can account for the energetics of assembly by a combination of buried surface area and proton reactions. As shown in Table I, the assembly reaction is apparently linked to a net absorption of 1.7 protons (i.e., the difference in protons released in the assembly reactions for oxy and deoxy molecules). This value for the apparent number of protons reacted is derived from the pH dependence of the dimer-tetramer association constants, and may represent a lower limit. The corresponding enthalpic difference is accurately known to be 33 kcal (3.9 ± 1.6 kcal for the oxy reaction and −28.9 ± 0.5 kcal for the deoxy reactions.) If this enthalpic difference between reactions VI and VII (scheme 2) were due solely to the heat of reaction with 1.7 protons it would require an enthalpy of 20 kcal/mol of protons released. This value although correct in sign is much larger than any ionization heats for amino acid groups having pK_a values in the region of pH 7.4 (17). Thus the apparent proton ionization per se could not account for these large enthalpic effects. In addition the sign of the experimentally-determined entropy linkage (Table I) ${}^4\Delta S_2 - {}^0\Delta S_2$ is opposite to that for a proton-driven reaction as discussed in the previous section.

If we consider the possible sum of effects due to change in buried surface area, salt bridge formation, and proton reaction we have inconsistency of the experimental values with the expected entropy sign for all three effects. These considerations suggest that none of these factors or their combination is the dominant contribution.

Hydrogen Bonds

The pattern obtained for observed thermodynamic values of subunit assembly is entirely consistent with a greater role of hydrogen bonding in stabilizing the deoxy tetramer. Hydrogen bond formation leads to increasing negative values of both enthalpic and entropic terms and that is exactly what we find in human hemoglobin. In considering the structural basis for such an effect, it is of interest that five hydrogen bonds are broken at the $\alpha^1\beta^2$ interface upon oxygenation and the same number of new ones formed. If these reactions do not occur isoenergetically (i.e., if there is a higher total energy for the oxygenation-sensitive hydrogen bonds of the deoxy molecule) then one would predict the observed pattern of effects.

Recent structural work (26) has led to the identification of four conspicuous hydrogen bonds within subunit chains of the deoxy molecule (Tyrβ145–Valβ98 and Tyrα140–Valα93) which are broken upon ligation. It seems likely that these intrachain hydrogen bonds may be disrupted upon dissociation into dimers as they are situated near the $\alpha^1\beta^2$ interface. Any oxygenation-sensitive structure alteration will be reflected in the energetics of dimer-tetramer assembly regardless of its structural localization. Thus it is quite possible that the intrachain hydrogen bonds involving the penultimate tyrosines β145 and α140 are dominant contributors to the observed pattern of thermodynamic effects.

Dynamic Considerations

It is possible that the energetics of the linkage scheme 1 involve significant contributions from the differences in distributions of vibrational states between the various molecular species. Heat capacity measurements which are presently lacking on the hemoglobin system would be of value in assessing such effects. Although the van't Hoff enthalpies presented in this discussion show little variation between 10° and 37°C, Benzinger (38) has shown that the integral of heat capacities from absolute zero is required for a totally definitive evaluation. The heat capacity may be relatively constant over a limited range and yet contribute an additive term to both the measured ΔH and ΔS which is unrelated to the bonding interactions per se of the reactions for which ΔG is measured. These terms, arising from molecular fluctuations, can give rise to enthalpy-entropy compensation as discussed by Lumry (39). There is presently no reason to believe that fluctuation behavior is the only source of such compensation effects in proteins, or that they dominate the enthalpy-entropy pattern for hemoglobin. Sturtevant (35) has estimated some contributions to protein interactions arising from vibrational states and found that they may contribute significantly to entropic effects. One interpretation of the hemoglobin results presented here is that the assembly of deoxy tetramers leads to a much greater reduction in vibrational degrees of freedom than occurs in the assembly of oxyhemoglobin. This might account for the large negative entropy and enthalpy observed.

These considerations illustrate both the power and limitations of thermodynamic analysis: if the pattern of energetic effects is determined by fluctuational behavior then all attempted correlations with bonding interactions derived from the static crystallographic analysis is invalid. In the present discussion we have limited ourselves to tests of consistency. If the fluctuations are overwhelming then our conclusions regarding inconsistency with dominance by hydrophobic, salt bridge, or protonation reactions are of course still valid.

BOHR PROTON REACTIONS ARE A MAJOR SOURCE OF ENERGETIC EFFECTS IN HEMOGLOBIN TETRAMERS

Several lines of evidence strongly suggest proton reactions as a major source of enthalpic effects in the ligation of tetramers. Consider the experimentally determined enthalpies of Table II. On the right hand column are listed the average values for van't Hoff and calorimetric enthalpies which have been corrected for heats of Bohr proton release (15). The intrinsic values of oxygenation for tetramers (reaction III) are estimated from the van't Hoff values by subtracting the heats of proton release (5.7 kcal/mol O_2 bound) from the measured heat of oxygenation (-8.6 kcal/mol O_2). The heats of proton release are calculated from the results of Antonini et al. (40). Both of these studies provide the same value of 11.0 kcal/mol of H^+ released at pH 7.4, 25°C. This value multiplied by n_{H^+} has been subtracted from the measured enthalpies to yield ΔH_Q^{corr}. Table II represents a summary of a wider range of determinations for both oxygen and CO ligands, and contains results from both calorimetric and van't Hoff methods, which were found to be in excellent agreement (15).

A significant point is that the mean values per mol of oxygen bound for reactions I, II, and III, (I, -15.8 ± 1; II, -16.6 ± 0.9; III, -14.3 ± 0.8) are not distinguishable to within the experimental errors. Thus it appears that within these limits the corrections for Bohr protons reaction account almost entirely for the difference between apparent heats of oxygen binding by tetrameric hemoglobin and those of isolated chains and dimers. The intrinsic enthalpy per heme site has been estimated from the mean of these values to be -15.6 kcal/mol O_2 At pH

9.5 where the Bohr effect is absent the calorimetrically determined heat of carbon monoxide binding is $-22.4 \pm .2$ kcal/mol of CO bound (15). This value when corrected for the differential heat of replacement of CO by O_2 (-4.0 kcal) and the heat of dissolving O_2 (-2.9 kcal/mol) yields a value of -15.5 kcal/mol O_2 (aq) which is identical to the intrinsic value obtained at pH 7.4 after correcting the experimental values for heat of proton release (15). For the overall heat of oxygenation ΔH_{obs} we may write

$$\Delta H_{obs} = \Delta H_{int} + n_{H^+} \cdot \Delta H_{H^+}, \tag{2}$$

where ΔH_{int} is the intrinsic enthalpy and ΔH_{H^+} is the enthalpy change per mole of protons released. This formula which has been verified experimentally for the overall oxygenation process is applicable to the four oxygenation steps individually. Otherwise additional heat effects would have to be present which exactly compensate the stepwise deviations from Eq. (3) in order to bring about conformity of their sums to Eq. (3). Stepwise conformity to Eq. (3) and the enthalpy values of Table III lead to a prediction of approximately equal proton release for the first three oxygenation steps (i.e., 0.7 mol H^+/mol O_2 bound) and very little Bohr effect at the last step. (16).

The experimental value of ΔH_{int} (-15.6 ± 1 kcal/mol) is similar to the value obtained for the heat of oxygenation of myoglobin as well as the mean value of isolated α and β chains.

Bohr Proton Release as a Source of the Cooperative Free Energy in Hemoglobin Tetramers

The observed variation in binding free energies ΔG for the successive oxygenation steps of tetrameric hemoglobin is shown on the right side of scheme 1 and in Table III. This variation is the primary manifestation of cooperativity and much of the effort in hemoglobin research has been directed toward understanding its molecular basis. A commonly used measure of cooperativity is the Wyman interaction energy ΔG_I (42) which equals the difference between binding free energies for the last and first steps (43). This difference accounts for the major part of the total variation in the ΔG of binding and is a good reflection of the overall enhancement in binding affinity which occurs during the successive ligation steps. From the values in Table III the calculated value of ΔG_I is -3.9 ± 0.5 kcal under the conditions specified. Resolving ΔG_I into its enthalpic and entropic contributions,

$$\Delta G_I = \Delta H_I - T\Delta S_I, \tag{3}$$

we find values of ΔH_I and $T\Delta S_I$ of (-9.7 ± 3.8) kcal and (-5.9 ± 3.9 kcal), respectively. Thus the interaction free energy is comprised of major contributions from both enthalpic and entropic terms. From the correlations between heats of proton release and oxygen binding drawn in the previous section, we see that the interaction enthalpy $\Delta H_I = -9.7$ kcal may be largely attributable to the heat of Bohr proton release.

In considering the entropic contributions to the interaction free energy we cannot partition it into protonation linked and nonprotonation linked effects since we do not have an estimate for the entropy of proton release at the first oxygenation step. In spite of this limitation we may obtain some insight from considering once more the signs of the expected and observed effects. Since all entropies of proton ionization of amino acids have negative values the effect of proton release alone would be to make the values of $T\Delta S$ for the first oxygenation step more negative than the value for the last step where very little proton release occurs. This would lead to a positive value for the interaction entropy ΔS_I whereas the experimentally determined value is actually negative. Thus it seems that the negative proton ionization term is

overcompensated by a positive entropy of other processes which accompany the binding of the first oxygen molecule. Such positive entropies accompanying oxygenation provide further evidence against a dominant role of buried surface area or of salt bridge formation stabilizing the deoxy tetramer relative to the tetramer in its oxygenated states. The sign of this effect is, however, consistent with a dominant role of hydrogen bonding in stabilizing the deoxy tetramer. It should be noted that this calculation pertains to transitions occurring within the tetrameric molecule as opposed to any additional effects which might accompany the tetramer-dimer dissociation process.

DISCUSSION

Using subunit dissociation as a means of probing the changes in thermodynamic properties which accompany ligation appears to be useful in the case of human hemoglobin. The results obtained for assembly and ligand binding reactions provide a self-consistent pattern of thermodynamic effects. In the first part of this discussion we have summarized the model-independent findings to which all mechanistic theories must conform. Next we have attempted to draw certain general correlations between thermodynamic forces and observed structural transitions which accompany oxygenation. These correlations are negative in the sense that they are tests for consistency and therefore can only be used to argue against theories, but not to verify them. We find evidence against a dominant role of hydrophobic interactions, salt bridges, and proton reactions in stabilizing the deoxy tetramer relative to the oxy molecule. The energetic results are seen to be highly consistent with a dominant role of hydrogen bond formation or of alterations in distributions of vibrational states.

In the third section of this study we have attempted to evaluate the possible role of Bohr proton effects in contributing to the energetics of cooperativity. The striking quantitative correspondence between the heats of Bohr proton release and apparent reduction in enthalpy of oxygenation for tetramers seems unlikely to be a coincidence. Certainly the enthalpy of interaction ΔH_1 may be accounted for entirely by the Bohr effect. On the other hand the corresponding entropy effects are of opposite sign. Unless the enthalpic correlation is coincidental there must be another source of entropic effects which overwhelm the entropic effect of proton release. The sign would be correct for hydrogen bond involvement of the type discussed earlier. These considerations lead to the suggestion that the dominant driving forces for cooperativity may be a combination of hydrogen bond formation, preferentially stabilizing the tetramer with large negative ΔS and small ΔH, and Bohr proton release, yielding large positive enthalpies and moderate but negative entropies. The magnitude of the negative entropies required by the hydrogen bonding interactions would be no larger than the value observed for the oxygenation-linked entropy of stabilization for deoxy vs oxy tetramers. Such a model involving a combination of hydrogen bonds and Bohr proton effects would appear to be the simplest way of accounting for the pattern of thermodynamic effects in a manner which is also consistent with the presently available structural information.

The experimental and theoretical information developed for this system has implications which are broader than the question of hemoglobin mechanism itself. In addition to the insights which can be obtained regarding normal human hemoglobin, these studies may serve as useful models for attempts to understand ligand-linked polymerization in other proteins.

This work has been supported by grants PCM 78-97582 from the National Science Foundation and grant GM 24486 from the National Institutes of Health. This is contribution No. 1055 from the McCollum Pratt Institute.

Received for publication 7 December 1979.

REFERENCES

1. Ackers, G. K., and H. R. Halvorson. 1974. The linkage between oxygenation and subunit dissociation in human hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4312–4316.
2. Atha, D. J., and G. K. Ackers. 1974. Calorimetric determination of heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. *Biochemistry.* **13**:2376–2382.
3. Rudolph, S. A., S. O. Boules, D. F. Dresden, and S. J. Gill. 1972. A calorimetric study of the binding of carbon monoxide to myoglobin. *Biochemistry.* **11**:1098–1101.
4. Gaud, H. T., B. D. Barisas, and S. J. Gill. 1974. Enthalpy changes for hemoglobin–ligand interactions revised calorimetric data. *Biochem. Biophys. Res. Commun.* **50**:1389–1394.
5. Mills, F. C ., M. L. Johnson, and G. K. Ackers. 1976. Oxygenation linked subunit interactions in human hemoglobin: experimental studies on the concentration dependence of oxygenation curves. *Biochemistry.* **15**:5350–5362.
6. Johnson, M. L., H. R. Halvorson, and G. K. Ackers. 1976. Oxygenation-linked subunit interactions in human hemoglobin: analysis of the linkage functions for constitutent energy terms. *Biochemistry.* **15**: 5363–5371.
7. Gersonde, K., L. Noll, H. T. Gaud, and S. J. Gill. . 1976. A calorimetric study of the CO Bohr effect of monomeric haemoglobins. *Eur. J. Biochem.* **62**:577–582.
8. Gaud, H. T., S. J. Gill, B. G. Barisas, and K. Gersonde. 1975. Heat of carbon monoxide binding by hemoglobin M iwate. *Biochemistry.* **14**:4584–4589.
9. Valdes, R., L. Vickers, H. R. Halvorson, and G. K. Ackers. 1979. Reciprocal effects in human hemoglobin: direct measurement at partial oxygen saturation. *Proc. Natl. Acad. Sci. U.S.A.* **75**: 5493–5497.
10. Ip, S. H. C., and G. K. Ackers. 1977. Thermodynamic studies on subunit assembly in human hemoglobin: temperature dependence of the dimer–tetramer association constants for oxygenated and unliganded hemoglobins. *J. Biol. Chem.* **252**:82–87.
11. Valdes, R., Jr., and G. K. Ackers. 1977. Thermodynamic studies on subunit assembly in human hemoglobin: self-association of oxygenated chains (α^{SH} and β^{SH}). *J. Biol. Chem.* **252**:74–81.
12. Valdes, R., and G. K. Ackers. 1978. Self-association of hemoglobin β chains is linked to oxygenation. *Proc. Natl. Acad. Sci. U.S.A.* **75**:311–314.
13. Chu, A. H., D. W. Pettigrew, B. W. Turner, and G. K. Ackers. 1980. Stability of the $\alpha^1\beta^2$ contact in human hemoglobin: implications for the mechanism of cooperativity. Proceedings of the Conference on Interactions Between Iron and Oxygen in Home Proteins, Airlie, Va. Elsevier-North Holland. New York. In press.
14. Mills, F. C ., and G. K. Ackers. 1979. Quaternary enhancement in binding oxygen by human hemoglobin. *Proc. Natl. Acad. Sci.* **76**: 273–277.
15. Mills, F. C., G. K. Ackers, H. Gaud, and S. J. Gill. 1979. Thermodynamic studies on ligand binding and subunit association of human hemoglobins. Enthalpies of binding O_2 and CO to subunit chains of hemoglobin A. *J. Biol. Chem.* **254**:2875–2880.
16. Mills, F. C., and G. K. Ackers. 1979. Thermodynamic studies on ligand binding and subunit association of human hemoglobins. Temperature dependence of the linkage between dimer-tetramer association and oxygenation state. *J. Biol. Chem.* **254**:2881–2887.
17. Rosemeyer, M. A., and F. R. Huehns. 1967. On the mechanism of the dissociation of haemoglobin. *J. Mol. Biol.* **25**:253–273.
18. Perutz, M. F. 1970. Stereochemistry of cooperative effects in haemoglobin. *Nature (Lond.).* **228**:726–734.
19. Perutz, M. F. 1976. Structure and mechanism of hemoglobin. *Brit. Med. Bull.* **32**:195–208.
20. Johnson, M. L., and G. K. Ackers. 1977. Resolvability of adair constants from oxygenation curves measured at low hemoglobin concentration *Biophys. Chem.* **7**:77–80.
21. Imai, K. 1979. Thermodynamic aspects of the cooperativity in four step oxygenation equilibria of hemoglobin. *J. Mol. Biol.* **133**: 233–247.
22. Kilmartin, J. V ., K. Imai, R. T. Jones, A. R. Faruqui, J. Fogg, and J. Baldwin. 1978. Role of Bohr group salt bridges in cooperativity in hemoglobin. *Biochim. Biophys. Acta.* **543**:15–25.
23. Lumry, R., and S. Rajender. 1970. Enthalpy-entropy compensation phenomena in water solutions of proteins and small molecules: a ubiquitous property of water. *Biopolymers* **9**:1125–1227.
24. Fermi, G. 1975. Three-dimensional Fourier synthesis of human deoxyhaemoglobin at 2.5 Å resolution: refinement of the atomic model. *J. Mol. Biol.* **97**:237–256.
25. Ladner, R. C., E. J. Heidner, and M. F. Perutz. 1977. The structure of horse methaemoglobin at 2.0 Å resolution. *J. Mol. Biol.* **114**:385–407.
26. Baldwin, J. L., and C. Chothia. 1979. Haemoglobin: the structure changes related to ligand binding and its allosteric mechanism. *J. Mol. Biol.* **129**:175–220.
27. Chothia, C., S. Wodak, and J. Janin. 1976. Role of subunit interfaces in the allosteric mechanism of hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **73**:3793–3997.
28. Lee, B., and F. M. Richards. 1971. The interpretation or protein structures: estimation of static accessibility. *J. Mol. Biol.* **55**: 379–400.

29. Nozaki, Y., and C. Tanford. 1971. The solubility of amino acid and two glycine peptides in aqueous ethanol and dioxane solutions. *J. Biol. Chem.* **246**:2211–2217.
30. Chothia, C. H., and J. Janin. 1975. Principles of protein-protein recognition. *Nature (Lond.).* **256**:705–708.
31. Schroeder, E., Wollmer, A., Kubicki, H. D., and Ohlenbusch, B. 1975. Proton-dependent dissociation equilibrium of hemoglobin. I. A 700-nanometer light scattering study on horse methemeoglobin in the pH range 4.8 to 7.2. *Biochemistry.* **15**:5693–5697.
32. Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Prot. Chem.* **14**:1–63.
33. Tanford, C. 1973. The Hydrophobic Effect. John Wiley & Sons, New York.
34. Edsall, J., and J. Wyman. 1958. Biophysical Chemistry. Academic Press, Inc., New York, 450–457.
35. Sturtevant, J. 1977. Heat capacity and entropy changes in process processes involving proteins. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2236– 2240.
36. Fersht, A. R. 1972. Conformational equilibria in α- and δ-chymotrypsin: the energetics and importance of the salt bridge. *J. Mol. Biol.* **64**:497–509.
37. Sober, H. A., and R. A. Harte, editors. 1968. Handbook of Biochemistry Selected Data for Molecular Biology. Chemical Rubber Company, Cleveland, Ohio. J–51.
38. Benzinger, T. H. 1971. Thermodynamics, chemical reactions and molecular biology. *Nature (Lond.).* **229**:100–102.
39. Lumry, R. 1980. Interpretation of calorimetric data from cooperative systems. *Proc. Natl. Conf. Bioenergetics Thermodynamics. Para, Italy. 1979. In press.*
40. Antonini, E., J. Wyman, M. Brunori, C. Fronticelli, E. Bucci, and A. Rossi-Fanneli. 1965. Studies on the relations between molecular and functional properties of hemoglobin. *J. Biol. Chem.* **240**:1096– 1103.
41. Chipperfield, J. R., L. Rossi-Bernardi, and F. J. W. Roughton. 1967. Direct calorimetric studies on the heats of ionization of oxygenated and deoxygenated hemoglobin. *J. Biol. Chem.* **242**:777–783.
42. Wyman, J. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Advan. Prot. Chem.* **19**:223–285.
43. Saroff, H. A., and A. P. Minton. 1972. The Hill plot and the energy interaction in hemoglobin. *Science (Wash., D. C.)* **175**:1253–1255.

DISCUSSION

Session Chairman: Alan N. Schechter *Scribe*: Preston Hensley

SCHECHTER: We have an extended comment from Allen Minton.

MINTON: An alternative estimate of the energetic contribution of oxygen-linked proton ionization to the free energy of cooperative interaction between subunits may be provided as follows: Let us define a quantity called the free energy of cooperative intersubunit interaction associated with the *i*th oxygenation step as equal to the difference between the standard state free energy of oxygenation for that step (corrected for the change in number of binding sites) and the standard state free energy of oxygenation for the fourth and final step.

$$\Delta G_i^{CI} \equiv \Delta G_i^{\circ} - \Delta G_4^{\circ}$$

The total free energy of cooperative oxygen-linked intersubunit interaction is then defined as

$$\Delta G^{CI} \equiv \sum_{i=1}^{4} \Delta G_i^{CI} = \sum_{i=1}^{4} \Delta G_i^{\circ} - 4\Delta G_4^{\circ}$$

From the linkage scheme presented by Ackers, the value of ΔG^{CI} may be calculated to be 10.16 kcal/mol or ~17 RT.

Using the method of Saroff and Minton (1972, *Science* **175**:1253), a simple expression may be derived for the contribution of oxygen-linked proton ionization to the total free energy of cooperative oxygen-linked intersubunit interaction:

$$\Delta G^{CI(H)} = -RT \ln \frac{\Pi_i (1 + k_i^{\text{oxy}}[\text{H}])^{n_i^{\text{oxy}}}}{\Pi_i (1 + k_i^{\text{deoxy}}[\text{H}])^{n_i^{\text{deoxy}}}}$$