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## SOME ASPECTS OF COOPERATIVITY IN HUMAN HEMOGLOBIN

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The cooperativity in hemoglobin can be described by the Hill parameter  $n$ , the free energy of interaction  $\Delta F_I$  and the allosteric free energy  $\Delta F_A$ . By this latter is meant here the free energy change associated with the transition from the deoxy to the oxy conformation in hemoglobin. In this paper some general relations between  $n$ ,  $\Delta F_I$  and  $\Delta F_A$  are given. A method is presented by which  $\Delta F_A$  can be calculated from oxygenation data.

## 1. Introduction

In the cooperative behaviour of the oxygen binding by hemoglobin an important part is played by the salt bridges which constrain the deoxy form and which break up upon going to the relaxed oxy conformation [1, 2]. The change in free energy associated with the transition from the deoxy to the oxy conformation will be defined here as the allosteric energy  $\Delta F_A$ . This free energy change was called the inter-subunit bonding energy by Noble [3] who calculated a value of 8 kcal/tetramer for it from the difference in ligand affinity between deoxyhemoglobin and the  $\alpha$  and  $\beta$  chains, whereas Perutz [1] estimated a value between 6 and 12 kcal/tetramer, based on the presence of six salt bridges. On the other hand, Wyman [4] has introduced the concept of free energy of interaction,  $\Delta F_I$ , which is quite different from  $\Delta F_A$  [5, 6]. Besides  $\Delta F_A$  and  $\Delta F_I$ , the Hill parameter  $n$  is frequently used to describe the cooperative effects in hemoglobin. The object of this paper is to show some relations between  $n$ ,  $\Delta F_A$  and  $\Delta F_I$  and further to give an estimate of the magnitude of  $\Delta F_A$ , based on oxygenation data of human hemoglobin.

2. The free energy of interaction  $\Delta F_I$ 

Our discussion will partially be based on experimental oxygenation curves and therefore we need parameters

which satisfactorily describe such a curve. We chose the Adair scheme [7] for this purpose. In this model the fractional saturation  $Y$  is given by:

$$Y = \frac{k_1 p + 3k_1 k_2 p^2 + 3k_1 k_2 k_3 p^3 + k_1 k_2 k_3 k_4 p^4}{1 + 4k_1 p + 6k_1 k_2 p^2 + 4k_1 k_2 k_3 p^3 + k_1 k_2 k_3 k_4 p^4} \quad (1)$$

where  $k_1$ ,  $k_2$ ,  $k_3$  and  $k_4$  are the intrinsic association constants for the reaction  $\text{Hb}(\text{O}_2)_{i-1} + \text{O}_2 \rightleftharpoons \text{Hb}(\text{O}_2)_i$  ( $i = 1$  to 4) and  $p$  the partial oxygen pressure. It should be realized that eq. (1) will be used in this paper merely as a mathematical description of an oxygen saturation curve. The Adair scheme has been chosen since the available experimental oxygenation curves are analyzed according to this scheme. The fact that equivalent binding sites are assumed in the derivation of eq. (1) provides no impediment to the use of it and we are not concerned with the physical meaning of  $k_1$  to  $k_4$ . The only limits we impose on the Adair model in this section is that  $k_4 \geq k_3 \geq k_2 \geq k_1$ . The models mostly used for a description of the interactions in hemoglobin, viz., the Monod–Wyman–Changeux and the Koshland–Németi–Filmer model fulfil this condition [6].

The Hill plot is defined as  $\log[Y/(1-Y)]$  against  $\log p$  and the slope  $n$  at  $Y = 0.5$  at the half saturation pressure  $p_{1/2}$  is called the Hill parameter.

We now will discuss the relation between  $n$  and  $\Delta F_I$ . Wyman [4] has shown that when  $Y$  approaches 0 or 1,  $n$  should become 1 independently of any model. Furthermore, he defined  $\Delta F_I$  as the free energy of interaction per heme which can be calculated from the distance

between the asymptotes of a Hill plot. It has been shown that in terms of the Adair model  $\Delta F_1 = RT \ln(k_4/k_1)$  [6, 8]. The cooperativity, however, as measured by  $n$  will certainly also depend on  $k_2$  and  $k_3$ , so it will be evident that  $\Delta F_1$  can only partially describe allosteric effects. To illustrate this point we investigated the dependence of  $n$  on  $\Delta F_1$ .

Let  $k_2 = ak_1$ ,  $k_3 = bk_2$ ,  $k_4 = c^{-1}k_1$  and  $k_1 p_{1/2} = x$ . Then it follows from the definition of  $n$  and from eq. (1):

$$n = (6x - 2a^2bx^3 + 4)/(3x + 3ax^2 + a^2bx^3 + 1), \quad (2)$$

where  $x$  follows from

$$a^2bc^{-1}x^4 + 2a^2bx^3 - 2x - 1 = 0. \quad (3)$$

Thus the Hill parameter is determined only by the relative magnitudes of the Adair constants. For each value of  $\Delta F_1$ ,  $n$  may take a range of values, depending on  $k_2$  and  $k_3$ . However,  $n$  reaches a maximum when  $k_1 = k_2$  and  $k_3 = k_4$ . In that case eq. (2) reduces to the simple equation:

$$n = 4(c^{1/2} + 1)/(4c^{1/2} + 3c + 1). \quad (4)$$

In fig. 1, curve A gives this relation between  $n$  and  $\Delta F_1$ . On the other hand,  $n$  becomes minimal for a fixed value of  $\Delta F_1$  when  $k_1 = k_2 = k_3$  or  $k_2 = k_3 = k_4$ . In fig. 1,

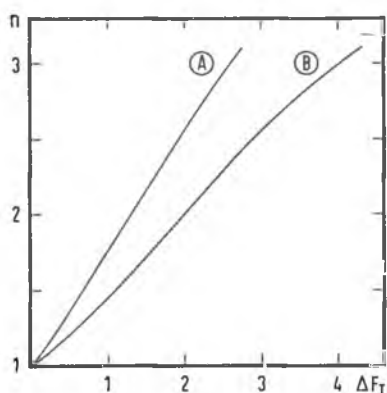


Fig. 1. Dependence of  $n$  on  $\Delta F_1$  (kcal/heme). Curve A represents the maximum and curve B the minimum value of  $n$  for a given  $\Delta F_1$  under the restrictions mentioned in the text. In the calculations a temperature of  $25^\circ$  was assumed.

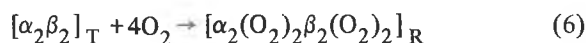
curve B represents this minimal  $n$  as a function of  $\Delta F_1$ . Thus as long as  $k_4 \geq k_3 \geq k_2 \geq k_1$  which seems to hold for human hemoglobin (at least in the absence of allosteric effectors as 2,3-diphosphoglyceric acid) [8, 9], any observed combination of  $n$  and  $\Delta F_1$  should fall in the region between curves A and B in fig. 1. For example  $n$  for human hemoglobin at neutral pH is found mostly near 2.7. From the curves presented in fig. 1 it follows that in that case  $\Delta F_1$  should vary between 2.2 and 3.3 kcal per heme as indeed usually is found. The figure further shows that an increase in  $\Delta F_1$  does not necessarily correspond with an increase in  $n$  and vice versa.

### 3. The allosteric free energy $\Delta F_A$

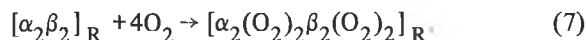
As stated in the introduction  $\Delta F_A$  stands for the standard free energy change of the transition



where  $\alpha_2\beta_2$  represents the non ligated hemoglobin tetramer and T and R the tensed deoxy and the relaxed oxy conformation, respectively. When ligand binding can be described by the two-state model [8],  $\Delta F_A$  is equal to  $RT \ln L$ ,  $L$  being the allosteric constant. Our aim is to obtain an expression for  $\Delta F_A$  in terms of Adair constants by considering the reactions

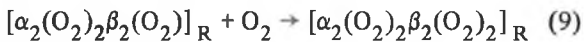
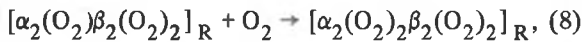


and



The free energy change associated with reaction (6) is the total free energy of oxygenation  $\Delta F_O$ . The free energy change of reaction (7) will be represented by  $\Delta F_R$ . It will be clear that  $\Delta F_A$  is equal to  $\Delta F_O - \Delta F_R$ .  $\Delta F_O$  is simply given by  $4RT \ln p_m$ ,  $p_m$  being the median oxygen pressure [4] whereas  $p_m$  is related to the Adair constants by the relation  $p_m^4 = (k_1 k_2 k_3 k_4)^{-1}$  [6, 8]. For the determination of  $\Delta F_R$  we will assume that before the last ligation step takes place, the molecule has switched over/from the T to the R state. In the model proposed by Perutz [1] a conformational change has been suggested after the binding of the second ligand. Kinetic experiments [10, 11], non-linear relationships

between fractional saturation and structural changes of hemoglobin as observed by electron paramagnetic resonance [12], nuclear magnetic resonance [13], ultraviolet spectroscopy [14] and the release of 2,3-diphosphoglyceric acid [15, 16] seem to support indeed that at least the binding of the last ligand takes place when hemoglobin is in the relaxed conformation. Theoretical considerations support this view [6, 8, 17–19]. Indicating the affinity constants of the reactions



by  $a_4$  and  $b_4$ , respectively,  $\Delta F_{\text{R}}$  becomes  $-RT \ln a_4^2 b_4^2$  and so

$$\Delta F_{\text{A}} = RT \ln a_4^2 b_4^2 p_{\text{m}}^4. \quad (10)$$

Unfortunately, values of  $a_4$  and  $b_4$  are lacking for oxygen as a ligand. Only with *n*-butyl isocyanide as a ligand have these constants been determined [20]. Here it was found that  $a_4$  is about two times  $b_4$ .

However, for  $k_4$  we can write  $k_4 = 2a_4 b_4 / (a_4 + b_4)$  and with  $a_4 = f b_4$  eq. (10) becomes

$$\Delta F_{\text{A}} = RT \ln (k_4^4 p_{\text{m}}^4) + \Delta F_{\text{f}}, \quad (11)$$

where  $\Delta F_{\text{f}} = 2RT \ln [(f+1)^2 / 4f]$ . It should be noted that  $\Delta F_{\text{f}}$  is always positive. Eq. (11) reduces to

$$\Delta F_{\text{A}} = RT \ln (k_4^4 p_{\text{m}}^4) = RT \ln (k_4^3 / k_1 k_2 k_3) \quad (12)$$

when  $f = 1$ . There are several arguments suggesting that  $a_4$  and  $b_4$  do not differ more than a factor 2–3. First, the isolated  $\alpha$  and  $\beta$  chains have equal or nearly equal oxygen affinity [21, 22], although an unequivocal interpretation is obscured by the different association behaviour of the isolated chains; moreover, the affinity of the isolated  $\beta$  chains has been reported to be equal to  $k_4$  [22]. It is also pertinent that on modification of hemoglobins or on total dissociation of modified hemoglobins the cooperativity mostly reduces but  $n$  never becomes smaller than 1 [23–25], whereas a difference of a factor two in affinity would result in an  $n$  of about 0.9 in absence of any cooperativity.

Studies on artificial cyano- or aquomet intermediates of human hemoglobin indicate a small non-equivalence

of the chains. From reported values of  $n$  and  $p_{1/2}$  one can easily calculate the affinity constants  $a_2$  and  $b_2$  for the binding of the second molecule of oxygen of the intermediates  $\alpha_2\beta_2^+$  and  $\alpha_2^+\beta_2$ . It was found that  $b_2$  is about 1.5 to 2.5 times  $a_2$  [21, 26]. On the other hand, the measurements of Maeda et al. [27] on cyanomet intermediates result in values for  $a_2$  and  $b_2$  of 3.9 and 3.4 mmHg<sup>-1</sup>, respectively, whereas  $k_4 = 4.0$  mmHg<sup>-1</sup> under identical experimental conditions [8]. In the case of non-equivalence  $\Delta F_{\text{A}}$  calculated using eq. (12) will be too small, but  $\Delta F_{\text{f}}$  amounts to only 0.14 kcal when  $f = 2$  and to 0.34 kcal when  $f = 3$ . In view of the absolute magnitude of  $\Delta F_{\text{A}}$  this is not a serious error so we will use eq. (12) as a very good approximation.

We now want to make some remarks about  $\Delta F_{\text{A}}$ . By rewriting eq. (12) in the form

$$\Delta F_{\text{A}} = \Delta F_{\text{I}} + RT \ln (k_4^2 / k_2 k_3), \quad (13)$$

it follows that  $\Delta F_{\text{A}} = \Delta F_{\text{I}}$  when  $k_2 = k_3 = k_4$  and  $\Delta F_{\text{A}} = 3\Delta F_{\text{I}}$  when  $k_1 = k_2 = k_3$ . So  $\Delta F_{\text{I}} \leq \Delta F_{\text{A}} \leq 3\Delta F_{\text{I}}$ . The limits  $\Delta F_{\text{A}} = \Delta F_{\text{I}}$  and  $\Delta F_{\text{A}} = 3\Delta F_{\text{I}}$  correspond with a minimal value of  $n$ , whereas the maximal value of  $n$  may be observed when  $\Delta F_{\text{A}} = 2\Delta F_{\text{I}}$ . Since  $\Delta F_{\text{I}}$  ranges from 2.2 to 3.3 (see above),  $\Delta F_{\text{A}}$  should vary between 2.2 and 9.9 kcal/tetramer. It should be noted here that  $\Delta F_{\text{A}}$  has been defined per tetramer and  $\Delta F_{\text{I}}$  per heme.

It should further be recognized that the occurrence of Adair parameters in the several equations does not mean that the validity of these equations depends on the validity of the Adair equation (1). If a different starting model was used other formulae would appear. However, applied to the same experimental data, the same value of  $\Delta F_{\text{A}}$  should be found. In other words, the value of  $\Delta F_{\text{A}}$  is of course independent of any model. The difference between the way of calculating  $\Delta F_{\text{A}}$  as presented here and that of Noble [3] is evident. The approximation introduced by Noble is that in eq. (10)  $p_{\text{m}}$  is equated to  $p_{1/2}$  and  $a_4$  to  $b_4$ , taking for the last two the affinity of the isolated chains, thus assuming that this affinity does not change if an isolated chain is embodied in the tetrameric relaxed hemoglobin molecule.

It should be realized that  $\Delta F_{\text{A}}$  changes when experimental conditions as pH, temperature, ionic strength, protein concentration and concentration of allosteric

effectors are varied. We briefly want to discuss the temperature and pH effect. The temperature effect is relatively small: in the region 20–30°,  $\Delta F_A$  does not change significantly according to the results of Imai and Tyuma [28]. The pH dependence is rather large and results from the change in  $p_{1/2}$  with pH. As long as  $k_4$  remains constant, which seems to be the case in pH region 7.0 to 7.8 [22], this change in  $\Delta F_A$  is given by  $4RT(\Delta \ln p_m)$ . This means that  $\Delta F_A$  decreases continuously from pH 7 to higher pH. For example, from the data of Bunn and Guidotti [29] one finds a decrease in  $\Delta F_A$  of about 1.7 kcal going from pH 7.0 to 7.8 (0.03 M bis-tris, 0.01 M Cl<sup>-</sup>, equating  $p_m$  to  $p_{1/2}$ ).

Applying eq. (12) to the Adair constants reported by Roughton and Lyster [9] for human hemoglobin (pH 7.0, 0.6 M phosphate buffer, 19°) and to the data of Imai [8] (pH 7.4, 0.05 M bis-tris buffer, 25°) one can easily calculate a value for  $\Delta F_A$  of 5.7 kcal and 4.7 kcal, respectively. The difference of 1 kcal between the two values can be attributed mainly to the difference in pH of the two sets of data.

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