BIOPHYSICAL CHEMISTRY 1 (1973) 130–133. © NORTH-HOLLAND PUBLISHING COMPANY

SOME ASPECTS OF COOPERATIVITY IN HUMAN HEMOGLOBIN

Lambert H.M. JANSSEN and Simon H. DE BRUIN
Department of Biophysical Chemistry, University of Nijmegen, Nijmegen, The Netherlands

Received 14 May 1973
Revised manuscript received 30 August 1973

The cooperativity in hemoglobin can be described by the Hill parameter n, the free energy of interaction ΔF_I and the allosteric free energy ΔF_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, ΔF_I and ΔF_A are given. A method is presented by which ΔF_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 ΔF_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 α and β 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, ΔF_I, which is quite different from ΔF_A [5, 6]. Besides ΔF_A and ΔF_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, ΔF_A and ΔF_I and further to give an estimate of the magnitude of ΔF_A, based on oxygenation data of human hemoglobin.

2. The free energy of interaction ΔF_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 $Hb(O_2)_{i-1} + O_2 \rightleftharpoons Hb(O_2)_i$ ($i = 1$ 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–Némethy–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 ΔF_I. Wyman [4] has shown that when Y approaches 0 or 1, n should become 1 independently of any model. Furthermore, he defined ΔF_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 $\Delta F_I = RT\ln(k_4/k_1)$ [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 ΔF_I can only partially describe allosteric effects. To illustrate this point we investigated the dependence of n on ΔF_I.

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 ΔF_I, 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 ΔF_I. On the other hand, n becomes minimal for a fixed value of ΔF_I when $k_1 = k_2 = k_3$ or $k_2 = k_3 = k_4$. In fig. 1, curve B represents this minimal n as a function of ΔF_I. Thus as long as $k_4 \geqslant k_3 \geqslant k_2 \geqslant k_1$ 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 ΔF_I 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 ΔF_I should vary between 2.2 and 3.3 kcal per heme as indeed usually is found. The figure further shows that an increase in ΔF_I does not necessarily correspond with an increase in n and vice versa.

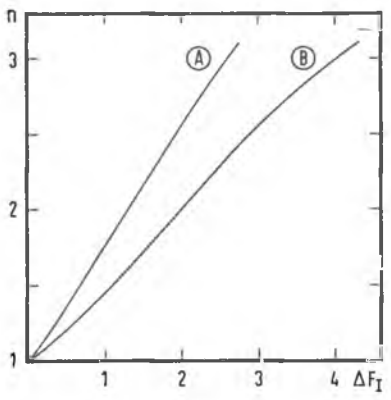

Fig. 1. Dependence of n on ΔF_I (kcal/heme). Curve A represents the maximum and curve B the minimum value of n for a given ΔF_I under the restrictions mentioned in the text. In the calculations a temperature of 25° was assumed.

3. The allosteric free energy ΔF_A

As stated in the introduction ΔF_A stands for the standard free energy change of the transition

$$[\alpha_2\beta_2]_T \rightarrow [\alpha_2\beta_2]_R, \quad (5)$$

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], ΔF_A is equal to $RT\ln L$, L being the allosteric constant. Our aim is to obtain an expression for ΔF_A in terms of Adair constants by considering the reactions

$$[\alpha_2\beta_2]_T + 4O_2 \rightarrow [\alpha_2(O_2)_2\beta_2(O_2)_2]_R \quad (6)$$

and

$$[\alpha_2\beta_2]_R + 4O_2 \rightarrow [\alpha_2(O_2)_2\beta_2(O_2)_2]_R \quad (7)$$

The free energy change associated with reaction (6) is the total free energy of oxygenation ΔF_O. The free energy change of reaction (7) will be represented by ΔF_R. It will be clear that ΔF_A is equal to $\Delta F_O - \Delta F_R$. ΔF_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 $p_m^4 = (k_1k_2k_3k_4)^{-1}$ [6, 8]. For the determination of ΔF_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

$$[\alpha_2(O_2)\beta_2(O_2)_2]_R + O_2 \rightarrow [\alpha_2(O_2)_2\beta_2(O_2)_2]_R, \quad (8)$$

$$[\alpha_2(O_2)_2\beta_2(O_2)]_R + O_2 \rightarrow [\alpha_2(O_2)_2\beta_2(O_2)_2]_R \quad (9)$$

by a_4 and b_4, respectively, ΔF_R becomes $-RT\ln a_4^2 b_4^2$ and so

$$\Delta F_A = RT\ln a_4^2 b_4^2 p_m^4. \quad (10)$$

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 $a_4 = fb_4$ eq. (10) becomes

$$\Delta F_A = RT\ln(k_4^4 p_m^4) + \Delta F_f, \quad (11)$$

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

$$\Delta F_A = RT\ln(k_4^4 p_m^4) = RT\ln(k_4^3/k_1 k_2 k_3) \quad (12)$$

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 α and β 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 β 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 $mmHg^{-1}$, respectively, whereas $k_4 = 4.0$ $mmHg^{-1}$ under identical experimental conditions [8]. In the case of non-equivalence ΔF_A calculated using eq. (12) will be too small, but ΔF_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 ΔF_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 ΔF_A. By rewriting eq. (12) in the form

$$\Delta F_A = \Delta F_I + RT\ln(k_4^2/k_2 k_3), \quad (13)$$

it follows that $\Delta F_A = \Delta F_I$ when $k_2 = k_3 = k_4$ and $\Delta F_A = 3\Delta F_I$ when $k_1 = k_2 = k_3$. So $\Delta F_I \leqslant \Delta F_A \leqslant 3\Delta F_I$. The limits $\Delta F_A = \Delta F_I$ and $\Delta F_A = 3\Delta F_I$ correspond with a minimal value of *n*, whereas the maximal value of *n* may be observed when $\Delta F_A = 2\Delta F_I$. Since ΔF_I ranges from 2.2 to 3.3 (see above), ΔF_A should vary between 2.2 and 9.9 kcal/tetramer. It should be noted here that ΔF_A has been defined per tetramer and ΔF_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 ΔF_A should be found. In other words, the value of ΔF_A is of course independent of any model. The difference between the way of calculating ΔF_A as presented here and that of Noble [3] is evident. The approximation introduced by Noble is that in eq. (10) p_m is equated to $p_{I/2}$ 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 ΔF_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°, ΔF_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 ΔF_A is given by $4\,RT\,(\Delta \ln p_m)$. This means that ΔF_A decreases continuously from pH 7 to higher pH. For example, from the data of Bunn and Guidotti [29] one finds a decrease in ΔF_A of about 1.7 kcal going from pH 7.0 to 7.8 (0.03 M bis–tris, 0.01 M Cl^-, 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 ΔF_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.

References

[1] M.F. Perutz, Nature 228 (1970) 726.
[2] M.F. Perutz, Nature 237 (1972) 495.
[3] R.W. Noble, J. Mol. Biol. 39 (1969) 479.
[4] J. Wyman, Advan. Protein Chem. 19 (1964) 223.
[5] H.A. Saroff and A.P. Minton, Science 175 (1972) 1253.
[6] L.H.M. Janssen and S.H. de Bruin, Intern. J. Peptide Protein Res. 5 (1973) 27.
[7] G.S. Adair, J. Biol. Chem. 63 (1925) 529.
[8] K. Imai, Biochemistry 12 (1973) 798.
[9] F.J.W. Roughton and J. Lyster, Hvalradets Skr. 48 (1965) 185.
[10] U. Schmelzer, R. Steiner, A. Mayer, T. Nedetzka and H. Fasold, European J. Biochem. 25 (1972) 491.
[11] J.M. Salhany, D.H. Mathers and R.S. Eliot, J. Biol. Chem. 247 (1972) 6985.
[12] T. Asakura, Biochem. Biophys. Res. Commun. 48 (1972) 517.
[13] W.H. Huestis and M.A. Raftery, Biochem. Biophys. Res. Commun. 49 (1972) 1358.
[14] K. Imai and T. Yonetani, Biochem. Biophys. Res. Commun. 50 (1973) 1055.
[15] R. MacQuarrie and Q.H. Gibson, J. Biol. Chem. 246 (197 5832.
[16] R. MacQuarrie and Q.H. Gibson, J. Biol. Chem. 247 (1972) 5686.
[17] J.J. Hopfield, R.G. Shulman and S. Ogawa, J. Mol. Biol. 61 (1971) 425.
[18] J. Herzfeld and H.E. Stanley, Biochem. Biophys. Res. Commun. 48 (1972) 307.
[19] R.T. Ogata and H.M. McConnell, Biochemistry 11 (1972) 4792.
[20] J.S. Olson and Q.H. Gibson, J. Biol. Chem. 246 (1971) 5241.
[21] M. Brunori, G. Amiconi, E. Antonini, J. Wyman and K.H. Winterhalter, J. Mol. Biol. 49 (1970) 461.
[22] I. Tyuma, K. Shimizu and K. Imai, Biochem. Biophys. Res. Commun. 43 (1971) 423.
[23] E. Antonini, J. Wyman, R. Zito, A. Rossi-Fanelli and A. Caputo, J. Biol. Chem. 236 (1961) PC 60.
[24] J.V. Kilmartin and J.A. Hewitt, Cold Spring Harbor Symp. Quant. Biol. 36 (1972) 311.
[25] J.A. Hewitt, J.V. Kilmartin, L.F. ten Eyck and M.F. Perutz, Proc. Natl. Acad. Sci. US 69 (1972) 203.
[26] R. Banerjee and R. Cassoly, J. Mol. Biol. 42 (1969) 351.
[27] T. Maeda, K. Imai and I. Tyuma, Biochemistry 11 (1972) 3685.
[28] K. Imai and I. Tyuma, Biochem. Biophys. Res. Commun. 51 (1973) 52.
[29] H.F. Bunn and G. Guidotti, J. Biol. Chem. 247 (1972) 2345.