Analysis of the effect of bezafibrate on the oxygen dissociation curve of human hemoglobin

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Analysis of the effects of a range of concentrations of the antihyperlipoproteinemia drug, bezafibrate, on the oxygen dissociation curve of dilute hemoglobin solutions indicates a 4:1 binding stoichiometry with a site binding constant of 434 mol^{-1} for deoxyhemoglobin. Analysis of the combined effects of a range of bezafibrate and 2,3-diphosphoglycerate (DPG) concentrations indicates that 2 of the 4 bezafibrate sites are in the vicinity of the DPG binding site, and competitive with DPG binding, and 2 of the sites are remote from the DPG binding site.

2,3-Diphosphoglycerate *Bezafibrate* *Oxygen dissociation curve* *Hemoglobin* *Right-shift*

1. INTRODUCTION

A recent publication [1] described the right-shifting effect of an antihyperlipoproteinemia drug in current use, bezafibrate, on the oxygen dissociation curve of hemoglobin. Unlike most other right-shifting agents which are highly charged anions, this compound was able to cross the red cell membrane and gave right-shifts in whole blood as well as on hemoglobin solution. The potential clinical benefits for such a compound were discussed [1]. A more detailed analysis of the effects of bezafibrate on the oxygen dissociation curve of dilute hemoglobin solutions, both alone and in combination with the natural effector, DPG [2], is presented here. It is shown that bezafibrate binds at multiple sites on the hemoglobin tetramer with a relatively weak association constant (434 mol^{-1}) and it is therefore doubtful whether the binding is sufficiently specific for hemoglobin compared to other proteins for the compound to be useful as a right-shifting agent in vivo.

Abbreviations: DPG, 2,3-diphosphoglycerate; IHP, *myo*-inositol hexaphosphate; bezafibrate, 2-[4-(2-*p*-chlorobenzamidoethyl)phenoxyl]-2-methylpropionate

2. MATERIALS AND METHODS

Human hemoglobin solutions of low phosphate content were prepared as in [3] and stored as 2-cm^3 aliquots at −20°C. Bezafibrate was a gift from Boehringer and DPG was purchased as the Tris salt from Calbiochem. Both compounds were used without further purification.

Oxygen dissociation curve measurements of dilute hemoglobin solutions (23 μM on a tetramer basis, 5.4 ml solution) were carried out at 37°C using a commercially available automatic apparatus (Hemox-Analyzer, TCS Medical Products Division, Southampton, PA). Hemoglobin solutions containing the required concentrations of compounds plus 35 mM KCl in 100 mM Hepes buffer adjusted to pH 7.4 were prepared in the optical cell (path length 15 mm). They were equilibrated in air for 10 min before bubbling nitrogen (British Oxygen Company 'white spot') through the stirred solution to deoxygenate it, which typically required about 30 min. Twenty μl of a 1.5% solution of silicone emulsion (diluted 1 in 20 from a stronger solution purchased from BDH) was also added to prevent frothing. The dissociation curves were measured as continuous curves on an X–Y

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plotter and in digital form (~150 points/curve) using an A–D converter (Interactive Structures Inc.) and an Apple IIe microcomputer. For subsequent data analysis 10 points per curve were selected at appropriate intervals along the saturation axis and theoretical models were fitted to the data using a standard non-linear iterative procedure as in [4]. P_m values (median oxygen pressure, the pressure at which the concentrations of deoxy- and oxyhemoglobin are equal) were calculated from the results of fitting the Adair equation to each data set [5]. Replicate determinations gave an associated error in P_m of ± 2% (standard deviation/mean).

3. RESULTS

The effect of a range of bezafibrate concentrations on the oxygen dissociation curve of dilute hemoglobin solution is shown in fig.1. The compound produces large right-shifts at high concentrations comparable to the shifts obtained with the most potent allosteric effector known at present, IHP [4]. However, bezafibrate differs from IHP in two important respects; much higher concentrations are necessary to produce similar effects and the range of effect for bezafibrate occurs over a narrower concentration range compared with other effectors such as IHP or DPG. Fig.2 shows

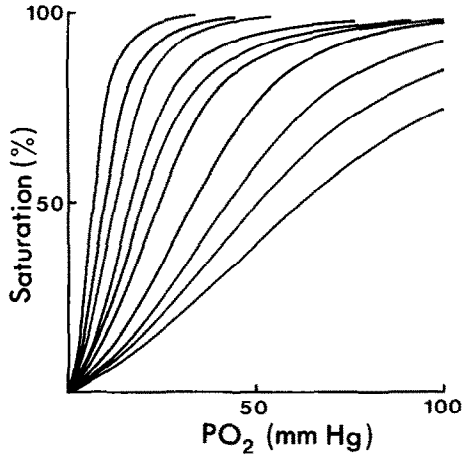

Fig.1. Effect of a range of bezafibrate concentrations on the oxygen dissociation curve of dilute (23 μM) hemoglobin solutions; 100 mM Hepes buffer (pH 7.4), 35 mM KCl, T = 37°C. Bezafibrate concentration from left to right: 0, 1, 2, 3.5, 5, 7, 10, 15, 20, 30 mM.

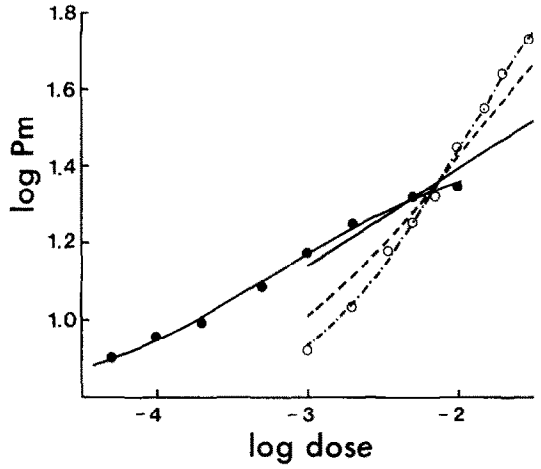

Fig.2. Plot of log P_m *vs* log concentration for DPG and bezafibrate, conditions as for fig.1: (●) DPG; (○) bezafibrate. The lines are the best-fit curves from fitting eq.1 to the data with: (——) n = 1, (– – –) n = 2, (·–·–·) n = 4 where n is the number of binding sites.

plots of log P_m against log concentration for DPG and bezafibrate and illustrates the steeper slope of the bezafibrate curve, suggesting multiple binding sites and cooperativity in binding for this compound.

For a compound binding at n equivalent sites on the hemoglobin tetramer eq.1 holds [5]:

$$\log P_m = \log P_{m_{stripped}} + \frac{n}{4} \log \left\{ \frac{(1 + dK_D)}{(1 + dK_O)} \right\} \quad (1)$$

where d is the free compound concentration and K_D and K_O are the association constants for binding to deoxy- and oxyhemoglobin, respectively (for $n > 1$ the individual sites are assigned the same site association constants and it is assumed that n is the same for oxy- and deoxyhemoglobin). For bezafibrate the lowest concentration used (1 mM) was more than 40 times the hemoglobin concentration (0.023 mM) so the total compound added was equated with the free concentration. Eq.1 was fitted non-linearly for values of n fixed at 1, 2 and 4 or allowed to float and the results are shown in table 1. The most satisfactory fit to the data is clearly obtained for n = 4, and the corresponding values for K_D and K_O are 434 ± 14 mol^{-1} and 19.0 ± 2.5 mol^{-1}, respectively. When the value of n was allowed to vary it was found to be not significantly different from 4.

Table 2 shows the effect of a range of DPG and

Table 1

Results of fitting eq.1 with various binding stoichiometries to log P_m data for bezafibrate

Binding site number (n)	K_D (mol^{-1})	K_O (mol^{-1})	Sum of squares
1	25500 (13200)	0	1.0380
2	1780 (21)	0	0.1680
4	434 (14)	19.0 (2.5)	0.0059
4.73 (1.43)	364 (108)	29.4 (19.2)	0.0055

Eq.1 was fitted to the data illustrated in fig.2 for bezafibrate with the binding stoichiometry fixed at the values indicated or allowed to float. For $n = 1$ and 2 the value of K_O was not significantly different from zero. The sum of squares is the sum of the squares of residuals between observed and calculated log P_m values. Approximate estimated standard errors are shown in parentheses beneath each best-fit parameter value. The best fit is obtained for $n = 4$

bezafibrate concentrations on the P_m for hemoglobin solutions when the two effectors are present in the same solution. From a single measured curve in the presence of 5 mM bezafibrate and 10 mM DPG and some preliminary crystallographic data, authors in [1] inferred different binding sites for the two effectors and a resulting synergy of effect. A more rigorous analysis is possible with the present data. If the two compounds bind at the same site, such that the binding of one excludes the other, then the equation corresponding to eq.1 is (where subscript 1 refers to DPG and 2 to bezafibrate and $\Delta\log P_m$ is the difference between treated and control curves):

$$\Delta\log P_m = \frac{1}{4}\log\left\{\frac{d_1K_{D1} + (1 + d_2K_{D2})^4}{d_1K_{O1} + (1 + d_2K_{O2})^4}\right\} \quad (2)$$

If the compounds bind at completely different sites then the appropriate equation is:

$$\Delta\log P_m = \frac{1}{4}\log\left\{\frac{(1 + d_1K_{D1})(1 + d_2K_{D2})^4}{(1 + d_1K_{O1})(1 + d_2K_{O2})^4}\right\} \quad (3)$$

A third situation may be identified, one of mixed competition where 2 of the 4 sites for bezafibrate are competitive with DPG binding and 2 are remote from the DPG site. The appropriate equation is then:

$$\Delta\log P_m = \frac{1}{4}\log\left\{\frac{(1 + d_2K_{D2})^2[d_1K_{D1} + (1 + d_2K_{D2})^2]}{(1 + d_2K_{O2})^2[d_1K_{O1} + (1 + d_2K_{O2})^2]}\right\} \quad (4)$$

Table 2

Observed and calculated log P_m values for DPG and bezafibrate in combination

DPG conc. (mM)	Bezafibrate conc. (mM)	Observed log P_m	Calculated log P_m		
			eq.2	eq.3	eq.4
1	1	1.25	1.19	1.33	1.26
1	2	1.32	1.20	1.44	1.31
1	5	1.41	1.29	1.65	1.42
1	10	1.57	1.46	1.84	1.54
2	1	1.30	1.25	1.40	1.32
2	2	1.36	1.26	1.51	1.38
2	5	1.46	1.31	1.71	1.48
2	10	1.59	1.46	1.90	1.58
5	1	1.39	1.32	1.48	1.40
5	2	1.47	1.32	1.58	1.45
5	5	1.56	1.35	1.79	1.55
5	10	1.64	1.46	1.98	1.64
Residual sum of squares:			0.200	0.531	0.003

The log P_m values were determined as described in the text for the DPG and bezafibrate concentrations indicated. The calculated values were obtained using eq.2 (competitive), eq.3 (non-competitive) and eq.4 (mixed competition) with K_D and K_O values of 3.53×10^4 and 87.8 mol^{-1} for DPG and 434 and 19.0 mol^{-1} for bezafibrate. The log P_m for stripped hemoglobin was 0.81. The residual sum of squares between observed and calculated log P_m values show that eq.4 representing 2 sites competitive with DPG binding and 2 non-competitive, gives the best fit to the data

The K_D and K_O values for bezafibrate and DPG obtained from fitting eq.1 to the data for these compounds alone, may be substituted into eq.2–4 to calculate the expected log P_m values for these alternative binding situations. It is clear that the mixed competitive model of eq.4 gives the closest agreement between observed and calculated log P_m values and this is emphasized by the last figure in each column for the sum of squares of residuals between observed and calculated values. The data

therefore indicate only partial synergy of effects for DPG and bezafibrate with 2 of the 4 binding sites competitive with DPG binding and two remote from the DPG binding site.

4. DISCUSSION

Most right-shifting compounds so far described are highly charged anionic compounds and bind at a single site, the DPG binding site, in deoxyhemoglobin [4,6–9]. Recently, however, anionic compounds which also possess a hydrophobic component have been shown to bind at more than one site. β-Naphthyl triphosphate was reported to bind in a 2:1 molar ratio [10] and the dye, erythrosin, was observed to bind to a large number of binding sites in both deoxy- and oxyhemoglobin, but with 4 molecules bound more strongly than the rest [11]. In addition it was shown [10] that one of the β-naphthyl triphosphate binding sites is competitive with DPG binding and 2 of the 4 strong erythrosin binding sites are competitive with IHP (and thus DPG) binding [12].

The steep slope of the log P_m *vs* log-dose curve for the hydrophobic anion bezafibrate clearly demonstrates multiple sites for the binding of the compound to deoxyhemoglobin and 4 sites gave the best fit to the data. The site binding constant of 434 mol^{-1} represents weak binding compared with DPG (3.53×10^4 mol^{-1} under the same conditions) and the data give no information on any further binding which is not oxygen-linked, as observed for erythrosin. In fact, clofibrate, an analogue of bezafibrate was reported to have up to 12 binding sites to deoxyhemoglobin [13]. However, any such additional binding would not significantly affect the results of the present analysis due to the high compound to hemoglobin ratios employed.

The combination experiments with DPG indicate competition for binding at 2 of the 4 bezafibrate binding sites. The binding site for DPG at the β-terminal amino region is highly positively charged but there are extensive hydrophobic areas on each side of this region. Modelling indicates that there is room for two molecules of bezafibrate to bind in an antiparallel fashion with their charged ends interacting with the terminal amino groups themselves and/or adjacent histidine residues (β2 and β143) and the hydrophobic ends extending into the hydrophobic areas particularly near residues Leu-81β, Phe-85β and Val-137β. The identity of the remaining two oxygen-linked sites is less clear, although the binding of inorganic anions [14] and certain pyridoxal derivatives [15–17] at the α-terminal amino region suggests this as a likely candidate. Again two possible binding sites may be identified comprising the terminal amino groups of one α-chain and arginine 141 of the other for the ionic component and residues Trp-37β, Pro-95α and Tyr-140α as part of a hydrophobic component. Crystallographic data for clofibrate [13] revealed only binding in the center of the hemoglobin tetramer and this was also indicated for bezafibrate [1]. However, the crystallizations were performed under conditions of high salt concentration which would inhibit the ionic binding at the α- and β-terminal amino regions and favour purely hydrophobic bonding as observed.

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