Reduction in potency and reversal of left-shifting activity of BW12C with the major and minor components of chicken hemoglobin

Raymond Wootton, David K. Stammers and Ralph D. White

Department of Biochemistry, Wellcome Research Laboratories, Langley Court, Beckenham BR3 3BS, England

Received 15 July 1986

The effects of the left-shifting, anti-sickling compound BW12C (5-(2-formyl-3-hydroxyphenoxy)pentanoic acid) on the oxygen saturation curve of whole chicken blood and the isolated major (AII) and minor (AI) components of chicken hemoglobin have been studied. The results support the postulated major binding mode for BW12C to human hemoglobin of bridging between the α-chain terminal amino groups in the oxy conformation with an important hydrophobic component contributed mainly by Pro 77α residues. In chicken AII (Pro 77α→Ser) BW12C still left-shifts at high concentrations but its potency is greatly reduced (at least 10-fold). In chicken AI (Pro 77α→Ser and Val 1α→Met) BW12C is a right-shifter with a potency comparable to that of 2,3-diphosphoglycerate suggesting that binding at the β-chain termini in the deoxy conformation is now dominant with α-chain binding no longer significant.

BW12C *Oxygen saturation curve* *(Chicken blood)* *Left-shift* *Hemoglobin* *Anti-sickling*

1. INTRODUCTION

BW12C, 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid, is a potent left-shifter of the oxygen saturation curve of human hemoglobin (HbA) and an inhibitor of the sickling of erythrocytes from homozygous sickle cell blood in vitro at reduced oxygen pressures [1,2]. The compound was designed to bind preferentially to the oxy conformation of HbA at the α-chain terminal amino region forming a Schiff base interaction with one amino group (Val 1α) and an ionic interaction with the other. In addition, modelling indicated that the tetramethylene side-chain could form a favorable hydrophobic interaction with hydrophobic residues at this site particularly Pro 77α and the potency of BW12C suggests that this is the case.

Preferential interaction of BW12C with the α-terminal amino groups in the oxy conformation has been established [3] but in order to provide evidence for the involvement of the Pro 77α residues the literature [4,5] was searched for mutant human hemoglobins or hemoglobins from alternative species with suitable sequence differences. Studies of the effects of different hemoglobins on the action of a series of right-shifting compounds previously provided evidence for binding at the β-chain terminal amino region as postulated [6]. No mutant human hemoglobins containing suitable modifications in the vicinity of the α-chain termini were found but chicken hemoglobins appeared interesting. Chicken erythrocytes contain two main hemoglobin types, chicken major (AII, ~75%) and chicken minor (AI, ~20%) [7]. The major component has the Pro 77α groups replaced by serine residues [8] and the minor component in addition has the Val 1α groups replaced by methionine residues [9]. The effects of BW12C on chicken blood and isolated chicken hemoglobins are reported here.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

Chicken blood from freshly killed White Leghorn chickens was collected into heparinised tubes. The hematocrit was determined as 30% which is lower than for normal human blood (43%). A mixture of chicken hemoglobins was prepared using the method of Paterson et al. [10]. This was then dialysed against 10 mM Hepes (Hopkins and Williams), pH 7.0, and applied to a carboxymethyl-cellulose column (CM-32, Whatman) equilibrated in the same buffer. Contaminating proteins were removed by washing with running buffer followed by 10 mM Hepes at pH 7.5 and the minor and major chicken hemoglobins were eluted by 10 mM Hepes, pH 8.0, and 10 mM Hepes, pH 8.0, plus 50 mM NaCl, respectively. Organic phosphates were removed by passage down a Sephadex G-25 column equilibrated in 0.1 M NaCl, 1 mM Hepes, pH 7.2. Phosphate contents were determined [10] as 0.07 and 0.13 mol total phosphate per mol hemoglobin for minor and major components, respectively. Inositol hexaphosphate was purchased as the sodium salt (BDH) and used as supplied. BW12C was prepared by Dr G. Kneen as described in published UK patent specification no.2053218, Kneen, G. (The Wellcome Foundation Ltd).

Oxygen saturation curve measurements on whole blood were performed in the association mode at 37°C using a Hem-O-Scan (Aminco, Silver Springs, MD) as described in [2]. Saturation curves for dilute hemoglobin solutions (23 μM on a tetramer basis, 5.4 ml of total solution 35 mM KCl, 100 mM Hepes; pH 7.4) were measured in the dissociation mode using a Hemox Analyzer (TCS Medical Products Division, Southampton, PA) as in [2].

3. RESULTS

The effect of a range of concentrations of BW12C on the oxygen association curve of fresh chicken blood is shown in fig.1a, and fig.1b gives corresponding curves for normal human blood for comparison [2]. As noted previously [2] the high affinity of BW12C for human hemoglobin results in essentially stoichiometric binding in whole blood, and at lower doses the curves show a biphasic appearance due to the two functionally

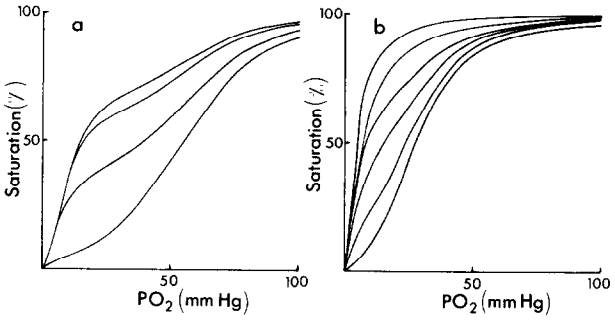

Fig.1. Effect of BW12C on the oxygen association curve for fresh whole chicken (a) and human (b) blood. Concentrations of BW12C (mM) from right to left: (a) 0, 1.5, 3, 4.5; (b) 0, 2, 4, 6, 8, 10.

different forms of hemoglobin present, i.e. left-shifted, BW12C-reacted hemoglobin and unreacted hemoglobin. In human blood, 10 mM BW12C is required to react fully with the hemoglobin representing a binding stoichiometry of ~4:1 and at this concentration the curve is no longer biphasic. In chicken blood the biphasic nature of the curves is even more pronounced due to the higher initial P_{50}. At the reduced hematocrit of the sample of chicken blood used (30%) a maximal effect of BW12C would be expected at ~7 mM but in fact 4.5 mM BW12C produces a maximal effect with 6 and 9 mM giving the same curve. Also, the curve is still biphasic and appears to correspond to ~70% of a left-shifted component and ~30% of a component which is not left-shifted.

These proportions show an obvious correspondence with the amounts of the major and minor hemoglobin components of chicken blood [7] and these were therefore isolated and stripped of phosphate for further study. The effects of BW12C on the oxygen dissociation curves of dilute solutions of chicken major and minor hemoglobins are shown in fig.2a and b, respectively. As expected from the sequence change Pro 77α→Ser, BW12C is less potent in its action on chicken major compared with human hemoglobin. 0.1 mM BW12C is sufficient to left-shift fully in human hemoglobin [2] whereas at this dose BW12C is only starting to produce effects in chicken major. Qualitative differences are also apparent, however, since at lower doses BW12C causes a right-shift at the top of the curve with chicken major, and a full

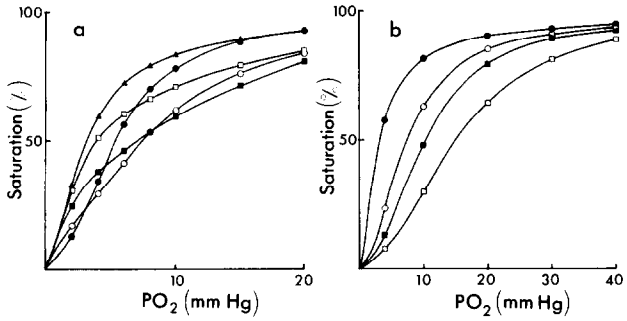

Fig.2. Effect of BW12C on the oxygen dissociation curve of dilute (23 μM) solutions of (a) chicken major hemoglobin and (b) chicken minor hemoglobin. Concentrations of BW12C (mM) – a: (●) 0, (○) 0.1, (■) 0.2, (□) 0.35, (▲) 1.0; b: (●) 0, (○) 0.1, (■) 0.2, (□) 0.5. T = 37°C, 100 mM Hepes buffer, pH 7.4, 35 mM KCl. The data were recorded as continuous curves. The symbols are added merely as an aid to identification.

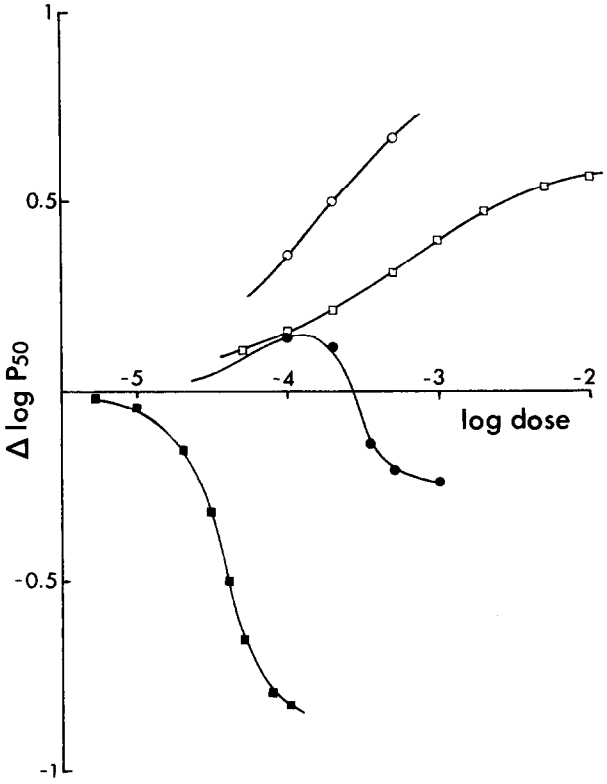

Fig.3. Plot of $\Delta\log P_{50}$ ($\log P_{50}$ treated minus $\log P_{50}$ control) against log dose for (●) BW12C with chicken major, (○) BW12C with chicken minor, (■) BW12C with human, (□) 2,3-diphosphoglycerate with human. Conditions as in fig.2.

left-shift is only produced for higher doses. The different potencies for the interaction of BW12C with human and chicken major are illustrated in fig.3 which plots the difference in log P_{50} between control and treated curves against log dose. In this representation BW12C appears about an order of magnitude less potent on chicken major compared with human, but the difference may be considerably larger since at the lower doses of BW12C used for human hemoglobin the free concentrations will be much lower than the total dose plotted in fig.3 [2].

The whole blood curves (fig.1a) suggested that BW12C might be without effect on chicken minor hemoglobin. In fact fig.2b shows that considerable right-shifting activity is observed. Fig.3 compares the right-shifting effect of BW12C in chicken minor with that of 2,3-diphosphoglycerate in human hemoglobin measured under the same conditions [11] and shows that the potencies are comparable. The fact that the top ends of the whole blood curves were not right-shifted may be explained if BW12C is binding to the β-terminal amino groups in the deoxy conformation, since the presence of the very tightly bound natural effector, inositol pentaphosphate, in chicken blood would preclude any additional shift by BW12C. In agreement with this the P_{50} for chicken minor hemoglobin in the presence of 0.1 mM inositol hexaphosphate was found to be the same (33 mmHg) in the absence or presence of 0.5 mM BW12C.

4. DISCUSSION

Although BW12C was designed to bind preferentially at the α-chain terminal amino region in the oxy conformation of human hemoglobin, binding to α- and β-chain terminal amino groups in the oxy and deoxy conformations via Schiff base formation has been observed [3]. In addition, binding to the ϵ-amino groups of the many lysine residues is likely, but extensive studies with pyridoxal derivatives have indicated a distinct preference for Schiff base formation at the amino termini [12–14] due presumably to the more favorable pK values for these groups compared with lysine residues [15]. Three distinct com-

ponents of the mechanism for the left-shifting effect of BW12C in whole blood may therefore be envisaged: (i) overall preferential binding at the α-chain terminal amino region in the oxy conformation; (ii) rupture of the anion-mediated α-chain amino termini salt bridges with the guanidinium groups of the carboxy terminal arginine residues of the opposite α-chains in the deoxy conformation [16] on Schiff base formation, leading to a direct increase in oxygen affinity of the deoxy conformation and reduction in cooperativity; (iii) direct competition for binding at the β-chain terminal region in the deoxy conformation with 2,3-diphosphoglycerate.

The present results provide supportive evidence for the postulated binding mode at the α-chain terminal amino region in the oxy conformation. Chicken hemoglobins, of course, contain many differences in sequence from human hemoglobin, but the replacement of Pro 77α by serine seems the likely cause for the loss of potency of BW12C in chicken major since there are no other significant changes in sequence from human hemoglobin in this region. The results indicate that the loss of this hydrophobic residue leads to a reduction in binding at the α-chain terminus in the oxy conformation with a consequently greater importance of other binding modes. In fact, the shapes of the curves may be explained if binding at the β-chains in the deoxy conformation predominates at low concentrations leading to the right-shift at the P_{50}, but at higher concentrations binding to the α-chains in the deoxy conformation becomes dominant resulting in a left-shift due to the rupture of salt bridges.

The right-shifted curves with chicken minor hemoglobin suggest that binding at the β-terminus in the deoxy conformation is now the dominant binding mode, and reaction at this site is supported by the competition for binding with inositol hexaphosphate. The sequences of the β-chains of both forms of chicken hemoglobin are apparently identical [17,18], and the difference between the effects of BW12C on the major and minor components must therefore depend on their different α-chain sequences. The most obvious difference in the α-chain terminal amino region is the replacement Val 1α→Met, and this would be expected to disfavor binding at this locus since model studies of the Schiff base formation of pyridoxal and amino acids [19] showed an approx. 2-fold increase in binding constants for the amino acids valine and isoleucine, which are branched on the β-carbon atom, compared with norvaline and leucine.

There is a possible additional effect, however. Ser 138α in human hemoglobin lies fairly close (O–N distance 6.7 Å [20,21]) to the terminal amino group of the opposite α-chain in the deoxy conformation. In chicken major this is alanine, which should not cause any marked change, but in chicken minor it is a glutamic acid. The carboxylate group of this glutamic acid residue would be ideally positioned to form a salt bridge with the terminal amino group itself, particularly at the rather low KCl concentration (35 mM) used in the present experiments which would not favor the anion-mediated salt bridge between the terminal amino and the carboxy terminal arginine observed at high ionic strength [16]. This salt bridge formation with Glu 138α would then tend to block the reaction of the α-chain terminal amino groups with BW12C leaving binding at the β-terminus as the only remaining binding mode. In fact, precisely the opposite effects, albeit less pronounced, were observed in the reaction of a series of right-shifting compounds designed to bind preferentially at the β-terminus [6]. Here, the blocked β-terminal amino groups in fetal minor (HbF_1), glucosylated hemoglobin (HbA_{IC}) and the mutant Hb Raleigh changed right-shifting activity into left-shifts for a dialdehyde compound binding largely by Schiff base formation.

ACKNOWLEDGEMENTS

We would like to thank Janet Ingram, Martin Jennings and Carl Ross for technical assistance and Joan Hambidge for typing the manuscript.

REFERENCES

[1] Kneen, G. and White, R.D. (1981) Br. J. Pharmacol. 74, 965P.
[2] Beddell, C.R., Goodford, P.J., Kneen, G., White, R.D., Wilkinson, S. and Wootton, R. (1984) Br. J. Pharmacol. 82, 397–407.
[3] Merrett, M., Stammers, D.K., White, R.D., Wootton, R. and Kneen, G. (1986) Biochem. J., in press.
[4] Wrightstone, R.N. (1982) Hemoglobin 6, 258–346.

[5] Dayhoff, M.O. (1972) Atlas of Protein Sequence and Structure, vol.5, Silver Spring: National Biomedical Research Foundation and suppl.2 (1976) and 3 (1978).
[6] Beddell, C.R., Goodford, P.J., Stammers, D.K. and Wootton, R. (1979) Br. J. Pharmacol. 65, 535–543.
[7] Vandecasserie, C., Schnek, A.G. and Leonis, J. (1971) Eur. J. Biochem. 24, 284–287.
[8] Matsuda, G., Takei, H., Wu, K.C. and Shiozawa, T. (1971) Int. J. Protein Res. 3, 173–174.
[9] Takei, H., Ota, Y., Wu, K.C., Kiyohara, T. and Matsuda, G. (1975) J. Biochem. 77, 1345–1347.
[10] Paterson, R.A., Eagles, P.A.M., Young, D.A.B. and Beddell, C.R. (1976) Int. J. Biochem. 7, 117–118.
[11] Wootton, R. (1984) FEBS Lett. 171, 187–191.
[12] Benesch, R.E., Yung, S., Suzuki, T., Bauer, C. and Benesch, R. (1973) Proc. Natl. Acad. Sci. USA 70, 2595–2599.
[13] Arnone, A., Benesch, R.E. and Benesch, R. (1977) J. Mol. Biol. 115, 627–642.
[14] Schnackerz, K.D., Benesch, R.E., Kwong, S., Benesch, R. and Helmreich, E.J.M. (1983) J. Biol. Chem. 258, 872–875.
[15] Kaplan, H., Hamel, P.A., Chan, A.M.-L. and Oda, G. (1982) Biochem. J. 203, 435–443.
[16] O'Donnell, S., Mandaro, R., Schuster, T.M. and Arnone, A. (1979) J. Biol. Chem. 254, 12204–12208.
[17] Paul, C., Vandecasserie, C., Schnek, A.G. and Leonis, J. (1974) Biochim. Biophys. Acta 371, 155–158.
[18] Vandecasserie, C., Paul, C., Schnek, A.G. and Leonis, J. (1975) Biochimie 57, 843–844.
[19] Metzler, D.E. (1957) J. Am. Chem. Soc. 79, 485–490.
[20] Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Edgar, F.M., Brice, M.D., Rogers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542.
[21] Fermi, G. (1975) J. Mol. Biol. 97, 237–256.