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Characterization of the binding of the anti-sickling compound, BW12C, to haemoglobin

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The anti-sickling agent BW12C [Beddell, Goodford, Kneen, White, Wilkinson & Wootton (1984) Br. J. Pharmacol. **82**, 397–407] was designed to left-shift the oxygen saturation curve of haemoglobin (HbA) by preferential binding to the oxy conformation at a single site between the terminal amino groups of the α-chains through Schiff's base formation, ionic and hydrophobic interactions. In the present work, Schiff's base linkages formed with [^{14}C]BW12C were reduced with $NaBH_4$ and the α- and β-globin chains separated. Under oxy conditions at a molar ratio of 2:1, the covalently bound BW12C is localized almost exclusively on a single α-chain; tryptic digestion confirms the terminal amino group (α^1-valine) as the reaction site, in accord with the design hypothesis. However, about half the labelled BW12C is released on tetramer disruption, suggesting the presence of additional non-covalent binding. Under deoxy conditions, α- and β-chains are labelled approximately equally, and at higher molar ratios additional binding in both oxy and deoxy conditions is seen. Isoelectric-focusing studies under oxy conditions show a complex pattern of modified bands for both HbA and HbA_{1c} (blocked β-terminal amino groups) but no modification for HbA carbamylated at both α- and β-terminal amino groups or at the α-chains only, again confirming the α-terminal amino region as the main interaction site. Equilibrium dialysis measurements under oxy conditions indicate two strong binding sites with a binding constant of less than 10^{-6} M and a number of weaker binding sites. The present data thus confirm that BW12C binds at the intended locus but reveal additional non-covalent binding at an undefined site, and weaker binding through Schiff's base formation with other amino groups.

INTRODUCTION

Sickle cell disease is an hereditary disorder of the blood caused by the homozygous expression of a mutant gene which codes for the amino acid sequence of the β-chains of haemoglobin. Thus, the β^6-glutamate of normal haemoglobin (HbA) is replaced by valine in sickle haemoglobin (HbS) (Ingram, 1956). Whereas oxygenated HbS has a similar solubility to both oxygenated and deoxygenated HbA, deoxygenated HbS has a much decreased solubility (Perutz & Mitchison, 1950). Consequently, in regions of low oxygen tension HbS aggregates within the erythrocyte into long polymers which distort the membrane and cause the characteristic deformed shape associated with the sickle cell syndrome (for a review see Dean & Schechter, 1978). Anti-sickling activity might be expected, therefore, for a compound which left-shifts the oxygen saturation curve, thereby maintaining a greater proportion of soluble oxyHbS at low oxygen tensions, and a potent left-shifting compound, 5-(2-formyl-3-hydroxyphenoxy)pentanoic acid (BW12C, Fig. 1), has been designed with this objective (Beddell *et al.*, 1984). The design, based on available crystal structure data (Perutz *et al.*, 1968; Bolton & Perutz, 1970) incorporates the following features (Fig. 1): the aromatic aldehyde of BW12C forms a Schiff's base adduct with one α-chain terminal amino group (α^1-valine) which is stablized by hydrogen bonding to the *o*-hydroxyl of BW12C; the side chain carboxyl of the compound forms an ionic link with the terminal amino group of the other α-chain and the complex is stabilized by hydrophobic bonding between the BW12C side chain methylene groups and the two α^{77}-prolines. The geometric rearrangement which occurs in haemoglobin on deoxygenation is thought to disfavour BW12C binding and so the overall effect of the compound is to increase the oxygen affinity of haemoglobin, manifest as a left-shift of the blood-oxygen saturation curve. The α-chain terminal region is the same in HbA and HbS and so the increase in oxygen affinity is observed in both types but, in addition, BW12C inhibits the reversible sickling of erythrocytes from patients suffering from sickle cell disease (Kneen & White, 1981; Beddell *et al.*, 1984).

The purpose of the present experiments was to examine the way in which BW12C interacts with HbA in order to test the validity of the design hypothesis. To this end, the binding of BW12C to HbA has been examined using several techniques, including covalent labelling of the binding site using [*formyl*-^{14}C]BW12C and reduction with $NaBH_4$, isoelectric focusing and equilibrium dialysis.

MATERIALS AND METHODS

Chemicals

BW12C [5-(2-formyl-3-hydroxyphenoxy)pentanoic acid] was prepared as described in U.K. Patent Specification no. 2053218, Kneen, G. (The Wellcome Foundation Ltd.). 5-(2-[^{14}C]Formyl-3-hydroxyphenoxy) pentanoic acid (4.4 mCi/mmol, 98.5–99.9% radiochemical purity) was prepared from 2,6-di-

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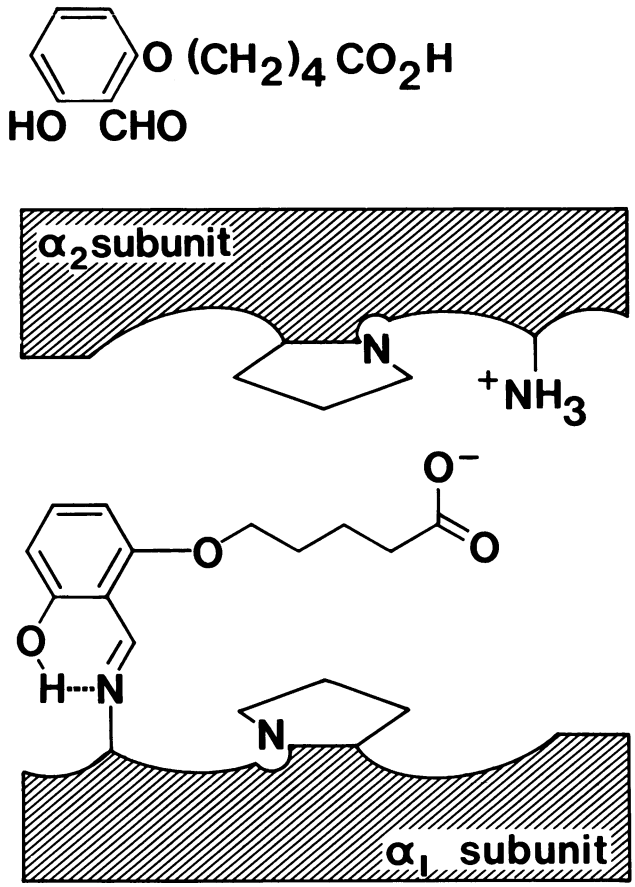

Fig. 1. Structure of BW12C and schematic representation of the postulated binding mode for BW12C at the α-chain terminal amino region in oxyHbA

methoxy[*carboxyl*-^{14}C]benzoic acid (ICI plc, Petrochemicals and Plastics Division, Billingham, Cleveland, U.K.) (25 mCi/mmol diluted to 5 mCi/mmol) via 2,6-dimethoxy[*carbonyl*-^{14}C]benzaldehyde in 12% overall yield. All other reagents were purchased from BDH (Poole, Dorset, U.K.) unless specified otherwise and were of Analar grade wherever possible.

Labelling of the covalent binding site

[*formyl*-^{14}C]BW12C was reacted with HbA (predominantly HbA_1, 0.54 mM in tetramer) isolated from human erythrocyte lysates (Paterson *et al.*, 1976) in 35 mM-potassium phosphate buffer (pH 7.3) at room temperature. After 20 min, $NaBH_4$ as a freshly prepared solution was added in a 100-fold molar excess over haemoglobin in order to reduce Schiff's base adducts. Reduction was allowed to proceed for 1 h whereupon the mixture was desalted by gel filtration on Sephadex G-25 equilibrated with 5 mM-sodium phosphate buffer (pH 6.7) containing 50 mM-2-mercaptoethanol. Experiments with deoxyHbA were carried out in a similar way but in a glove-box gassed with N_2 (British Oxygen Company, 'white spot').

Globin chains were separated in 8 M-urea by ion-exchange chromatography on carboxymethyl cellulose (Whatman CM-52) using a method based on that of Clegg *et al.* (1968) and the distribution of BW12C and globin was determined by measurement of radioactivity and absorbance at 280 nm. Labelled α-globin chains were desalted on Sephadex G-25 in 5% (v/v) formic acid containing 1 mM-dithiothreitol. The protein was then freeze-dried and aminoethylated with *N*-(iodoethyl)-trifluoroacetamide (Pierce AE-8) as described in Protein Digest (1979). Following dialysis against water for 24 h and further freeze-drying, the aminoethylated globin was digested for 16 h at 37 °C with 1% (w/w) porcine trypsin (Sigma) that had been treated previously with L-(tosylamido-2-phenyl)ethylchloromethyl ketone (Sigma, Poole, Dorset, U.K.) according to the procedure of Carpenter (1967) to remove chymotryptic activity. The freeze-dried digest was dissolved in 10% (v/v) formic acid and chromatographed on a column of Sephadex G-50 superfine, equilibrated with the same solvent. Peptides were located in the eluate by reaction with *o*-phthaldialdehyde (Koch-Light, Haverhill, Suffolk, U.K.) in the presence of 2-mercaptoethanol (Lai, 1977).

Peptide separation by h.p.l.c. was achieved with an Altex Ultrasphere ODS C-18 reversed-phase column as described by Fullmer & Wasserman (1979). Peptides were hydrolysed at 110 °C for 20 h in 6 M-HCl containing 0.1% (w/v) phenol and amino acid analysis was carried out with a Rank–Hilger Chromaspek amino acid analyser equipped with a fluorometric detection system using *o*-phthaldialdehyde.

Isoelectric focusing

Mixtures of haemoglobins (i.e. unreacted or reacted with BW12C) were separated by isoelectric focusing on narrow-range polyacrylamide gels containing ampholytes for the pH range 5.5–8.5. The preformed PAG-Ampholine system (LKB, Biochrom) was used, together with an LKB Multiphor with LKB 2103 power supply. Solutions of the haemoglobin (5%, w/v) were reacted with a stock solution of 40 mM-BW12C in 0.1 M-NaCl/1 mM-Hepes buffer, pH 7.2, to give molar ratios of BW12C to haemoglobin of 0.5:1 up to 4:1. Samples were diluted before application to the gels which, after focusing, were fixed and stained with Coomassie Blue. Isoelectric points were measured using HbA_1 and partially oxidized haemoglobins (IB_I and IB_{II}) as reference points (Bunn & Drysdale, 1971); a correction for non-linearity was applied. Equivalent experiments were carried out using [*formyl*-^{14}C]BW12C to assess the stoicheiometry of BW12C binding to haemoglobin in the different bands. Undiluted haemoglobin samples were used on the gels and the different bands were cut out and eluted with 0.5 ml of Drabkin's reagent to determine the total haemoglobin concentration as described by Evelyn & Malloy (1938). The samples were then decolourized with H_2O_2 and counted in a liquid-scintillation counter.

HbA_1 and HbA_{1c} were prepared from erythrocyte lysates by chromatography on columns of carboxymethyl-cellulose (Whatman CM-52) equilibrated with 10 mM-sodium phosphate, pH 6.8 and eluted with a pH gradient of 6.8–7.5. Carbamylated HbA_1 was prepared by the method of Nigen *et al.* (1974) and the extent of carbamylation was checked by isoelectric focusing as described earlier. HbA_1, selectively carbamylated at the α-chain terminal amino groups, was prepared as above except for the addition of 2,3-bisphosphoglycerate (10 mM) and a reaction time of 30 min.

Equilibrium dialysis

The binding of BW12C to haemoglobin was measured using a Dianorm (MSE Scientific Instruments, Crawley,

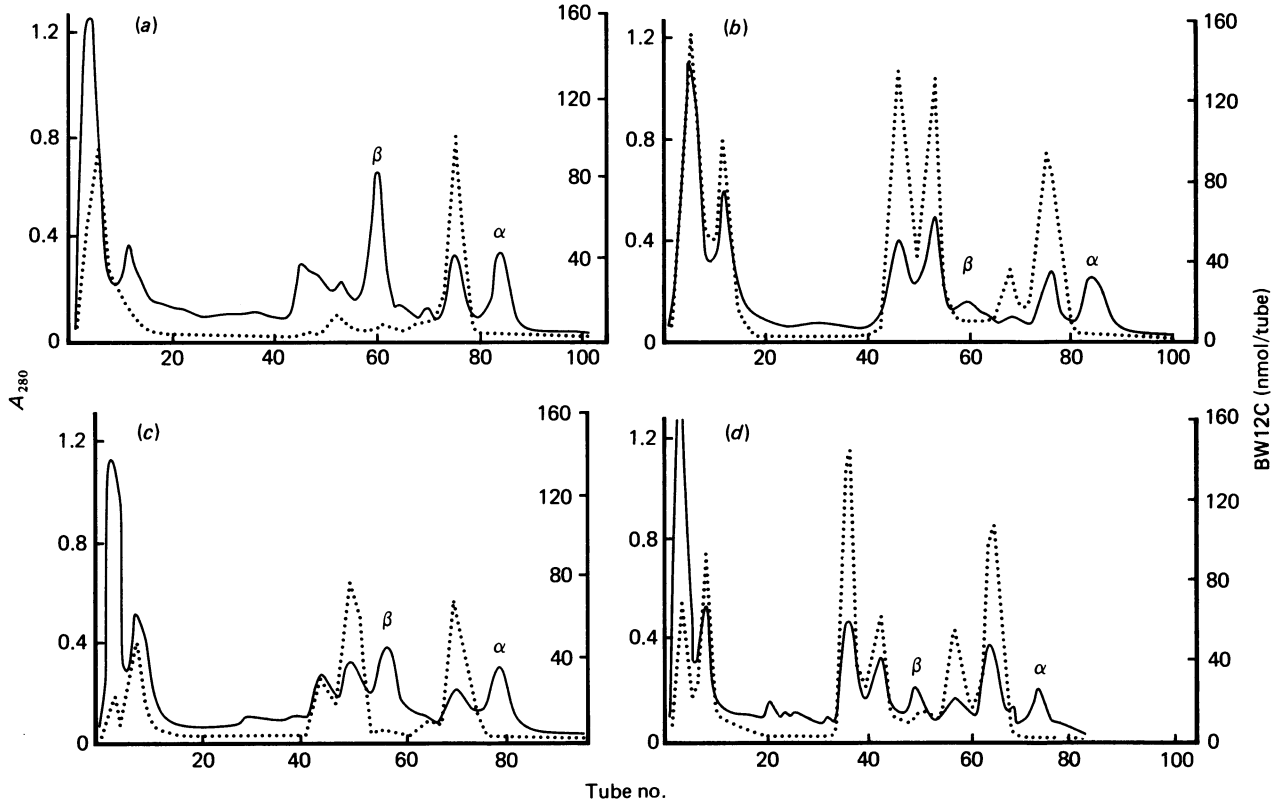

Fig. 2. Fractionation of the α- and β-globin chains after reaction with [^{14}C]BW12C followed by $NaBH_4$ reduction

The columns was equilibrated in 5 mM-sodium phosphate/50 mM-2-mercaptoethanol/8 M-urea (pH 6.7) and eluted with 250 ml of starting buffer followed by a linear gradient (total 400 ml) of buffer containing 5–40 mM-sodium phosphate (pH 6.7). (*a*) and (*b*), oxyHbA, BW12C/HbA = 2:1 and 10:1 respectively; (*c*) and (*d*), deoxyHbA, BW12C/HbA = 2:1 and 10:1 respectively. The bands are eluted in the order haem, β-chains and α-chains with BW12C-modified chains eluted earlier. —, A_{280}; ·····, BW12C (nmol/tube) (from radioactivity).

Sussex, U.K.) with Teflon cells (1 ml half cell capacity: 'micro') HbA solutions (0.04–4%, w/v) containing BW12C were placed on one side of the chamber and dialysed against buffer (100 mM-Hepes/33 mM-KCl, pH 7.4) for 16 h at 4 °C. Control experiments confirmed full equilibration after this time and quantitative recovery of BW12C from the system with no detectable binding to equipment components. In some experiments, BW12C and HbA were mixed under anaerobic conditions and dialysis was conducted under an atmosphere of N_2.

Oxygen dissociation curve measurements

Oxygen saturation curves of dilute haemoglobin solutions (23 μM in tetramer) were carried out at 37 °C in the dissociation mode with a HEMOX-ANALYZER (T.C.S. Medical Products Division, Southampton, PA, U.S.A.) as described previously (Beddell *et al.*, 1984).

RESULTS

Identification of the covalent binding sites

Reaction of labelled BW12C with oxyHbA followed by $NaBH_4$ reduction resulted in incorporation of radioactivity which was not removed by gel filtration. However, disruption of the tetramer by treatment with 8 M-urea resulted in extensive loss of label, manifest as a leading edge during the chromatographic separation of the globin chains (Fig. 2*a*). This indicates either that the borohydride reduction of any Schiff's base adducts was not quantitative or, as is most likely, some of the binding of BW12C to haemoglobin does not involve Schiff's base formation. Addition of labelled BW12C to HbA in molar ratios of up to 2:1 resulted in almost exclusive binding of BW12C to the α-globin chains. This was manifest as a new protein peak, eluting before the native α-globin chain and containing the radiolabel. Addition of the compound in molar ratios of 10:1 (and 5:1) resulted in the formation of significant proportions of labelled β- as well as α-chains (Fig. 2*b*). Under these conditions other, more negatively charged species (eluting earlier) corresponding to globin chains carrying more than one BW12C molecule were observed in increasing proportions. However, even at these high levels approximately half of the α-chains remained unlabelled.

When BW12C was reacted with deoxyHbA only a small amount of the label was lost upon disruption of the tetramer (Figs. 2*c* and 2*d*). Separation of chains indicated approximately equal labelling of α- and β-chains under deoxy conditions, even at low molar ratios of added BW12C to HbA. At higher ratios, considerably more than 50% of the total α-chain is modified, indicating that BW12C does not bind in the

same way as in oxyHbA. Thus, the oxygenated conformation is necessary for preferential binding to the α-chain. The binding of BW12C to the β-chains of deoxyHbA was considerably decreased in the presence of 2,3-bisphosphoglycerate and practically abolished in the presence of inositol hexakisphosphate, suggesting binding of BW12C to the β-amino termini and/or β^{82}-lysine residues.

A major ^{14}C-labelled peptide was isolated from a tryptic digest of α-globin chains which had been reacted with a 2:1 BW12C/HbA molar ratio under oxy conditions and unequivocally identified as originating from the *N*-terminal region. The composition (normalized to alanine) was valine (0.2), leucine (0.5), serine (1.0), proline (0.9), alanine (1.0), aspartate (0.8), lysine (1.0). Since isolation of this peptide by tryptic digest would have failed had BW12C been attached to the ε-amino group of lysine, then the only other likely point of attachment of the radiolabel is at the *N*-terminal group of valine and would account for the depletion. The shortfall in leucine is most likely due to the relative resistance of Val–Leu to hydrolysis under the conditions employed.

Isoelectric focusing studies

The results of the variation of BW12C concentration on the oberved isoelectric focusing patterns of oxyHbA$_1$ are shown in Fig. 3. Over the range of concentrations used, i.e. molar ratios of BW12C to HbA of 0.5, 1.0, 2.0 and 4.0, up to four new species are observed, all with more acidic isoelectric points than the predominant native HbA$_1$ (pI 6.96). At the lowest concentrations two bands in addition to unmodified haemoglobin are observed with pI values of 6.91 and 6.82. At intermediate concentrations a third band is present at pI 6.88, whilst a fourth band with a pI of 6.78 is visible at the highest concentrations used. Similar results were obtained with carbonmonoxyhaemoglobin. The BW12C:haemoglobin ratios for these bands in decreasing order of pI were determined as 1, 2, 2 and 3 by the use of [*formyl*-^{14}C]BW12C, thus representing a complex binding pattern.

Carbamylated HbA$_1$ ($\alpha_2^c\beta_2^c$) has a more acidic isoelectric point than HbA$_1$, consistent with the modification of all four ionizable terminal amino groups. Half-carbamylated HbA$_1$ ($\alpha_2^c\beta_2$) has the same isoelectric point as HbA$_{1c}$, both having lost two ionizable terminal amino groups, the former on the α-chains and the latter on the β-chains. With both $\alpha_2^c\beta_2^c$ and $\alpha_2^c\beta_2$ no changes in isoelectric point were observed on addition of BW12C (Fig. 3). Addition of BW12C to solutions of HbA$_{1c}$ induced changes in the isoelectric point with a pattern of modification very similar to that for HbA$_1$, albeit shifted to lower pH because of the more acidic pI of HbA$_{1c}$ compared with HbA$_1$ (Fig. 3).

BW12C was also found to have no effect on the oxygen saturation curve of $\alpha_2^c\beta_2^c$ at a concentration (100 μM) giving a fully left-shifted curve with HbA (Beddell *et al.*, 1984). A small left-shift, particularly at low saturation values, was observed for $\alpha_2^c\beta_2$ at a low BW12C concentration (10 μM) but increasing the concentration had no further left-shifting effect; this result is explicable in terms of a small amount of unreacted HbA$_1$ in the samples of $\alpha_2^c\beta_2$.

The results are thus consistent with the reaction of BW12C at the α-chain terminal amino groups of oxygenated HbA$_1$; binding of the compound is only seen when the α-chain terminal amino groups are unmodified. No detectable binding to the β-chain *N*-termini occurs under the conditions used, since blocking these groups whilst leaving the α-chain *N*-termini unblocked does not alter the overall binding pattern. However, since BW12C is charged, only strong binding would be detectable by this method due to the migration of any free BW12C to the cathode (Zaugg *et al.*, 1977).

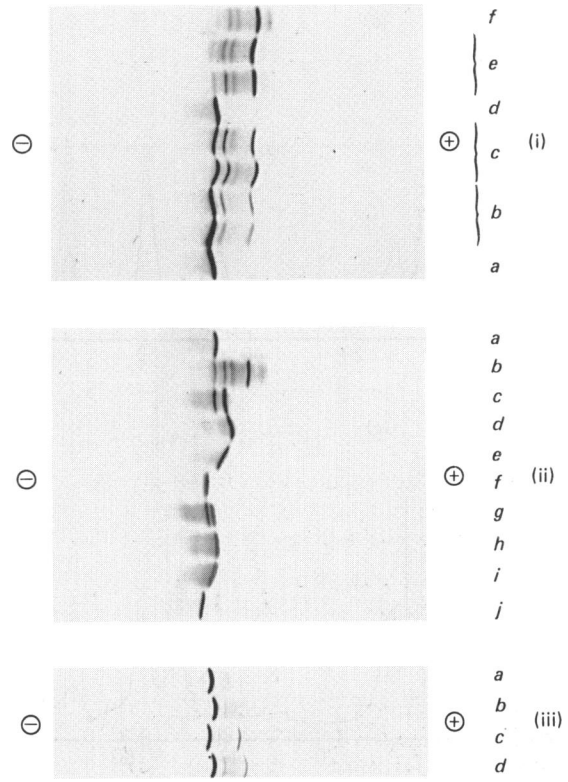

Fig. 3. Isoelectric focusing of BW12C-modified haemoglobins

The loadings were 0.2% (w/v) haemoglobin solutions with a molar ratio of BW12C to haemoglobin of 4:1 unless otherwise indicated. NaCN (10 mM) was added to the samples before application to the gel. (i) HbA plus BW12C in molar ratios: *a*, 0; *b*, 0.5; *c*, 1; *d*, 0; *e*, 2; *f*, 4. (ii) *a*, HbA$_1$; *b*, HbA$_1$+BW12C;' *c*, HbA$_1$+$\alpha_2^c\beta_2^c$; *d*, $\alpha_2^c\beta_2^c$; *e*, $\alpha_2^c\beta_2^c$+BW12C; *f*, HbA$_1$; *g*, HbA$_1$+$\alpha_2^c\beta_2$; *h*, $\alpha_2^c\beta_2$; *i*, $\alpha_2^c\beta_2$+BW12C; *j*, HbA$_1$. (iii) *a*, HbA$_1$; *b*, HbA$_{1c}$; *c*, HbA$_1$+BW12C (1:2); *d*, HbA$_{1c}$+BW12C (1:2).

Equilibrium dialysis

The relationship between the molar ratio of bound BW12C to HbA concentration (HbA = 6.3 μM) and the free BW12C concentration, measured using equilibrium dialysis under oxygenated conditions, is plotted in Fig. 4. The data clearly reveal more than one binding site and indicate two strong binding sites plus weaker binding. Additional data for higher HbA concentrations (HbA = 0.63 mM) show a continual increase in the amount of BW12C bound as the ratio of BW12C to HbA is increased up to 10 mol of BW12C/mol of Hb at 16 mM-BW12C.

The binding curve at the lower HbA concentrations is poorly defined since it lies at the limit of the

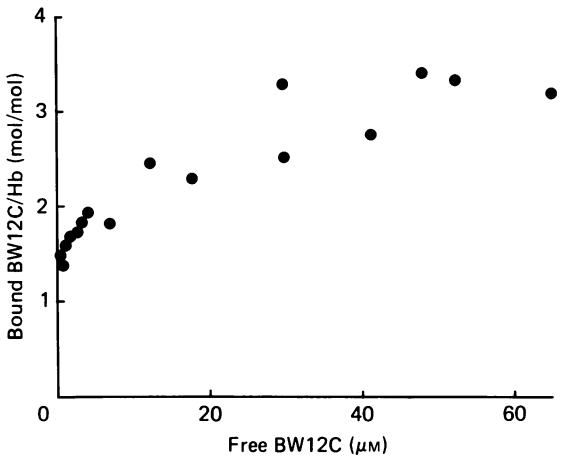

Fig. 4. Plot of the molar ratio of bound BW12C to haemoglobin concentration against free BW12C concentration measured by using equilibrium dialysis under oxy conditions

Conditions: 6.3 μM (0.04% w/v) haemoglobin, 100 mM-Hepes buffer, pH 7.4, 33 mM-KCl, BW12C 10–150 μM.

spectrophotometric detection of BW12C, and it is not possible to resolve individual binding constants for the two sites. However, these are clearly less than micromolar which represents tight binding as indicated previously (Beddell *et al.* 1984). Data obtained under deoxygenated conditions at high HbA concentrations (HbA = 0.63 mM) again show multiple binding of BW12C but do not permit the assignment of any detailed binding pattern.

DISCUSSION

Many aromatic aldehydes have now been shown to interact via Schiff's base formation with haemoglobin and left- or right-shift the oxygen saturation curve (Benesch *et al.*, 1969; Beddell *et al.*, 1976; Zaugg *et al.*, 1977). In particular, pyridoxal derivatives show a pronounced preference for Schiff's base formation at α- and β-chain terminal amino groups rather than the ε-amino groups of the many surface lysine residues (Benesch *et al.*, 1972, 1973; Arnone *et al.*, 1977) although a more recent paper (Benesch *et al.*, 1982) revealed some binding of pyridoxal phosphate to β^{82}-lysine residues at the 2,3-bisphosphoglycerate binding site (Arnone, 1972). The specificity for reaction at the terminal amino groups arises in part from the lower p*K* values for these groups compared with lysine amino groups (Kaplan *et al.*, 1982) but the detailed structure of the α- and β-terminal amino regions also influence binding. The highly charged binding site for 2,3-bisphosphoglycerate favours binding of dianions such as pyridoxal phosphate in deoxy conditions but monoanions, such as pyridoxal sulphate and pyridoxal-deoxymethylene phosphonate, favour the less charged α-terminal amino region, particularly under oxy conditions (Arnone *et al.*, 1977; Schnackerz *et al.*, 1983).

The present data for the binding of BW12C, which exists largely in the monoanionic form at neutral pH, reveal a pronounced preference for binding at the α-terminal amino region under oxy conditions but this specificity is lost under deoxy conditions with approximately equivalent binding to α- and β-chains. At high BW12C to haemoglobin molar ratios additional binding to the β chains occurs under oxy conditions and also binding at lysine residues in either oxy or deoxy. The overall Schiff's base binding pattern is thus complex and the mechanism of the left-shifting effect is correspondingly complex with three different effects contributing to whole-blood activity. These are (i) overall preferential binding to the oxy conformation, (ii) binding at the α-chain terminal amino groups in the deoxy conformation with a consequent rupture of salt bridges (O'Donnell *et al.*, 1979) leading to a direct increase in oxygen affinity of the deoxy conformation, and (iii) direct competition for binding with 2,3-bisphosphoglycerate at the β-terminal amino region in the deoxy conformation.

The design of BW12C was based on the assumption of a single strong binding site in oxyHbA. The observation that in oxyHbA only one α-chain reacts even at high BW12C to haemoglobin ratios is consistent with this, and the potency of the compound (Beddell *et al.*, 1984) and tight-binding observed from dialysis measurements suggests a substantial contribution to the binding from hydrophobic interactions; BW12C binds considerably more tightly than pyridoxal derivatives which also bind through a similar Schiff's base formation and ionic interactions (Benesch *et al.*, 1973; Arnone *et al.*, 1977). In contrast, the equilibrium dialysis measurements clearly indicate two strong binding sites in oxyHbA, and the complexity of the band pattern from isoelectric focusing for BW12C-treated HbA_1 ad HbA_{1c}, together with the considerable loss of BW12C on tetramer disruption after borohydride reduction, are also compatible with additional strong non-Schiff's base binding in oxyHbA. The location of this additional binding remains unknown and attempts to observe the binding crystallographically were without success. No crystallization occurred for carbonmonoxy-HbA solutions in the presence of BW12C under the usual conditions (Perutz, 1968) and preformed crystals of carbonmonoxy-HbA disintegrated when soaked in solutions containing BW12C.

Clinical studies with BW12C are now progressing and have shown that considerable concentrations of BW12C are tolerated with no detectable effects except on the oxygen saturation curve of blood samples determined *ex vivo* (Fitzharris *et al.*, 1985; Keidan *et al.*, 1986). A dose of 20 $mg \cdot kg^{-1}$ gave a maximum erythrocyte concentration of approx. 2 mM and an oxygen saturation curve corresponding to the presence of 16% of BW12C-modified HbA. Plasma levels were 15-fold lower indicating that, although BW12C binds to a number of sites on haemoglobin and will thus bind to other proteins, its functionally important binding is sufficiently tight to lead to pronounced specificity for haemoglobin *in vivo*.

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REFERENCES

Arnone, A. (1972) Nature (London) **237**, 146–149
Arnone, A., Benesch, R. E. & Benesch, R. (1977) J. Mol. Biol. **115**, 627–642
Beddell, C. R., Goodford, P. J., Norrington, F. E., Wilkinson, S. & Wootton, R. (1976) Br. J. Pharmacol. **57**, 201–209

Beddell, C. R., Goodford, P. J., Kneen, G., White, R. D., Wilkinson, S. & Wootton, R. (1984) Br. J. Pharmacol. **82**, 397–407
Benesch, R. E., Benesch, R. & Yu, C. I. (1969) Fed. Proc. Fed. Am. Soc. Exp. Biol. **28**, 604
Benesch, R. E., Benesch, R., Renthal, R. D. & Maeda, N. (1972) Biochemistry **11**, 3576–3582
Benesch, R. E., Yung, S., Suzuki, T., Bauer, C. & Benesch, R. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 2595–2599
Benesch, R., Benesch, R. E., Kwong, S., Acharya, A. S. & Manning, J. M. (1982) J. Biol. Chem. **257**, 1320–1324
Bolton, W. & Perutz, M. F. (1970) Nature (London) **228**, 551–552
Bunn, H. F. & Drysdale, J. W. (1971) Biochim. Biophys. Acta **229**, 51–57
Carpenter, F. H. (1967) Methods Enzymol. **11**, 237
Clegg, J. B., Naughton, M. A. & Weatherall, D. J. (1968) Nature (London) **219**, 69–70
Dean, J. & Schechter, A. N. (1978) N. Engl. J. Med. **299**, 752–763, 804–811 and 863–870
Evelyn, K. A. & Malloy, H. T. (1938) J. Biol. Chem. **126**, 655–662
Fitzharris, P., McLean, A. E. M., Sparks, R. G., Weatherley, B. C., White, R. D. & Wootton, R. (1985) Br. J. Clin. Pharmacol. **19**, 471–481
Fullmer, C. S. & Wasserman, R. H. (1979) J. Biol. Chem. **254**, 7208–7212
Ingram, V. M. (1956) Nature (London) **178**, 792–794
Kaplan, H., Hamel, P. A., Chan, A. M.-L. & Oda, G. (1982) Biochem. J. **203**, 435–443
Keidan, A. J., Franklin, I. M., White, R. D., Joy, M., Huehns, E. R. & Stuart, J. (1986) Lancet **i**, 831–834
Kneen, G. & White, R. D. (1981) Br. J. Pharmacol. **74**, 965P
Lai, C. Y. (1977) Methods Enzymol. **47**, 236–243
Nigen, A. M., Njikam, N., Lee, C. K. & Manning, J. M. (1974) J. Biol. Chem. **249**, 6611–6616
O'Donnell, S., Mandaro, R., Schuster, T. M. & Arnone, A. (1979) J. Biol. Chem. **254**, 12204–12208
Paterson, R. A., Eagles, P. A. M., Young, D. A. B. & Beddell, C. R. (1976) Int. J. Biochem. **7**, 117–118
Perutz, M. F. (1968) J. Cryst. Growth **2**, 54–56
Perutz, M. F. & Mitchison, J. M. (1950) Nature (London) **166**, 677–679
Perutz, M. F., Muirhead, H., Cox, J. M. & Goaman, L. C. G. (1968) Nature (London) **219**, 131–139
Protein Digest (1979), August issue, Pierce, Rockford, IL
Schnackerz, K. D., Benesch, R. E., Kwong, S., Benesch, R. & Helmreich, E. J. M. (1983) J. Biol. Chem. **258**, 872–875
Zaugg, R. H., Walder, J. A. & Klotz, I. M. (1977) J. Biol. Chem. **252**, 8542–8548

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