Photoaffinity labelling of cyanomethaemoglobin with derivatives of tryptophan and 5-bromotryptophan

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Tryptophan and 5-bromotryptophan (5-BrTrp) are relatively potent inhibitors of sickle-haemoglobin polymerization. The binding sites of these compounds to normal and sickle haemoglobin (HBA and HBS) have been suggested, but not firmly established, through the use of spin-labelled derivatives and/or computer modeling. In the present study we approached the problem by utilizing the technique of photoaffinity labelling. The cyanomet forms of HBA and HBS were subjected to photoaffinity labelling with *N*^α-(4-azidotetrafluorobenzoyl)tryptophan and *N*^α-(1-ethyl-2-diazomalonyl)-5-bromotryptophan respectively. Both

irradiated samples of HBA and HBS were denatured, digested with trypsin, and then separated by reversed-phase HPLC. A labelled tryptic peptide was isolated from the photolabelling of HBS with *N*^α-(1-ethyl-2-diazomalonyl)-5-bromotryptophan. The peptide was identified to be Val¹(α)-Lys⁷(α), with the label attached to Val¹(α), by virtue of amino acid analysis and sequencing, in conjunction with fast-atom-bombardment MS. The binding mode of *N*^α-(1-ethyl-2-diazomalonyl)-5-bromotryptophan is proposed and its relevance to the potency of the 5-BrTrp-based anti-sickling agents is discussed.

INTRODUCTION

Sickle-cell anaemia is a genetic disease that is caused by a haemoglobin mutation [Glu⁶(β) → Val]. The mutant haemoglobin (HBS) molecules polymerize when concentrated or under 'deoxy' conditions, which distorts the erythrocyte membrane and causes the characteristic sickle shape, leading to the symptoms of the disease. Chemical compounds that inhibit this process are potential candidates for use as therapeutic agents. Among many compounds studied that delay the onset of polymer formation through non-covalent interactions [1–9], tryptophan and 5-bromotryptophan (5-BrTrp), as well as the corresponding dipeptides, have been found to be among the more potent polymerization inhibitors, and the potency of the dipeptide of 5-BrTrp is on the threshold of being clinically useful [5,9]. Although the binding sites were determined crystallographically for one of the dipeptides, namely succinyl-Trp-Trp (STT) [10], the binding sites of the building blocks of these dipeptides, namely tryptophan and 5-BrTrp, have only been suggested, but not fully established, by the use of spin-labelled derivatives and computer modelling [11,12] (C. J. Yuan, L. Kar, P. Z. deCroos, B. L. Currie and M. E. Johnson, unpublished results). Alternative approaches would either confirm the existing proposed sites or identify new ones, which should be of great value to the further development of novel anti-sickling agents. The photoaffinity-labelling technique may provide such an alternative vehicle, since the technique has proved quite useful in understanding the interactions between biological ligands and receptors [13,14]. In the present paper, we describe the photoaffinity labelling of both normal haemoglobin (HBA) and HBS by using two photochemically active derivatives of tryptophan and 5-BrTrp respectively. We found that the cyanomet forms of both HBA and HBS [HiCN(A) and HiCN(S)] were reasonably stable under UV irradiation in comparison with other forms. We report here the results of the labelling experiments, subsequent peptide mapping by reversed-phase HPLC

and identification of a labelled peptide isolated from the solution of HiCN(S) irradiated in the presence of *N*^α-(1-ethyl-2-diazomalonyl)-5-bromotryptophan (compound 7, Scheme 2 below), using conventional protein-chemistry techniques in conjunction with fast-atom-bombardment MS (FABMS).

EXPERIMENTAL

Materials and methods

Trypsin [tosylphenylalanylchloromethane (TPCK)-treated, type VIII] was purchased from Sigma. Methyl perfluorobenzoate, trichloromethyl carbonate (triphosgene) and 2-ethoxy-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) were obtained from Aldrich. All other chemicals were of the highest purity available. Buffer solutions were prepared with distilled deionized water, and the pH values of the solutions were measured with a Corning digital pH-meter. Melting points were measured on a Thomas-Hoover Unimelt apparatus without correction.

¹H-, ¹³C-, and ¹⁹F-NMR spectra were acquired on a Varian Associates XL-300 NMR spectrometer. Tetramethylsilane was used as an internal reference for ¹H- and ¹³C-NMR experiments in organic solvents, whereas trifluoroacetic acid (TFA; in [²H]-chloroform) was used as an external reference for ¹⁹F-NMR experiments. IR spectra were recorded on a Perkin-Elmer Fourier-transform IR spectrometer. An IBM model 9420 UV-visible spectrophotometer was used for recording UV-visible spectra. HPLC analyses were performed on a Waters Associates HPLC system equipped with a Waters 484 UV-visible absorbance detector; some re-purification runs were carried out with a Rainin HPLC system equipped with a UV-DII dual-wavelength detector. Mass spectra were acquired on a Finnigan MAT 90 double-focusing mass spectrometer. For FABMS experiments, xenon was used, under an acceleration voltage of 8 kV, to generate molecular ions, and glycerol was employed as the

Abbreviations used: HBS, sickle-cell haemoglobin; HBA, human normal adult haemoglobin; HiCN(A), cyanomet form of HBA; HiCN(S), cyanomet form of HBS; HBA·CO, carbomonoxyhaemoglobin; HBA·O₂, oxyhaemoglobin; 5-BrTrp, 5-bromotryptophan; FABMS, fast-atom-bombardment MS; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodi-imide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride; STT, succinyl-Trp-Trp; BZF, bezafibrate; THF, tetrahydrofuran; HR-, high resolution; α-T₁, Val¹(α)-Lys⁷(α) (first α-chain tryptic peptide).

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matrix. Amino acid analysis was performed using pre-column derivatization with phenyl isothiocyanate, and peptide samples were hydrolysed with 6 M HCl for 1 h at 150 °C. Sequencing was conducted on an Applied Biosystems 477A protein sequencer.

All photoaffinity-labelling experiments and photochemical reactions were carried out in a microscale immersion-well photochemical reactor (Ace Glass Inc., Vineland, NJ, U.S.A.).

Evaluation of anti-gelling activities of the synthesized photo-labels [compound **4** (Scheme 1 below) and compound **7** (Scheme 2 below)] was conducted by using a modification [9] of the method of Mazhani et al. [15].

Chemical synthesis

4-Azidotetrafluorobenzoyl chloride (compound **1**, Scheme 1 below)

4-Azidotetrafluorobenzoic acid (compound **2**, Scheme 1 below) [16] (0.80 g, 3.4 mmol) was dissolved in 24 ml of thionyl chloride and the solution was heated at 58 °C for 22 h. The reaction solution was evaporated by aspirator to remove unchanged thionyl chloride to yield a yellowish oil (0.78 g, 90 %), which was used in the next step without further purification: IR [16]: 2125, 1764 cm⁻¹.

N^α-(4-Azidotetrafluorobenzoyl)tryptophan methyl ester (compound **3**, Scheme 1 below)

Method 1. The chloride (compound **1**) (600 mg, 2.37 mmol) was added dropwise to a mixture of DL-tryptophan methyl ester hydrochloride (603 mg, 2.37 mmol) and triethylamine (330 µl, 2.37 mmol) in 13 ml of dichloromethane. The second equivalent of triethylamine (330 µl, 2.37 mmol) was added to the reaction mixture after it was stirred at 25 °C for 19 h, and the stirring was continued for another 6 h. The reaction mixture was then mixed with 40 ml of dichloromethane and washed with 3 % HCl (three times) and saturated KHCO₃ (three times) respectively. The solution was dried (over anhydrous Na₂SO₄) and evaporated to give 900 mg of crude product (87 %), which was recrystallized from chloroform [m.p. 122–124 °C (decomp.)]: ¹H-NMR ([²H]₆]chloroform) δ 8.29 (s, 1 H), 7.50 (d, 1 H, *J* 7.8 Hz), 7.28 (d, 1 H, *J* 7.8 Hz), 7.15 (t, 1 H, *J* 7.5 Hz), 7.07 (t, 1 H, *J* 7.4 Hz), 6.97 (d, 1 H, *J* 2.3 Hz), 6.64 (d, 1 H, *J* 7.4 Hz), 5.08 (m, 1 H), 3.72 (s, 3 H), 3.43 (m, 2 H); ¹H-NMR ([²H]₆]dimethyl sulphoxide) δ 10.92 (s, 1 H), 9.43 (d, 1 H, *J* 7.6 Hz), 7.51 (d, 1 H, *J* 7.6 Hz), 7.34 (d, 1 H, *J* 7.6 Hz), 7.16 (s, 1 H), 7.08 (t, 1 H, *J* 7.6 Hz), 6.99 (t, 1 H, *J* 7.6 Hz), 4.70 (m, 1 H), 3.65 (s, 3 H), 3.20 (m, 2 H); ¹³C-NMR ([²H]₆]dimethyl sulphoxide) δ 171.45, 157.19, 144.6 (m), 141.4 (m), 138.3 (m), 136.09, 126.93, 123.76, 121.01, 118.44, 117.86, 111.43, 108.93, 53.71, 52.10, 26.79; ¹⁹F-NMR ([²H]₆]dimethyl sulphoxide) δ -61.69 (m, 2 F), -70.98 (m, 2 F); IR (KBr) 3349, 2128, 1669 and 1483 cm⁻¹; UV (ethanol): λ_{max} 258 (4.40). Analysis: Found: C, 47.00; H, 2.59; N, 14.09. Calc. for C₁₉H₁₃F₄N₅O₃·0.5 CHCl₃: C, 47.31; H, 2.75; N, 14.15.

Method 2. Triethylamine (119 µl, 0.85 mmol) and EEDQ (230 mg, 0.935 mmol) were added, respectively, to a mixture of the acid (compound **2**) (200 mg, 0.85 mmol) and L-tryptophan methyl ester hydrochloride (217 mg, 0.85 mmol) in 7 ml of dichloromethane. After stirring under nitrogen at 25 °C for 1 h, the reaction mixture was mixed with another 30 ml of dichloromethane and washed with water (once), 3 % HCl (three times) and saturated NaHCO₃ (three times). The aqueous phases were extracted with dichloromethane, and all the organic phases pooled. The combined dichloromethane solution was dried (Na₂SO₄) and evaporated *in vacuo* to give 350 mg of crude product (95 %), which was recrystallized in benzene to yield white needles, m.p. ~ 125 °C, crystals darkened; 134–135 °C,

decomp. The NMR and IR spectra were the same as those of the DL-racemate obtained with Method 1.

L-*N*^α-(4-Azidotetrafluorobenzoyl)tryptophan (compound **4**, Scheme 1 below)

The methyl ester, L-compound **3** (56 mg, 0.13 mmol) was dissolved in 4 ml of methanol, followed by the addition of 2 M NaOH (2 ml). The resulting solution was stirred at 25 °C for 20 min. The solution was then cooled in an ice/water bath and acidified with 2 M HCl to pH < 1; an extensive loose white precipitate was produced. After mixing with 6 ml of water, the suspension was extracted with dichloromethane (twice). The aqueous phase was mixed with an equivalent amount of saturated NaCl solution and extracted with dichloromethane. The organic phases were combined, dried (over anhydrous Na₂SO₄), and evaporated to dryness; the residue was crystallized in chloroform/benzene (10:1, v/v) to yield white crystals (38 mg, 69 %), m.p. 83–85 °C (decomp.): ¹H-NMR ([²H]₆]dimethyl sulphoxide) δ 10.90 (s, 1 H), 9.30 (d, 1 H, *J* 7.8 Hz), 7.55 (d, 1 H, *J* 7.6 Hz), 7.34 (d, 1 H, *J* 7.6 Hz), 7.16 (d, 1 H, *J* 2.1 Hz), 7.08 (t, 1 H, *J* 7.6 Hz), 6.99 (t, 1 H, *J* 7.6 Hz), 4.66 (m, 1 H), 3.40–3.05 (m, 2 H); ¹⁹F-NMR ([²H]₆]dimethyl sulphoxide) δ -61.63 (m, 2 F), -71.09 (m, 2 F); IR (KBr) 3414, 2126, 1663 and 1487 cm⁻¹; UV (50 mM PBS, pH 7.0): λ_{max} 261 (4.29).

Ethyl-2-diazomalonyl chloride (compound **5**, Scheme 2 below)

A 10 g portion of ethyl diazoacetate (56.7 mmol) was added to a solution of 8.6 g (29.2 mmol) of triphosgene in 60 ml of benzene. The resulting solution was heated at 55 °C for 3.5 h, during which time evolution of bubbles were observed. The solvent was removed by aspiration and the residue was distilled *in vacuo*. The yellow fraction at 13.3 Pa (0.1 mmHg) and 69–71 °C was collected (5.3 g, 69 %): ¹H-NMR ([²H]chloroform) δ 4.36 (q, 2 H, *J* 7.0 Hz), 1.35 (t, 3 H, *J* 7.0 Hz).

N^α-(1-Ethyl-2-diazomalonyl)tryptophan (compound **6**, Scheme 2 below)

Tryptophan (156 mg, 0.76 mmol) was suspended in 4 ml of tetrahydrofuran (THF) at 25 °C, followed by addition of ethyl-2-diazomalonyl chloride (compound **5**) (100 µl, 0.76 mmol). The resulting mixture was stirred under nitrogen for 68 h. Triethylamine (106 µl, 0.76 mmol) in 2 ml of THF was then added dropwise; the precipitation decreased and the solution turned moderately yellow. After stirring for another 5 h at 25 °C, the solution was mixed with 50 ml of dichloromethane and washed with 10 % acetic acid (three times). The washed solution was dried (over anhydrous Na₂SO₄) and evaporated; the residue was taken up in a small volume of ethyl acetate/acetic acid (40:1, v/v) and separated on a silica-gel column with ethyl acetate/acetic acid (40/1, v/v) as eluant. The major fraction isolated (121 mg, 46 %) was found to be the desired product: ¹H-NMR ([²H]₆]dimethyl sulphoxide) δ 10.95 (s, 1 H), 7.98 (d, 1 H, *J* 7.5 Hz), 7.50 (d, 1 H, *J* 7.8 Hz), 7.33 (d, 1 H, *J* 7.8 Hz), 7.14 (d, 1 H, *J* 2.1 Hz), 7.06 (t, 1 H, *J* 7.8 Hz), 6.96 (t, 1 H, *J* 7.8 Hz), 4.68 (t, 1 H, *J* 7.5 Hz), 4.20 (q, 2 H, *J* 7.2 Hz), 3.24 (d, 2 H, *J* 5.7 Hz), 1.20 (t, 3 H, *J* 7.2 Hz); NMR ([²H]chloroform) δ 9.01 (s, 1 H, br), 8.22 (s, 1 H), 8.20 (s, 1 H, br), 7.58 (d, 1 H, *J* 7.8 Hz), 7.29 (d, 1 H, *J* 8.1 Hz), 7.16 (t, 1 H, *J* 8.1 Hz), 7.08 (t, 1 H, *J* 7.8 Hz), 7.04 (d, 1 H, *J* 2.4 Hz), 4.96 (m, 1 H), 4.25–4.15 (m, 2 H), 3.45–3.30 (m, 2 H), 1.35–1.20 (m, 3 H); ¹³C-NMR ([²H]chloroform) δ 175.89, 136.08, 127.33, 123.11, 122.05, 119.46, 118.59, 111.20, 109.54, 61.82, 53.37, 27.58, 14.19; UV (50 mM PBS, pH 7.0): λ_{max} 254 (3.93); high resolution (HR)-FABMS *m/z* 345.1211; Calc. for C₁₆H₁₆N₄O₅ + H, 345.1199.

Among the two minor fractions, one (28 mg, 14%) was determined to be N^α -(1-ethyl-2-diazomalonyl)tryptophanyltryptophan [the nature of another fraction (17 mg) was unknown]: NMR ($[\text{D}_6]\text{acetone}$) δ 10.07 (s, 1 H), 10.04 (s, 1 H), 8.15 (d, 1 H, J 8.0 Hz), 7.66 (d, 1 H, J 8.1 Hz), 7.52 (d, 1 H, J 7.7 Hz), 7.43 (d, 1 H, J 7.2 Hz), 7.36 (d, 1 H, J 8.1 Hz), 7.35 (d, 1 H, J 7.7 Hz), 7.23 (d, 1 H, J 3.0 Hz), 7.10 (d, 1 H, J 2.7 Hz), 7.09–7.04 (m, 2 H), 7.03–6.94 (m, 2 H), 4.90–4.78 (m, 2 H), 4.24 (q, 2 H, J 7.4 Hz), 3.34–3.17 (m, 4 H), 1.27 (t, 3 H, J 7.4 Hz); ^{13}C -NMR ($[\text{D}_6]\text{acetone}$) δ 173.17, 171.50, 164.30, 137.36, 137.22, 128.62, 128.59, 124.77, 124.62, 124.46, 124.30, 122.05, 122.00, 119.55, 119.45, 119.41, 119.16, 112.01, 111.98, 110.59, 110.51, 62.26, 54.75, 53.82, 29.13, 27.98, 14.46; FABMS m/z positive mode $[M+H]^+$ 531, negative mode $[M-H]^-$ 529; Calc. for $\text{C}_{27}\text{H}_{28}\text{N}_6\text{O}_6$, 530.

N^α -(1-Ethyl-2-diazomalonyl)-5-bromotryptophan (compound 7, Scheme 2 below)

The title compound was synthesized using the same procedure outlined for the preparation of compound 6, except that the purification of the desired compound (compound 7) was much easier. Washing of the crude residue with a small amount of cold acetone afforded an analytically pure white powder (60%), m.p. 213–216 °C (decomp.): ^1H -NMR ($[\text{D}_6]\text{dimethyl sulphoxide}$) δ 10.32 (s, 1 H, br), 8.11 (d, 1 H, J 5.4 Hz), 7.73 (d, 1 H, J 1.2 Hz), 7.33 (d, 1 H, J 8.6 Hz), 7.27 (d, 1 H, J 2.1 Hz), 7.18 (dd, 1 H, J_1 8.6 Hz, J_2 2.1 Hz), 4.92–4.86 (m, 1 H), 4.24 (q, 2 H, J 7.1 Hz), 3.45–3.30 (m, 2 H), 1.25 (t, 3 H, J 7.1 Hz); IR (KBr) 3435, 2141, 1696, 1624, and 1539 cm^{-1} . Analysis: Found: C, 45.44; H, 3.55; N, 13.05. Calc. for $\text{C}_{16}\text{H}_{15}\text{N}_4\text{O}_5\text{Br}$: C, 45.41; H, 3.57; N, 13.24.

Photolysis of N^α -(4-Azidotetrafluorobenzoyl)tryptophan methyl ester (3) in methanol

The title compound (3) (40 mg, 8.1×10^{-2} mmol) was dissolved in 15 ml of methanol that had been bubbled with nitrogen for 30 min. The solution was irradiated at 254 nm and the progress was monitored by UV measurement. After irradiation, the solution was evaporated and the residue was separated on an analytical silica-gel TLC plate with ethyl acetate/hexane (1/1, v/v) as eluant. The major product isolated (> 50%) was found to be N^α -(4-aminotetrafluorobenzoyl)tryptophan: ^1H -NMR ($[\text{D}_6]\text{chloroform}$) δ 8.28 (s, 1 H, br), 7.53 (d, 1 H, J 7.1 Hz), 7.34 (d, 1 H, J 8.0 Hz), 7.16 (t, 1 H, J 7.1 Hz), 7.08 (t, 1 H, J 8.0 Hz), 7.02 (d, 1 H, J 2.4 Hz), 5.09 (m, 1 H), 4.23 (s, 2 H, br), 3.70 (s, 3 H), 3.43 (m, 2 H); ^{19}F -NMR ($[\text{D}_6]\text{chloroform}$) δ -67.18 (m, 2 F), -85.57 (m, 2 F); electron-impact MS: 409 (M^+ , 9), 201 (33), 192 (27), 130 (100); HR-FABMS m/z 410.1125; Calc. for $\text{C}_{19}\text{H}_{14}\text{F}_4\text{N}_3\text{O}_3 + \text{H}$, 410.1128.

Preparation of cyanomethaemoglobins (HiCNs)

HiCN(A). HiCN(A) was prepared from carbomonoxyhaemoglobin (HBA·CO), in which form isolated haemoglobin [17] was temporarily stored. The HBA·CO was first converted into oxyhaemoglobin (HBA·O₂) as described by Di Iorio [18]. Then 4.58 ml of concentrated HBA·CO (concn. 3.15 mM; all concentrations referred to in the present paper are those of the haemoglobin tetramer) was diluted with 16.02 ml of 5.0 mM PBS, and the resulting solution (0.7 mM) was cooled in an ice/water bath and then irradiated with a floodlamp (approx. 25.4 cm above the solution) while O₂ was passed slowly on to the solution. After oxygenation was conducted for 90 min, the irradiation was terminated and a solution (116 ml) consisting of K₃Fe(CN)₆ (0.608 mM), KCN (0.77 mM), and KH₂PO₄

(1.03 mM) [18] was added to the HBA·O₂ solution. The resulting solution was rotated in the ice/water bath for another 2.5 h, and then kept at 4 °C overnight. The HiCN solution thus formed was concentrated at 4 °C to approx. 3–4 ml and desalted with a Sephadex G-25 gel-filtration column (Pharmacia) with 5.0 mM PBS as eluant.

HiCN(S). HiCN(S) was prepared from HBS·CO using the same procedure, except 50 mM PBS was used as eluant in the desalting step.

Irradiation of HiCN(A) in the presence of photolabel 4

An HiCN(A) solution (0.964 ml, 0.50 mM) was added to 15 ml of 50 mM PBS, pH 7.0, that was contained in the microscale photochemical reactor and pre-bubbled with nitrogen for 30 min, followed by dropwise addition of compound 4 [20 mg (47.5 mmol)] in ethanol (300 μl). The final concentration of compound 4 was 2.9 mM, while that of HiCN(A) 0.029 mM. While under gentle magnetic stirring and cooling with circulation of tap water through the immersion well, the resulting solution was irradiated at 254 nm for a period of 61 min, and the process was monitored by removing samples (30 μl) for UV-visible measurement. After irradiation, the solution was filtered through filter paper and concentrated by centrifuging at 4 °C; the concentrated solution was desalted through gel-filtration (Sephadex G-25).

Irradiation of HiCN(S) in the presence of photolabel 7

The procedure was largely the same as outlined above, except that 100-fold (versus the HBS tetramer) of compound 7 in 600 μl of ethanol was added dropwise to 16.0 ml of a 3.07×10^{-2} mM solution of HiCN(S) in 50 mM PBS, pH 7.0. The resulting solution had final concentrations of 3.0 mM and 0.030 mM for compound 7 and HiCN(S) respectively; it was left at room temperature for 30 min before being irradiated at 254 nm for a total period of 21 min.

Enzymic digestion of heat-denatured haemoglobin samples

The haemoglobin samples (HB·CO or HiCN) in 5.0 mM PBS, after concentrating to ~ 0.5 mM, were denatured by heat before being digested with trypsin [19–21]. Hence an HiCN solution (241 μl) was heated at 100 °C for 4 or 8 min. To the denatured HiCN was added, respectively, solutions of NaOH (0.05 M, 70 μl), NH₄HCO₃ (4.38 mg/ml, 260 μl), and trypsin (4.0 mg/ml, 36 μl). The resulting solution, which had a pH of 8.6, was incubated at 37 °C for 15 h with gentle magnetic stirring. A second aliquot of the trypsin solution (18 μl) was added to the HiCN solution, and the incubation continued for another 9 h. The digestion was then quenched by lowering the pH to ~ 3 and/or freezing to -10 °C.

HPLC analysis and separation

The digest solution was filtered through a filter unit (0.45 μm pore size), and an aliquot (5–150 μl) was injected into the Waters HPLC system equipped with a Vydac C-18 reversed-phase column [4.6 mm $\times$ 250 mm; 30 nm (300 Å) pore size; 10 μm particle size], with UV detection at 220 or 290 nm. The mobile phases consisted of solution A (0.1% aq. TFA) and solution B [0.1% TFA and 5% (v/v) water in acetonitrile]; a linear gradient from 0 to 38% B was run over a period of 80 min. Peaks of interest collected from the separation were re-purified using the same column, but with a different binary gradient system consisting of 10 mM ammonium acetate and acetonitrile as

solution A and B respectively. Some re-purification runs were also performed with a Rainin MV C-18 column [4.6 mm $\times$ 250 mm; 10 nm (100 Å) pore size; 5 μ m particle size] on the Rainin HPLC system with the capability of dual-wavelength detection.

Computer modelling of photolabel 7

The co-ordinates of deoxyHBS [22] were obtained from the Brookhaven Protein Data Bank [23]. Computations were performed with the DISCOVER and INSIGHTII (Biosym, San Diego, CA, U.S.A.) package on a Silicon Graphic Personal Iris workstation. D- and L-isomers of photolabel 7 were built using BUILDER and manually docked into the proposed symmetric binding site between the N-termini of two α -subunits, using the known X-ray structure of STT [10] as a template. The full valence force field potential was used to calculate the potential energy. Energy minimizations were carried out within the HBS-binding site with a distance dependent dielectric constant; a cut-off distance of 2 nm (20 Å) was used for both electrostatic interactions and van der Waals interactions. All hydrogen atoms were considered explicitly, and charges were introduced in the residues of arginine, lysine, aspartic acid, glutamic acid and the C- and N-termini. Energy minimizations consisted of a few steps of steepest descent followed by conjugate gradient minimization until the maximum derivative was less than 0.418 kJ/(mol $\cdot$ nm) [0.01 kcal/(mol $\cdot$ Å)]. During the energy minimization, the indole ring of photolabel 7 was tethered to that of the second tryptophan residue (from the N side) of STT initially, and then the label was minimized free from any constraints. STT was modelled in the symmetric binding site according to the published crystal structure [10]. Minimizations were done both with the protein fixed, and with residues within 1 nm (10 Å) of the binding site allowed to move. The results of both calculations were similar.

RESULTS

Synthesis of photolabels

The synthesis of compound 4 is outlined in Scheme 1. The photoactive group, 4-azidotetrafluorobenzoyl, was coupled to the affinity moiety (tryptophan) via its acyl chloride or directly

from the acid form with the aid of the coupling reagent EEDQ [24]. EEDQ has been reported to cause very little racemization [25] in comparison with the widely used DCC or EDC. The methyl ester (compound 3) was then easily hydrolysed to give the desired compound (4). Ethyl-2-diazomalonyl chloride (5) was prepared from chloroformylation of ethyl diazoacetate with trichloromethyl carbonate (triphosgene), which was introduced as a stable crystalline substitute for phosgene [26]. In the preparation of *N* $^{\alpha}$ -(1-ethyl-2-diazomalonyl)tryptophan (6) (as a model for the preparation of compound 7), the desired product was contaminated by two by-products ($\sim$ 10% total yield), which were removed by separation using silica-gel column chromatography [eluant: ethyl acetate/acetic acid, 40:1 (v/v)]. Compound 6 was isolated in 46% yield. One of the by-products was found to be the corresponding dipeptide derivative *N* $^{\alpha}$ -(1-ethyl-2-diazomalonyl)Trp-Trp; apparently part of the malonyl chloride served as an activating reagent for the carboxyl group, as illustrated in Scheme 2. The reaction of DL-5-bromotryptophan with ethyl-2-diazomalonyl chloride is similar to that of its model compound; two minor by-products also formed (TLC evidence). Purification of the desired compound (7), however, was easier, since the by-products could be washed away from the precipitated compound 7 with a small amount of acetone. Analytically pure compound 7 was obtained in 60% yield.

Photolysis of *N* $^{\alpha}$ -(4-Azidotetrafluorobenzoyl)tryptophan methyl ester (3) in methanol

Compound 3 (5.4 mM) was efficiently decomposed by UV light at 254 nm during the 20 min period, as evidenced by the decrease of the characteristic peak at 258 nm (for the azido group) and the accompanying red shift (λ_{max} , 267 nm) (spectra not shown). A major product (> 50%) isolated from the photolysis was found to be *N* $^{\alpha}$ -(4-aminotetrafluorobenzoyl)tryptophan methyl ester, as indicated by the NMR and high-resolution mass spectra.

Irradiation of HiCN(A) in the presence of *N* $^{\alpha}$ -(4-azidotetrafluorobenzoyl)tryptophan (4)

Under UV irradiation at 254 nm, photolabel 4 decomposed in the solution of HiCN(A) as a function of time. At the end of the

Scheme 1 Synthesis of the azide label, compound 4

Scheme 2 Synthesis of the photoaffinity label (compound 7) and its model compound (6)

Figure 1 Irradiation of HiCN(A) solution (0.029 mM) in the presence of compound 4 (2.9 mM)

The process was followed by UV-visible spectrophotometry: curve a, $t = 0$ min; curve b, $t = 11$ min; curve c, $t = 33$ min; curve d, $t = 66$ min.

irradiation (66 min), some of the photolabel still remained intact, as indicated by the fact that the characteristic peak at 261 nm was reduced, but not eliminated (Figure 1). To minimize damage to HiCN(A), longer irradiation to completely decompose 4 was not attempted.

Irradiation of HiCN(S) in the presence of *N*^ε-(1-ethyl-2-diazomalonyl)-5-bromotryptophan (7)

Compound 7 was efficiently photolysed at 254 nm in the HiCN(S) solution over a period of 21 min. At the end of that period, the

Figure 2 Irradiation of HiCN(S) solution (0.030 mM) in the presence of compound 7 (3.0 mM)

The process was followed by UV-visible spectrophotometry: curve a, $t = 0$ min; curve b, $t = 11$ min; curve c, $t = 21$ min.

Figure 3 HPLC separation conducted on a Rainin HPLC system equipped with a Vydac 4.6 mm $\times$ 250 mm C-18 column and a UV-D II dual-wavelength detector

The mobile phases consisted of a solution A [0.1% TFA in water] and a solution B [0.1% TFA and 5% (v/v) water in acetonitrile]. A linear gradient of 0–38% was run over an 80 min period with UV detection at 220 nm (upper curve) and 290 nm (middle and lower curves). Upper (220 nm) and middle (290 nm) curves, tryptic digest (150 μ l) of irradiated HiCN(S). Lower curve, tryptic digest (20 μ l) of unirradiated HiCN(S).

HiCN(S) was largely undamaged (Figure 2), while the peak at 254 nm (diazo) essentially vanished.

HPLC and amino acid analysis of the HiCN(A) irradiated in the presence of compound 4

HPLC analysis of the tryptic digest from the irradiated and heat-denatured HiCN(A) showed about seven small new peaks relative

Figure 4 Purification of fraction c

Fraction c, collected from three scale-up runs (one of the runs illustrated in Figure 3, upper and middle curves), was concentrated and purified on the Rainin HPLC system equipped with a Rainin MV C-18 column. A gradient of 20–50% B (acetonitrile) in A (10 mM ammonium acetate) was employed over 30 min. UV detection was done simultaneously at both 220 nm (upper trace) and 290 nm (lower trace). The purified fraction, which was eluted at 19.5 min, was subjected to amino acid analysis and sequencing and FAB/MS spectrometry.

Figure 5 FAB mass spectrum of purified fraction c (collected in Figure 4)

The inset shows the expanded region around the twin peaks of 1079 and 1081.

to that of a control run from the unirradiated HiCN(A), with UV detection at 220 nm or 290 nm (results not shown). These new peaks were collected and purified on the same C-18 column, but with a different gradient generated between 10 mM ammonium acetate and pure acetonitrile. The purified fractions were then subjected to amino acid analyses; the analyses revealed no amino acid composition that would reasonably be considered to match that of a peptide sequence in haemoglobin.

HPLC analysis and separation of the HBS irradiated in the presence of compound 7

HPLC analysis (15 μ l injection) of the tryptic digest from the irradiated and heat-denatured HiCN(S) showed three new peaks relative to that of a control run from unirradiated HBS, with UV detection at 220 nm or 290 nm. A ten-fold scale-up run showed

Figure 6 Appearance of individual amino acid in each cycle of the sequencing of purified fraction c

○, Val; □, Leu; ◇, Ser; △, Pro; +, Ala; ×, Asp.

the same result (Figure 3); fractions (a–c) were collected and, after concentration, injected into the same Vydac column, but with a different gradient system (ammonium acetate and acetonitrile) for further purification. We found, however, that fraction c stuck on to the column with this gradient system, while fractions a and b were purified without much difficulty. The purification of fraction c was then attempted with the same ammonium acetate/acetonitrile gradient system on a Rainin MV C-18 column that has smaller pore and particle sizes. Successful purification was achieved with this new column. Three scale-up separation runs (150 μ l injection) were then conducted on the Vydac column for the remaining digest solution. Fractions a–c collected from the scale-up runs were pooled, and fractions a and b were purified on the Vydac column, while fraction c was purified on the MV column (Figure 4).

Amino acid analysis, sequencing and FABMS measurement of the purified fractions a–c obtained from the labelling experiment with photolabel 7

Part of every purified fraction was subjected to amino acid analysis. Fractions a and b did not show a composition that would match the haemoglobin sequence at any location, while analysis of fraction c indicated that its composition matched the sequence from Val¹(α) to Lys⁷(α) resulting from the tryptic digestion (α -T₁). The FAB mass spectrum of fraction c showed a set of twin peaks at 1079.1 and 1081.1, in addition to weak fragment peaks (Figure 5). Amino acid sequencing of the same fraction (c), using Edman degradation, gave unusual results: nothing was observed in the first cycle, and then every amino acid present in α -T₁ showed up sequentially. However, approximately the same amount of every residue (as when it first occurred) showed up in every following cycle (Figure 6).

Estimation of labelling yield at Val¹(α)

The yield of the labelling at Val¹(α) was estimated by comparing the area of peak (detected at 290 nm) with the total area of all the native peaks (Figure 3, middle curve). Only the labelled Val¹(α) and those native peptides that bear tryptophan absorb UV light significantly at 290 nm (the absorbance of tyrosine, phenylalanine and histidine is negligible at this wavelength). It was determined, therefore, that approx. 5% of Val¹(α) was labelled during the irradiation.

Computer modelling of photolabel 7

Both D- and L-isomers were docked into the symmetric binding site by superposition of their indole rings with that of the second tryptophan residue in STT, since the major interactions of STT with HBS are hydrophobic contacts between the second tryptophan residue of STT and Pro⁷⁷(α), and hydrogen bonds between the carboxy group of the second tryptophan residue and the HN group of Leu²(α) as well as OH group of Ser¹³¹(α). The remaining

Figure 7 Stereo view of proposed interaction of compound 7 inside the left side of the symmetric site

The continuous line represents the L isomer; the broken line represents the D isomer.

moieties of the D- and L-isomers were adjusted to have other possible contacts with HBS, and the full structures were energy-minimized in the binding site to relax any strains and eliminate any collision with HBS. It appears that the central cavity entrance is large enough to accommodate two photolabels simultaneously without any contacts between the two labels. Detailed interactions between the photolabels and HBS are displayed in Figure 7. Both indole rings of the D- and the L-photolabels are located above the edge of the five-membered ring of Pro⁷⁷(α), contact the Met⁷⁶(α) side chain at the left side and approach the Val¹³⁵(α) side chain from behind. In addition to these hydrophobic interactions, several hydrogen bonds common to both D- and L-isomers were observed. The HN group of the indole ring interacted with the carbonyl group of Val⁷³(α), and the two oxygen atoms of the carboxy group (of tryptophan) hydrogen-bonded to the HN group of Leu²(α) and the OH group of Ser¹³¹(α) respectively. The indole ring was oriented in such a way that its bromine atom was pointing out into the solvent. The rest of the two isomers exhibited different orientation and contacts with HBS due to their different chiralities at C $_{\alpha}$. The ethyl group of the D-isomer was inserted deeply into the HBS central cavity, contacting Ala¹⁰³(α), while the same group of the L-isomer pointed outwards from the cavity to contact its own indole ring. The D-isomer had an additional hydrogen bond between the first malonyl CO group and the OH group of Ser¹³¹(α). The N-terminus of the α subunit was hydrogen-bonded to the second malonyl carbonyl group and salt-bridged to the tryptophan carboxyl group (in both the D- and the L-isomer), which helped to hold the N-terminus proximal to the potential carbene site for the consequent covalent attachment. Although the D-isomer had a few more contacts with HBS than the L-isomer, it appeared unfavourable for its ethyl group to extend into the central cavity in terms of electrostatic interactions. That region, surrounded by the side chains of Lys¹²⁷(α), Arg¹⁴¹(β), and the N-terminus of the α subunit, may be too polar to accommodate the ethyl group. The modelled distance between the N-terminus to the potential carbene site is ~ 0.4 nm (4 Å) for the D-isomer and ~ 0.5 nm (5 Å) for the L-isomer. The flexibility of the N-terminus should allow it to further approach the carbene site.

DISCUSSION

HiCN samples were used in all the labelling experiments, since we found that the cyanomet form was relatively stable under UV irradiation at 254 nm, while other forms, for example HB·CO, were not stable under the same conditions, as evidenced by a rapid decrease of the characteristic Soret band around 410 nm (results not shown). Previous studies on the photolysis of non-met forms of haemoglobin solutions with UV showed that the haemoglobin molecules were readily damaged [27,28]. The tertiary structures of all forms of haemoglobin are quite similar, while the quaternary structures differ in the interfaces between the subunits [29]. In that sense, HiCN is more like the oxy, rather than the deoxy, form. Thus the present approach may not detect those binding sites, if any, of tryptophan or 5-BrTrp at the interfaces of deoxyhaemoglobin, because of the necessity of using HiCN in the labelling experiment. However, NMR measurements with spin-labelled tryptophan analogues suggest that tryptophan and related compounds bind to the same sites in both HB·CO and deoxyhaemoglobin (C. J. Yuan, L. Kar, P. Z. deCros, B. L. Currie and M. E. Johnson, unpublished work).

One crucial factor that may determine success or failure of a photolabel is the reactivity of the photochemically produced intermediate from that label. A good photolabel based on a

carbene or nitrene is expected to give a high yield of covalent incorporation (insertion) into a receptor where it binds. Aryl azides are the most widely utilized photolabels, probably because of the ease of their synthesis and incorporation into various ligand structures. The normal phenyl azides, however, give essentially no C-H insertion products when photolysed in hydrocarbon solvents at room temperature [30]. On the other hand, functionalized perfluorophenyl azides were reported to give high yields of C-H insertion through the photochemically produced nitrenes [16]. Thus it appeared promising that a suitable photoaffinity reagent could be produced by linking the perfluoroazide functionality to tryptophan. Photoaffinity reagent **4** thus made was used to label HiCN(A). The peptide mapping by RP-HPLC of the irradiated HiCN(A) in the presence of **4** revealed about seven small new peaks relative to a control run from that of unirradiated HiCN(A) or HBA·CO (results not shown). Amino acid analysis indicated, however, that none of these new peaks would stem from compound-**4**-labelled peptides; they may be decomposed products from compound **4** and/or HiCN(A), because of minor, undesirable photochemical damage occurring during the photolysis. The reason that compound **4** was unable to label HiCN(A) can be deduced from the fact that compound **4** was found later to have no HBS anti-gelling activity at all. It was quite unexpected that the introduction of the 4-azidotetrafluorobenzoyl group on to the amino group of tryptophan would abolish the activity completely, but it may be that the rigidity of the long 4-azidotetrafluorobenzoyl group prevented proper binding of compound **4** to haemoglobin. The second photoaffinity reagent (**7**) employed was based on the combination of the photoactive group diazomalonyl (which is more flexible than 4-azidotetrafluorobenzoyl) and the affinity moiety 5-BrTrp; the resulting compound retained $\sim 50\%$ of the anti-gelling activity of the parent compound [9]. The diazomalonyl group was introduced as a photoaffinity label superior to its predecessor, α -diazooacetyl, in that, in contrast with the latter, the carbene intermediate produced from the diazomalonyl group does not undergo Wolff rearrangement [31–33], which leads to the formation of a less reactive ketene. Thus high yield of insertion (labelling) can be achieved by the use of the diazomalonyl group [34]. Moreover, the group can be readily coupled with biological ligands via amide or ester linkage through its acyl chloride intermediate. Peptide mapping of HiCN(S) irradiated in the presence of compound **7**, using the same procedures described above, showed three new peaks with UV detection at both 220 and 290 nm (Figure 3). Amino acid analysis showed that purified fraction c (from Figure 4) contained the composition of α -T₁, i.e. Val¹(α)-Lys⁷(α), while no interpretable composition was found for either fraction a or b. [Presumably, fractions a and b were photochemically decomposed products from compound **7** and/or HiCN(S).] From the analysis of fraction c it was also noted that no residue was missing as a consequence of the labelling (Table 1). There appears to be no doubt that the label was covalently attached to α -T₁, since the native peptide does not absorb UV light at 290 nm. Therefore the linkage between the label and the peptide must be labile towards the strong acid hydrolysis required for amino acid analysis. The labelled site was initially thought to be either on the side-chain oxygen atom of Ser³(α) or that of Asp⁶(α), since it was known that similar ether or ester type linkages decomposed under the same hydrolysis conditions [35].

FABMS analysis of purified fraction c showed a set of twin peaks at 1079.1 and 1081.1 as the base peaks (Figure 5), rather than 1123.4 and 1125.4 as would be expected from a 1:1 adduct between the labelling moiety and the native peptide. The mass difference of 44 strongly suggested that decarboxylation had occurred during the irradiation, since there was no doubt that the

Table 1 Amino acid composition of the purified fraction c (collected in Figure 4)

Amino acid	Composition (residues/mol)	
	Found	Theoretical*
Asx	0.9	1
Ser	0.9	1
Ala	1.1	1
Pro	1.0	1
Val	1.0	1
Leu	1.2	1
Lys	1.0	1

* For peptide Val¹(α)-Lys⁷(α) of HBS.

Figure 8 Proposed structure of the labelled peptide (α -T₁)

FAB/MS: twin peaks (m/z) at 1079 and 1081 were observed, which are equal to the values of $[M+H]^+$ peaks of the proposed structure.

label had been covalently attached to the peptide, as evidenced by the set of twin peaks (indicating the presence of ⁷⁹Br and ⁸¹Br, which exist in approximately equal abundance). This hypothesis is supported by the fact that decarboxylation is one of the common pathways resulting from photochemically excited tryptophan derivatives and peptides [36–38]. The peak of 1001.2, the only significant fragmentation peak, probably resulted from debromination of the labelled peptide during the ionization process. In order to locate the exact site of labelling, the same labelled peptide was also subjected to amino acid sequencing using conventional Edman degradation chemistry. The result, however, was a little surprising: the expected valine did not occur in the first cycle, but, from the second cycle on, every amino acid present in α -T₁ showed up sequentially. However, approximately the same amount of every residue (as that when it first appeared) was observed in each following cycle (Figure 6). This unusual 'lag' phenomenon can be rationalized if the label was attached to the amino group of Val¹(α) and the linkage between them was partially destroyed during each coupling/cleavage step (phenyl isothiocyanate, trimethylamine, 48 °C, 20 min/TFA, 48 °C, 5 min). This view is further supported by the fact that the sample amount submitted for sequencing was about 200 pmol, while the amount of amino acid residue appearing in each cycle was only about 20–30 pmol. A structure for the labelled peptide, therefore, can be proposed (Figure 8), based on the above information. The yield of the labelling was estimated to be approx. 5%; this is a significant modification, given the fact that the affinity moiety (5-BrTrp) has very low binding affinity towards haemoglobin ($K_D \sim 3$ mM) (C. J. Yuan, L. Kar, P. Z. deCroos, B. L. Currie and M. E. Johnson, unpublished work).

Previous X-ray studies by Perutz et al. [10] determined that

STT occupied sites at the entrance of the central cavity between the two α -subunits. Binding studies carried out by Votano and Rich [39] showed that approx. 2.3 molecules of STT bound to every haemoglobin tetramer. They reasoned that the value of 2.3 must represent one major symmetric site and one or more additional minor sites, since haemoglobin is a symmetric molecule related by a C₂ axis. Their result is in good agreement with that of Perutz et al. [10]; thus the symmetric site at the entrance of the central cavity must be the major binding site of STT. Furthermore, this symmetric site was also among the several binding regions of 5-BrTrp predicted by Manavalan et al. [12] using computer modelling. In the present study, therefore, it is very reasonable to assume that the photoaffinity label (7) may primarily bind to the same symmetric site. Indeed, if we dock compound 7 to the symmetric site in which the orientation of the 5-bromoindole moiety follows that of the indole ring of the second tryptophan residue in STT, a reasonable explanation for the observed labelling result can be obtained: the potential carbene site in compound 7 could be only ~ 0.4 – 0.5 nm (4–5 Å) away from the amino group of Val¹(α) (Figure 7). Since our photolabelling measurements were done with HiCN, the relevance to deoxy haemoglobin might be questioned. However, we have compared the probable binding-site regions between liganded and deoxy-haemoglobin and between met- and deoxy-haemoglobin and found only ~ 0.06 – 0.07 nm (0.6–0.7 Å) backbone root-mean-square deviation, indicating that the tertiary conformation in this region is quite stable. Similarly, NMR results indicate that a variety of indole-based inhibitors bind to liganded and deoxy haemoglobin at the same site (C. J. Yuan, L. Kar, P. Z. deCroos, B. L. Currie and M. E. Johnson, unpublished work). Thus binding to deoxyhaemoglobin can be expected to follow the pattern observed here.

Votano and Rich [39] also studied anti-gelling activity and binding affinity of another two peptides, namely succinyl-Phe-Phe and succinyl-Phe-Gly-Phe, in addition to STT. They found a correlation between the binding affinity (contributed mainly by the binding of all these three peptides to the same symmetric site) and the anti-gelling activity. They therefore concluded that the symmetric site must be involved in the polymerization process. Perutz et al. [10] determined, however, that the symmetric site was located in the entrance of the central cavity, far away from the important lateral and axial contact sites in the HBS fibre suggested by Love and co-workers [22,40,41]. Perutz et al. [10] also found that bezafibrate (BZF), a compound that promoted sickling, bound to a region between two α -subunits and one β -subunit that overlapped with the symmetric site. Thus Perutz et al. [10] found it difficult to ascribe the anti-gelling activity to binding at the major symmetric site, since both the anti-gelling compound (STT) and the pro-gelling compound (BZF) bound to two adjacent regions. In the present study, we found from the photoaffinity labelling evidence that label 7 (and very probably 5-BrTrp and tryptophan themselves) bound to the same symmetric site, probably in a fashion similar to that of STT (in terms of interactions with haemoglobin), as suggested by the preliminary computer docking analysis (Figure 7). We also note that the symmetric site occupied by STT is quite different from the BZF-binding site, although these two regions overlap slightly with each other. (The overlap is actually very small, according to our docking experiments (results not shown), based on the results of Perutz et al. [10].) It is therefore possible that binding to the symmetric site is primarily responsible for the anti-sickling activity of STT, 5-BrTrp and other tryptophan- and phenyl-alanine-based peptides, although we certainly cannot exclude the possibility that weak binding at sites other than the symmetric one may be mainly responsible for the anti-gelling activity. On

the other hand, all the compounds that bind to the BZF site are strong allosteric effectors [42], and since they decrease oxygen affinity significantly, they would thus all promote sickling.

In summary, we have located a binding region for 5-BrTrp (and possibly its dipeptide) through the use of its photoactive derivative, compound 7. The identification of the labelled peptide from that region was achieved by taking advantage of the inherent UV-absorbent property of the label (which provided a convenient tracer during the HPLC separations), and by virtue of FABMS, in addition to conventional protein-chemistry techniques. The binding mode of the photoaffinity label (7) has been proposed by using computer modelling. Further work is in progress to clarify the correlation between this major binding mode and the potency of the 5-BrTrp-based anti-sickling agents.

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