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A Mass Spectrometry Screening Method for Antiaggregatory Activity of Proteins Covalently Modified by Combinatorial Library Members: Application to Sickle Hemoglobin

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A homogeneous assay, based on electrospray mass spectrometry, is described for identifying compounds in a combinatorial library that covalently modify a protein and thereby enhance its solubility. The technique is based on measuring the distribution of modified proteins in the supernatant versus aggregate. Compounds having the greatest anti-aggregatory activity are those with the highest supernatant/aggregate ratio. Mass is used as a marker to identify which covalent modifier in the library is involved. An exploratory study is presented which demonstrates that the antisickling activity of a family of isothiocyanates, as measured by the standard C_{sat} assay, correlates well ($r^2 = 0.98$) with the mass spectrometry analysis of the supernatant/aggregate distribution. The technique has potential for screening libraries capable of covalently modifying other proteins of clinical interest, e.g., Alzheimer's, Huntington's, and various prion related diseases.

Most drugs express their biological activity through reversible interactions with receptors. Although there can be potential toxicity problems with a covalent approach to drug design, important drugs such as aspirin and phenoxybenzamine, which act through covalent modification of the cyclooxygenase[1] and the β-adrenergic receptor,[2] respectively, demonstrate that this approach can have merit. In principle, newer methods of combinatorial chemistry might be used to define chemical structure which would allow the targeting of receptors with specific covalent agents, providing a new and potentially important approach to modifying their biological activity. Recently, Mckendrick and co-workers described such an approach using mass spectrometry to rapidly determine irreversible proteinase inhibitors in combinatorial libraries.[3] Here we present an exploratory study on a homogeneous assay for screening libraries of substituted isothiocyantes for antisickling activity through covalent modification of sickle cell hemoglobin.

Sickle cell anemia results from the substitution of Val for Glu at position 6 in the hemoglobin β chain, and it is characterized by abnormal rigidity of erythrocytes at low oxygen tension due to intracellular polymerization of the sickle hemoglobin (HbS), which, in turn, can lead to occlusions in the microcirculation.[4] The polymerization of HbS is stabilized by an intermolecular hydrophobic contact through the interaction between donor Valβ6 and acceptor Pheβ85 and Leuβ88.[5,6] One approach to reduce the polymerization has been to disrupt the intermolecular interaction, or to destabilize the deoxy (T) state conformation, by direct covalent modification of the HbS protein. Several structurally unrelated compounds have been partially successful in this regard.[7]

Several methods have been developed for measuring the inhibition of sickle hemoglobin aggregation: equilibrium solubility, or saturation concentration (C_{sat}),[8] turbidity,[9,10] and oxygen affinity.[11] The equilibrium solubility method is widely used, measuring the effect of a compound on hemoglobin gelation under deoxygenation conditions (addition of sodium dithionite). Separation of the HbS solution from the semisolid gel by ultracentrifugation provides the equilibrium solubility by measuring spectrophotometrically the HbS remaining in solution.

If HbS were covalently modified with a compound which had antisickling activity, the modified HbS would tend to avoid polymerization and stay in solution. If so, one would expect that the distribution of modified HbS between supernatant and gel would reflect the antisickling activity of the covalent modifier. Further, the approach might lend itself to the simultaneous use of multiple agents in the same assay, i.e., a direct homogeneous assay for antiaggregatory activity. Herein, we present an electrospray mass spectrometry (ESMS) screening method for evaluating covalent modifiers of HbS for antisickling activity based on this hypothesis.

Recently, we reported that alkyl isothiocyanates selectively modify the Cysβ93 residue in Hb and that the modified HbSs showed varying degrees of saturation concentration in the C_{sat} assay.[12] The underlying mechanism of this antisickling activity is not clear. However, the Cysβ93 residue is located in the FG helix of the β-subunit, as are the Pheβ85 and Leuβ88 "receptor" residues,[13] which, as noted, may be involved in the sickling process.

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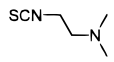
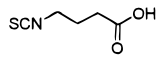
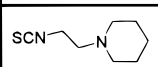
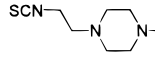
Table 1. Saturation Solubility of Isothiocyanates from the C_{Sat} Assay[a]

Compound	MW	Structure	$(C_{sat}-C_o)/C_o$[b] in %
1	130		28.7
			27.2
			26.5
2	145		9.8
			9.6
			13.5
3	170		24.1
			22.6
			24.2
4	261		-5.9
			-8.8
			-1.7

[a] Assayed by the method of De Croos.[23] [b] C_{sat} = saturation solubility of modified HbS, C_o = saturation solubility of unmodified HbS.

To test the above screening technique, we prepared four alkyl isothiocyanates, **1**−**4**, previously shown[12] to have a range of antisickling activity, as measured with the standard C_{sat} assay (Table 1). HbS was modified with each of these compounds (individually) and, as previously demonstrated, ESMS showed that the agents had greater than 95% selectivity for the β- over the α-subunit of the Hb tetramer. Each of these modified HbSs was purified by Sephadex G-25 column chromatography and then screened with the C_{sat} assay to evaluate their intrinsic antisickling activity. As shown in Table 1, HbS modified with amine isothiocyanates **1** and **3** show solubility increases of greater than 20% over unmodified controls, whereas the acid, **2**, shows about a 10% increase. Interestingly, HbS covalently modified with the piperazine isothiocyanate, **4**, showed progelling activity relative to controls.

In a separate experiment, HbS was modified with a mixture of these same four isothiocyanates. Aggregation was then induced in the resultant modified HbSs in the same fashion as described for the C_{sat} assays, and the supernatant and gel separated by ultracentrifugation. Both the supernatant and gel were diluted, and then analyzed by ESMS without further purification or isolation. The multiply charged peaks for the α- and β-subunits of HbS were spread over a range of 600−1200 *m*/*z*. Peaks in the 850−1200 *m*/*z* mass range were selected for maximum entropy deconvolution.[14] Figure 1 shows representative deconvoluted mass spectra for the supernatant (**S**) and gel (**G**) of Hb simultaneously modified with the four isothiocyanates **1**−**4**. Hb modified with compounds **1** and **2**, which differ in mass by 15 amu, were resolved to approximately 40% of baseline in the multiply charged spectra (16+) and can be seen to be resolved in the deconvoluted spectra (Figure 1). It will be noted that Hb modified with isothiocyanates **1**, **2**, and **3** show higher peaks in the supernatant than in the gel, while Hb modified with isothiocyanate **4** was higher in the gel than in the supernatant. Distribution of the β-subunits modified by the four compounds was quantified by dividing the peak intensity in the transformed spectra for a given modified protein in the supernatant relative to that in the gel. According to Roberts, peak height is a more accurate measure than area, when there is potential for interference from partially resolved peaks,

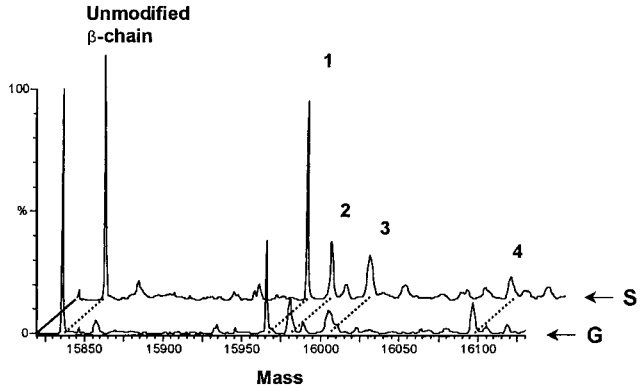


Figure 1. Calculated mass of covalently modified HbS from supernatant and gel of HbS modified with isothiocyanates **1**−**4**. Multiply charged peaks, corresponding to the β-subunit of covalently modified HbS in the 850−1200 *m*/*z* range, were subjected to maximum entropy deconvolution[14] to produce calculated spectra for both the supernatant (**S**) and gel (**G**). Peaks **1**−**4** correspond to the mass of HbS plus that of the covalent modifier **1**−**4** (structures given in Table 1).

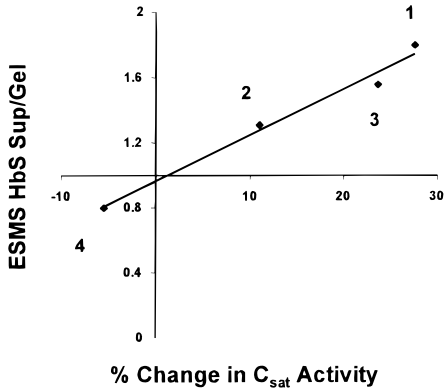


Figure 2. Comparison of antisickling activity as measured by mass spectrometry and the conventional C_{sat} assay. Numbers **1**−**4** refer to the isothiocyanate derivatives given in Table 1.

impurity, or noise.[15] The experiments were conducted in triplicate, with each ESMS analysis also run in triplicate.

A plot of the ratio of the peak intensity in the supernatant to that in the gel for each modified HbS derivative against the C_{sat} values, determined in the aforementioned individual assays for each modified HbS molecule, is given in Figure 2. As may be seen, the correlation between mass spectral analysis and the conventional C_{sat} assay is in good agreement ($r^2 = 0.98$). Further, the least squares line for the data passes through the *Y* axis at a value of 0.98, consistent with the expectation that a compound having neither antiaggregatory nor proaggregatory properties would have an equal distribution (ratio = 1.0) between the supernatant and the gel. Finally, it will be noted that HbS modified with the progelling isothiocyanate **4** was in higher concentration in the gel relative to the supernatant, consistent with prediction. The linear correlation of the ESMS ratio with the C_{sat} results for both pro- and anti-gelling compounds is a particularly encouraging validation of this new assay methodology.

On the basis of these exploratory observations, it may be concluded that ESMS could be used to deconvolute covalent libraries for intrinsic antisickling activity. Though quantitation using mass spectrometry can be difficult, the success in this case may be traced to the fact that, in both the gel and

the supernatant, the MS assay measures a ratio of the same covalently modified molecule; thus, problems due to ionization efficiency are largely eliminated (automatically normalized). Use of such a ratio also takes into account the possibility that different modifiers may give different yields of modified protein, measuring only the effect such modification has on the distribution between the aggregate and solution phase. At present, the technique is limited by instrument resolution. Thus, for proteins at the 16+ charge level, library members must be separated by approximately 16 amu to be differentiated with the instrumentation used herein. However, use of FT mass spectrometry could extend the analysis to unit mass and, coupled with automation, the technique should provide a true high throughput screen for covalent modifiers.

The technique may be of interest for screening libraries capable of covalently modifying other proteins of pathological interest as well, e.g., β-amyloid aggregation in Alzheimer's,[16] amyloid-like protein aggregates in Huntington's disease,[17] and prion protein aggregation in various prion related diseases.[18,19]

Experimental Section

Melting points were determined on a Thomas-Hoover capillary tube apparatus and are not corrected. NMR spectra were obtained on a Bruker Avance 300 spectrometer with tetramethylsilane as the internal standard. IR spectra were obtained on a Jasco FT/IR-410 spectrometer. Mass spectra were recorded with a Finnigan LCQ for APCI, MAT 90 for CI, and Micromass Quattro-II for HbS analyses. UV absorbances were recorded on a Jasco V550 spectrometer. Chemicals were purchased from commercial suppliers. Silica gel column chromatography was carried out on silica gel 60 purchased from Aldrich.

Syntheses. 2-(*N*,*N*-Dimethylamino)ethyl Isothiocyanate (1) was prepared according to the procedure of McElhinney[20] from *N*,*N*-dimethylethylenediamine (6.23 mL, 56.72 mmol): yield 2.58 g (35%); bp[10] 77−79 (lit. 80−84/12 mm); mp 34−35 (lit. 34−35); ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 6H, 2 × NCH_3), 2.61 (t, J = 6.6, 2H, CH_2), 3.60 (t, J = 6.6, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 43.83, 45.74, 58.87; IR (neat) cm^{-1} 2106, 2193; CIMS *m*/*z* 131 ([M + H]$^+$, 51), 72 (M − NCS, 100).

General Procedure for Preparation of Isothiocyanates 2, 3, and **4** from 4-aminobutyric acid (0.11 g, 1 mmol), 1-(2-aminoethyl)piperidine (0.13 g, 1 mmol) and **6** (0.22 g, 1 mmol, see synthesis under that for **4**), respectively, was according to the method of Kim.[21] To a stirred solution of the primary amine (1 mmol in 20 mL of CH_2Cl_2) was added di-2-pyridyl-thionocarbonate (0.23 g, 1 mmol) at room temperature, and the mixture was stirred for 20 h for **2** and 1 h for **3** and **4**. The solvent was then evaporated and the residue purified by column chromatography.

4-Isothiocyanobutyric Acid (2): eluent ethyl acetate: hexane = 1:1; yield 100 mg (69%); ^{1}H NMR ($CDCl_3$) δ 2.03 (q, J = 7.2, 2H, CH_2), 2.54 (t, J = 7.2, 2H, CH_2), 3.65 (t, J = 7.2, 2H, CH_2), 10.8 (bs, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 25.27, 31.06, 44.59, 179.05; IR (neat) cm^{-1} 1708, 2110, 2190; CIMS *m*/*z* 146 ([M + H]$^+$, 62), 128 (M − OH, 100), 87 (M − NCS, 50).

1-(2-Isothiocyanoethyl)piperidine (3): eluent ethyl acetate:hexane = 2:1; yield 82 mg (48%); ^{1}H NMR($CDCl_3$) δ 1.38−1.49 (m, 2H, CH_2), 1.5−1.62 (m, 4H, 2 × CH_2), 2.3−3.5 (m, 4H, 2 × CH_2), 2.64 (t, J = 6.6, 2H, CH_2), 3.59 (t, J = 6.6, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 24.53, 26.33, 43.49, 54.81, 58.42; IR (neat) cm^{-1} 2096, 2190, 2936; APCIMS *m*/*z* 171 ([M + H]$^+$, 100), 112 (M − NCS, 23).

***N*-(2-Isothiocyanoethyl)-*N*′-benzylpiperazine (4)** was prepared from the aminoethyl derivative **6** by the following sequence: *N*-benzyl-*N*′-cyanomethylpiperazine (**5**) was prepared by the method of Mull[22] from commercially available *N*-benzylpiperazine and chloroacetonitrile and purified by flash chromatography: ethyl acetate:hexane = 1:1; yield 8.9 g (72%); ^{1}H NMR ($CDCl_3$) δ 2.45−2.60 (bs, 4H, 2 × CH_2), 2.60−2.70 (bs, 4H, 2 × CH_2), 3.51 (s, 2H, CH_2), 3.52 (s, 2H, CH_2), 7.20−7.45 (m, 5H, ArH). This cyanomethyl derivative **5** was reduced to *N*-(2-aminoethyl)-*N*′-benzylpiperazine (**6**) by the method of Mull[22] using $LiAlH_4$: yield 6.2 g (70%); ^{1}H NMR ($CDCl_3$) δ 1.77 (s, 2H, NH), 2.40−2.65 (bs, 8H, 4 × CH_2), 2.42 (t, J = 6.3, 2H, CH_2), 2.79 (t, J = 6.3, 2H, CH_2), 3.51 (s, 2H, CH_2), 7.20−7.45 (m, 5H, ArH). The amine was converted to the isothiocyanate **4** by the general procedure described above: eluent ethyl acetate: hexane = 2:1; yield 150 mg (57%); ^{1}H NMR ($CDCl_3$) δ 2.42−2.60 (m, 8H, 4 × CH_2), 2.68 (t, J = 6.3, 2H, CH_2), 3.51 (s, 2H, CH_2), 3.58 (t, J = 6.3, 2H, CH_2), 7.20−7.35 (m, 5H, ArH); ^{13}C APT ($CDCl_3$) δ 43.40, 53.34, 53.38, 57.63, 63.40, 127.49, 128.64, 129.62; IR (neat) cm^{-1} 2103, 2190; APCIMS *m*/*z* 262 ([M + H]$^+$, 100).

C_{sat} Assay. C_{sat} assays were carried out as previously described[8] with the following modifications.[23] HbS·CO was oxygenated by slowly flushing O_2 into a round-bottom flask containing HbS·CO in an ice bath on a rotary evaporator while irradiated with a 150 W tungsten flood lamp. Complete oxygenation was confirmed by UV−visible spectroscopy using an extinction coefficient for the heme group of ϵ_{577nm} = 14.6 mM^{-1} cm^{-1}.[24] Modification of HbS·O_2 (1 mM final concentration; all Hb concentrations are in terms of tetramer) was carried out in 50 mM phosphate buffer at pH 7.3 with a 3:1 molar ratio of each isothiocyanate (1 mg/mL phosphate buffer), except for **6**, where dimethyl sulfoxide (less than 2% of final volume) was added, before adding to the Hb solution which was then incubated at 37 °C for 3 h.

Modified HbS·O_2 was separated from excess isothiocyanate by gel filtration using a Sephadex G-25 column (3 × 40 cm) preequilibrated with 50 mM phosphate buffer (pH 7.3) and concentrated by ultrafiltration in an Amicon (Bedford, MA) Centriflow Membrane Cone (MWCO = 2500) to 35% at 2500*g* in a Sorvall RC-5B centrifuge. At 4 °C, 250 μL of this HbS·O_2 was added to a 5 × 41 mm Beckmann Ultra-Clear centrifuge tube (Fullerton, CA) containing 80 μL of buffer (50 mM phosphate, pH 8.5) which was capped with a septum. Deoxygenation of HbS was initiated by addition of cold sodium dithionite solution (20 μL, 0.9 M) through the septum cap using a syringe (final HbS concentration = 25 g/dL). The tube was then incubated in a refrigerated circulation bath under anaerobic conditions for 2 h at 30 °C. The gelled sample (350 μL total volume) was centrifuged for 1 h at 20400*g* in a Beckman L8-70M

ultracentrifuge with a SW 55Ti rotor at 30 °C. After centrifugation, the supernatant was separated and converted to cyanomet-Hb with excess Drabkin's solution.[25] The concentration of cyanomet-Hb was determined spectrophotometrically with $\epsilon = 11\ mM^{-1}\ cm^{-1}$ at $\lambda_{max} = 540$ nm.[26]

Covalent Modification of Hb and MS Analyses. Modification of $HbS{\cdot}O_2$ (all Hb concentrations are in terms of the tetramer) was carried out in phosphate buffer (50 mM, pH 7.3) with each isothiocyanate in the library in a molar ratio of 0.4/1 with respect to HbS. $HbS{\cdot}O_2$ was added to a given isothiocyanate library, and the concentration of $HbS{\cdot}O_2$ was adjusted to 1 mM by adding buffer. The mixture was then incubated at 37 °C for 3 h. The modified $HbS{\cdot}O_2$ was purified by gel filtration using a Sephadex G-25 column preequilibrated with 50 mM phosphate buffer (pH 7.3) and concentrated by ultrafiltration in an Amicon Centriflow Membrane Cone to 35% at 2500*g* in a Sorvall RC-5B centrifuge. The modified HbS was then treated to dithionite reduction, and the supernatant and gel were separated as described above.

The supernatant was diluted with distilled water to about 2 mg/mL. The gel was dissolved with CO saturated, distilled water and also diluted with distilled water to about 2 mg/mL. Both the supernatant and gel solutions were then diluted with acetonitrile/water (1/1 = v/v) containing formic acid (0.2%) to a concentration of 0.5 μg/μL. The latter sample was then subjected to analysis using the Micromass Quattro II mass spectrometer: cone voltage = 30 V; source temperature = 70 °C; flow rate = 10 μL/min; capillary potential = 3.5 kV. The spectrometer was scanned from *m*/*z* 600−1300 in 10 s over a 3 min period. The mass scale was calibrated using horse heart myoglobin (Mr = 16 951.5).

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