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Quantitative determination of SH groups using ^{19}F NMR spectroscopy and disulfide of 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid

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A new method of measurement of thiol concentration by ^{19}F NMR spectroscopy is developed. The method is based on the detection of products of the exchange reaction of thiols with a newly synthesized fluorinated disulfide, 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid (BSSB). A significant broadening of the ^{19}F NMR signal of BSSB in the presence of thiols was observed and attributed to the exchange reaction between the parent disulfide and 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid. The rate constant for this reaction was found to be equal to $(63 \pm 11) \times 10^3$ M^{-1} s^{-1} at pH 7.0. The method was applied for the measurement of concentration of glutathione and albumin in rat blood. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: NMR; thiols; thiol measurements; ^{19}F; thiol-disulfide exchange; glutathione; fluorinated disulfides; benzoic acid

INTRODUCTION

The role of thiols in metabolic processes and in supporting intracellular and extracellular homeostasis *in vivo* is widely appreciated. Among low-molecular-weight thiols, glutathione, GSH, and cysteine are the most significant. Particularly GSH, presenting in various cells in mM concentration range, is considered to be the most important player in defining cellular 'redox state'.[1] The role of labile thiol groups in supporting the structure and function of proteins is also well documented. The main protein components of blood, hemoglobin, and albumin are important examples of macromolecular thiols, having in their structure two and one SH groups easily available for modification, correspondingly.[2–5]

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Currently existing methods of SH group registration (including photometric measurements using Ellman's reagent[6]) require optically transparent samples or prior isolation of SH-containing compounds[7–9] and, therefore, cannot be applied *in vivo*. Several years ago we proposed an electron paramagnetic resonance (EPR) spectroscopy approach for thiol detection, which has better potential for *in vivo* use.[10–12] The method is based on thiol–disulfide exchange reactions of stable biradical disulfides with thiols, which result in formation of monoradical products followed by significant changes in EPR spectrum. The possibility of working in nontransparent media and the high sensitivity of EPR are the most important advantages of this noninvasive technique. However biological reduction of the probes in EPR-silent products significantly limits the applications of the method. The development of fluorine-containing disulfides and application of the ^{19}F NMR technique for thiol detection overcomes the latter disadvantage of the EPR approach. Moreover, the NMR approach could enable imaging of SH-containing compounds in living organisms. In the present paper a fluorine-containing disulfide of 4-mercapto-2,3,5,6-tetrafluorobenzoic acid (BSSB) was synthesized. The mechanism of the reaction of BSSB with a number of thiols of biological importance was studied and applications of BSSB to measure SH compounds in rat blood is demonstrated.

EXPERIMENTAL

Chemicals

Disodium- and monopotassium phosphate, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), Chelex-100, glutathione (GSH), oxidized glutathione (GSSG), human serum albumin (ASH), and bovine hemoglobin (Hem) were obtained from Sigma. Deionized water was used for all experiments. Phosphate buffer was passed through a column with Chelex-100 to remove trace amounts of the transition-metal ions.

Synthesis of BSH and BSSB

4-Mercapto-2,3,5,6-tetrafluorobenzoic acid (BSH) was synthesized according to Ref. 13. 4,4′-Dithio-bis(2,3,5,6-tetrafluorobenzoic acid) (BSSB) was synthesized as follows: Solution of 0.51 g of bromine in 0.5 ml of acetic acid was added dropwise with stirring to a mixture of 1.36 g of the 4-mercapto-2,3,5,6-tetrafluorobenzoic acid and 4 ml of acetic acid. The reaction mixture was allowed to stand overnight. Then the solvent was partly removed (~80%). In this process, the product precipitated, and the precipitate was filtered off, washed with water, and dried to give 1.09 g of BSSB. The product was purified by sublimation at 170 °C (~1 mmHg), yielding about 80% purity. M.p. 211–213 °C. Found: m = 449.9254 $C_{14}H_2F_8O_4S_2$. Calculated: m = 449.9267.

NMR measurements

^{19}F NMR spectra were measured on a Bruker AVANCE 200 spectrometer in 5-mm tubes. All solutions were titrated to pH 7.0, and bubbled by Ar for 10 min before mixing and measurements. All samples contained 10% D_2O used as a lock. Standard $\pi/2$ pulse sequence with ^1H decoupling was used. Standard settings were as follows: number of scans, 32–512; sweep width, 30 ppm; LB = 0–10 Hz; acquisition time, 1.5 s; delay between scans, 5 s. Linewidths, LW, of individual ^{19}F NMR spectra lines of BSSB and BSH compounds were determined by fitting calculated spectra to the experimental ones using NUTS95 (Acorn NMR) program package.

Preparation of blood plasma and erythrocytes for determination of SH compounds

Wistar rats (male, 100 g) were obtained from Institute of Cytology and Genetics (Novosibirsk, Russia). Samples were prepared using a protocol described earlier with slight modifications.[11] Rats were anesthetized by diethylether. Blood was collected from the neck after injection of 6 μl of heparine sodium (5000 I.E./ml). About 5 ml of the blood was mixed with 250 μl of 0.5 M EDTA and centrifuged for 10 min at 700 g at 4 °C. Supernatant (plasma) was collected and used without further preparation. Sedimentated erythrocytes were frozen in liquid nitrogen. After defreezing, the sample was diluted by a phosphate buffer, pH 7.0, up to initial blood volume. 1.5 ml of the obtained mixture was added to 0.15 ml of 50% (w/v) TCA. The samples were centrifuged at 2500 g for 10 min and the supernatant was adjusted to pH 7.0 and used to determine glutathione concentration.

RESULTS

Thiol–disulfide exchange reactions of BSSB with physiologically relevant thiols

Fluorinated disulfide of benzoic acid, BSSB, was synthesized as shown in Fig. 1. It was expected that reaction of the BSSB with thiols would result in the splitting of the disulfide bond and formation of the corresponding monothiol, fluorinated benzoic acid, BSH.

Figure 2a shows the ^{19}F NMR spectrum of the disulfide, BSSB, in deuterated dimethylsulfoxide. The spectral pattern is characteristic for an AA′BB′ system[14] in agreement with the BSSB structure with nonequivalent fluorine nuclei. The values of spin–spin interaction constants and chemical shifts obtained by fitting the experimental spectra to calculated ones are listed in Table 1 and Fig. 2 captions. Note that the values of chemical shift strongly depend on the solvent and pH. The multiplet structure of the ^{19}F NMR spectrum of the BSSB in water was not observed because of line broadening. For spectra simulations, the parameters of spin–spin coupling obtained in DMSO were used.

Figure 3 shows the ^{19}F NMR spectra of BSSB in 0.1 M phosphate buffer, pH 7.0, measured before and after GSH addition. The appearance of the signals of two new products at 26.1 and 16.0 ppm (product P_1), and 31.2 and 20.43 ppm (product P_2) is clearly seen in Fig. 3b and c. Significant broadening of BSSB signal is also observed. It should be mentioned that the initial BSSB signal (Fig. 3a) was already broad compared with that in Fig. 2a. Linewidth of the product P_1 also depends on concentration of GSH and significantly decreases under high concentration of added glutathione (Fig. 3d). The signal of the product P_2 consists of two multiplets with well-resolved structure. Their LWs were not affected by variation in GSH concentration. In the excess of GSH, only the product, P_2, was observed (Fig. 3d).

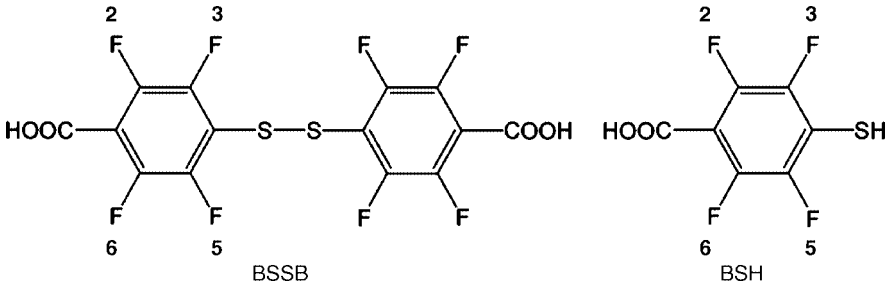

Figure 1. Chemical structures of the fluorinated compounds, BSSB and BSH.

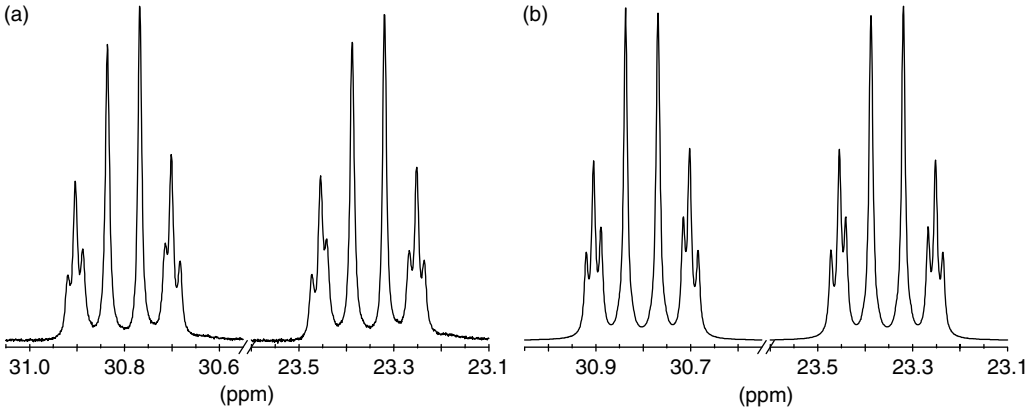

Figure 2. (a) ^{19}F NMR spectrum of the solution of the BSSB in DMSO-δ_6. (b) Calculated spectrum with parameters J_{ij} listed in Table 1. Values of chemical shifts of the signals of BSSB in DMSO-δ_6 are $\sigma(F^2) = \sigma(F^6) = 30.8$ ppm, $\sigma(F^3) = \sigma(F^5) = 23.3$ ppm relative to signal of C_6H_6.

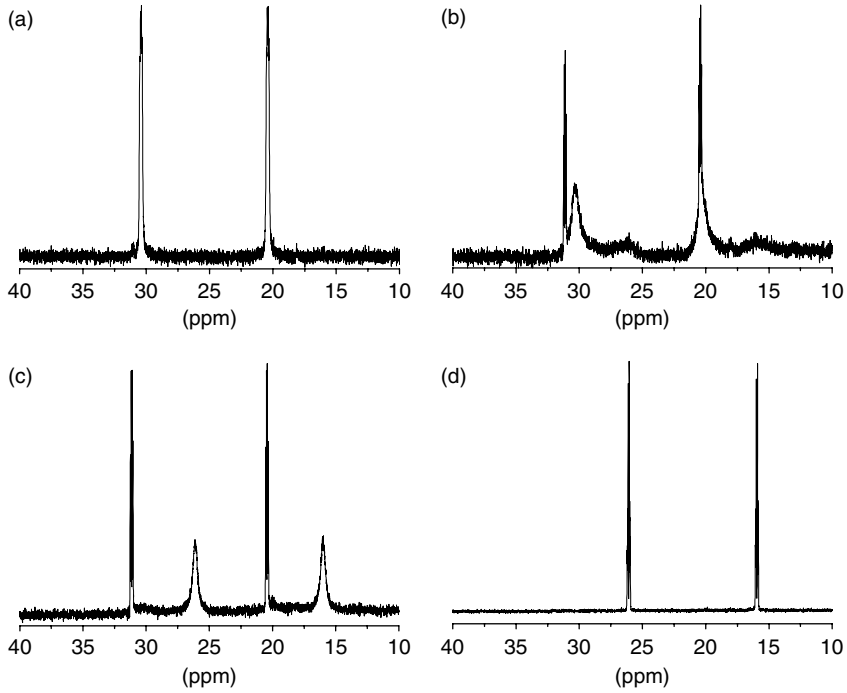

Figure 3. ^{19}F NMR spectra of 5-mM BSSB solution in 0.1 M phosphate buffer pH 7.0 measured before (a) and after addition of glutathione in final concentration 2 mM (b), 5 mM (c), and 10 mM (d). Number of scans is 32 for each spectrum.

Table 1. ^{19}F NMR spectra parameters of BSSB and BSH in DMSO and phosphate buffer ([a])

	BSSB				BSH			
$J_{Fi,Fj}$, Hz,	F_2	F_3	F_5	F_6	F_2	F_3	F_5	F_6
F_2		25	−12.6	−2.9		26.5[a]	−11.2[a]	−5.8[a]
F_3			−2.9	−12.6			−2.2[a]	−11.2[a]
F_5				25.0				26.6[a]
Chemical shifts	30.8	23.3	23.3	30.8	25.8	17.2	17.2	25.8
	30.4[a]	20.4[a]	20.4[a]	30.4[a]	26.1[a]	16.0[a]	16.0[a]	26.1[a]

[a] Data obtained in 0.1 M phosphate buffer, pH 7.0

The comparison of the spectrum shown in Fig. 3d with the spectrum of separately synthesized fluorinated benzoic acid, BSH, allows assigning the product P_1 to tetrafluoro-4-mercaptobenzoic acid (Fig. 1). The corresponding spectral parameters of this compound are listed in Table 1.

Figure 4 shows the dependence of concentration of the disulfide BSSB and its products, P_1 and P_2, on GSH concentration. The measurements were performed in 0.1-M phosphate buffer, pH 7.0, and concentrations were calculated using integral intensity of ^{19}F NMR signals of

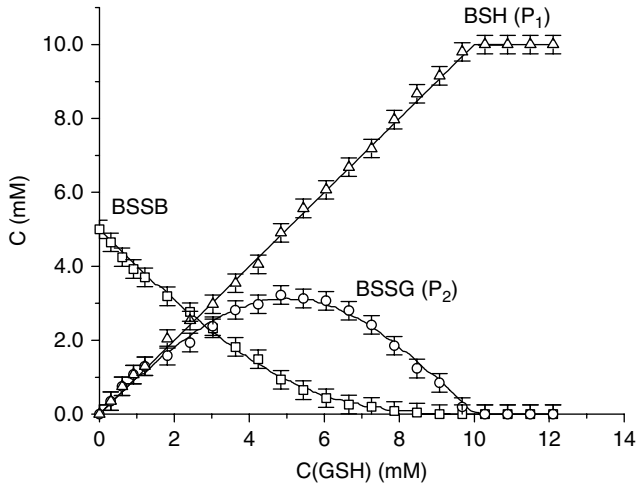

Figure 4. Dependence of the concentrations of the disulfide, BSSB (□), and its products, BSH (P_1, △), and BSSG (P_2, ○) formed in the reaction with glutathione, on GSH concentration. GSH was added to 5 mM solution of BSSB in 0.1 M phosphate buffer, pH 7.0, and NMR spectra were registered afterwards. Number of scans was 32 for each spectrum. The concentrations were calculated using integral intensities of the corresponding NMR signals. Solid lines correspond to numerous calculations using Eqns (3–7) with parameters $K_1, K_2 > 10^3$; $K_1/K_2 = 11.6$.

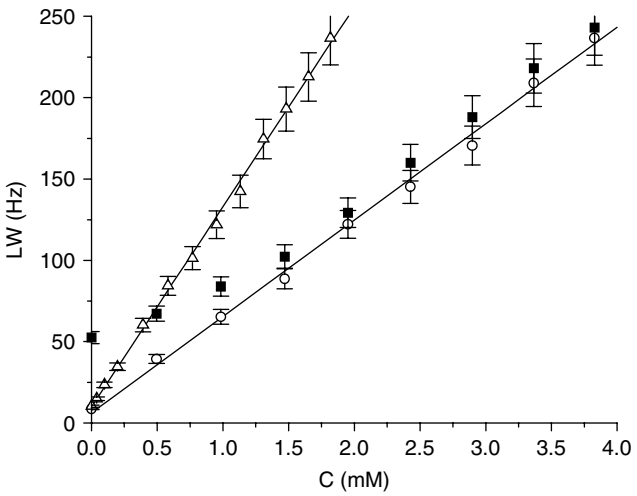

Figure 5. Dependence of linewidth, LW, of the individual ^{19}F NMR line of BSSB (○) and BSH (△) on concentration of added BSH and BSSB. BSH (BSSB) was added to a 5-mM solution of BSSB (BSH) in 0.1 M phosphate buffer, pH 7.0, and ^{19}F NMR spectra were measured. LW values were determined by fitting the calculated spectra using parameters listed in Table 1 to the experimental spectra yielding LW (*Experimental*). Solid lines correspond to linear approximation $LW_i = A_i + B_i \times C$ with parameters $A_{BSH} = 9.95 \pm 1.52$ Hz, $B_{BSH} = (123 \pm 1.5) \times 10^3$ Hz $\times$ M^{-1}; $A_{BSSB} = 6.27 \pm 4.2$ Hz, $B_{BSSB} = (59.2 \pm 0.85) \times 10^3$ Hz $\times$ M^{-1}. (■) – Dependence of linewidth of whole multiplet signal of BSSB on the concentration of BSH added.

the corresponding compounds. It is clearly seen that the concentration of formed BSH is equal to the concentration of added GSH in each experiment. The solid lines correspond to the best fit of the Eqns (3–7) to the experimental data (*Discussion* for details).

The line broadening of the NMR signals of BSSB and BSH might originate from the thiol–disulfide exchange between these two compounds. To test this hypothesis, the ^{19}F NMR spectra of a mixture of these compounds in phosphate buffer were measured. It was found that linewidth of each of the compounds is proportional to the concentration of other compound (Fig. 5). Linewidth of the whole multiplet became proportional to the concentration of added reagent only when its concentration was higher than 1.5 mM. The twofold difference in the slopes of the relationships should be noted, apparently attributed to the difference in stoichiometry of the corresponding thiol–disulfide exchange reactions (*Discussion*).

Figure 6 shows ^{19}F NMR spectra of 5 mM BSSB with 0.67 mM albumin (ASH; Fig. 6a) or 0.85 mM hemoglobin (Hem; Fig. 6b) in 0.1 M phosphate buffer, pH 7.0. An appearance of two new signals at 15.9 and 26.1 ppm is clearly seen. Moreover the additional new signal at 18.0 ppm appears in the case of hemoglobin addition. Spectra lines of all components are significantly broadened. The ratio of intensities of the signals of BSSB and product formed can be measured only in the case of addition of albumin and is equal to 14.4 ± 1.5 (Fig. 6a). An accurate determination of the corresponding ratio in the experiment with hemoglobin (Fig. 6b) is hardly possible due to strong line broadening and spectra overlapping.

The LW of individual lines of BSSB were determined by fitting the calculated spectra (*Experimental*) using spectral parameters listed in Table 1 to the experimental spectra. The obtained dependencies of LW of BSSB on concentration of added proteins is shown in Fig. 7. The data are in a good agreement with a linear approximation, $LW_{BSSB} = A_i + A_i \times C_i$ (i = ASH, Hem, GSH) with parameters A_i and B_i being specified in the caption. The slopes of the lines increase in the series ASH >Hem > GSH.

Using BSSB for the determination of concentration of SH groups in rat blood

Figure 8a, b shows ^{19}F NMR spectra measured after addition of BSSB solution to cellular media of erythrocytes and blood plasma, correspondingly. An ^{19}F NMR spectral multiplet line of a new product in the region of 31 ppm is clearly seen in Fig. 8a. The second multiplet line of the product overlaps with broad line of BSSB at 20.4 ppm. On the basis of previously discussed data, this product was assigned to BSSG (*Discussion*). Broad lines of formed BSH are also clearly seen. The ratio of integral intensities, BSSG/BSSB, is equal to 0.097 ± 0.024. Linewidth for individual line of BSSB was found to be equal to 33 Hz.

Addition of blood plasma to the solution of BSSB widens NMR lines of the latter one to 94 Hz (Fig. 8b). The ratio of integral intensities of BSH and BSSB on the spectrum can be estimated as 0.054 ± 0.014. Taking into account that albumin, ASH, is the main SH-containing compound in the

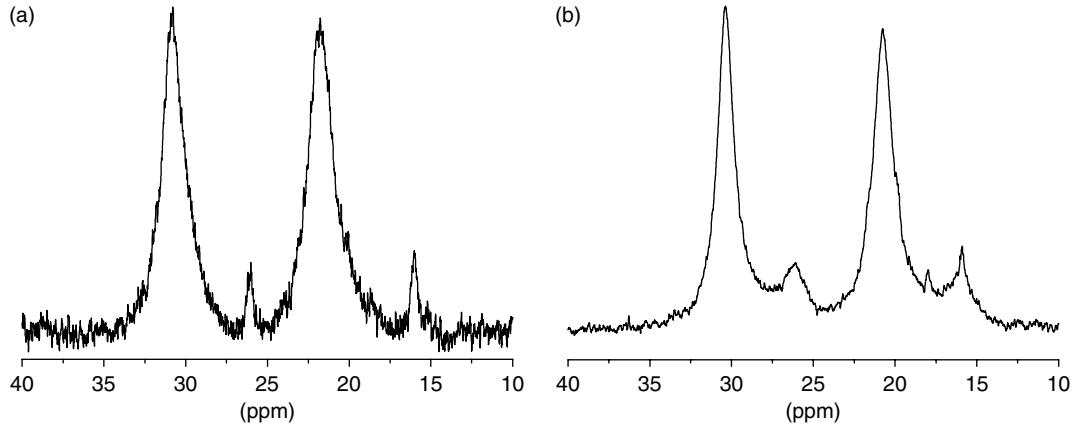

Figure 6. ^{19}F NMR spectra of 5-mM solution of BSSB in 0.1 M phosphate buffer, pH 7.0, measured after addition of 0.67 mM albumin (a) and 0.85 mM hemoglobin (b). Number of scans is 64 in (a) and 256 in (b).

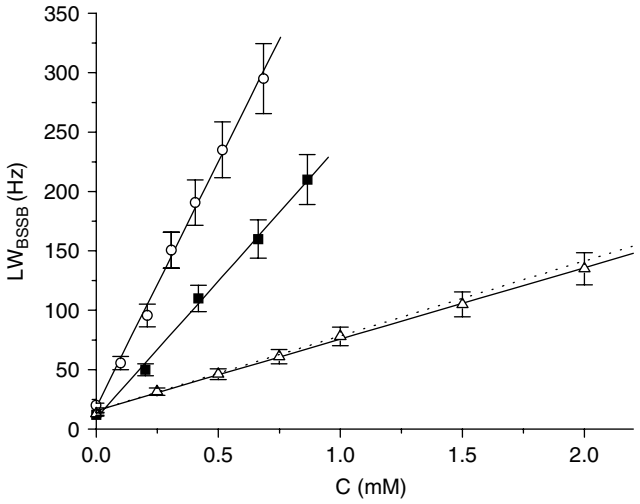

Figure 7. Dependence of LW of ^{19}F NMR line of BSSB on concentration of added albumin (ASH, ○), hemoglobin (Hem, ■), and glutathione (GSH, △). ASH and Hem were added to a 5-mM solution of BSSB in 0.1 M phosphate buffer pH 7.0, and NMR spectra were measured. Experimental data shown in Fig. 4 were used to plot dependence for glutathione (△). Solid lines correspond to linear approximation of the experimental data, $LW_{BSSB} = A_i + B_i \times C_i$ where $A_{ASH} = 18.5 \pm 4.0$ Hz, $B_{ASH} = (412 \pm 10) \times 10^3$ Hz $\times$ M^{-1}; $A_{Hem} = 9.35 \pm 3.6$ Hz, $B_{Hem} = (231 \pm 6.7) \times 10^3$ Hz $\times$ M^{-1}; $A_{GSH} = 15.5 \pm 1.1$ Hz, $B_{GSH} = (60.2 \pm 0.98) \times 10^3$ Hz $\times$ M^{-1}. Dotted line corresponds to straight line $LW_{BSSB} = A_{GSH} + k \times$ [GSH] with $k = 63 \times 10^3$ Hz $\times$ M^{-1}.

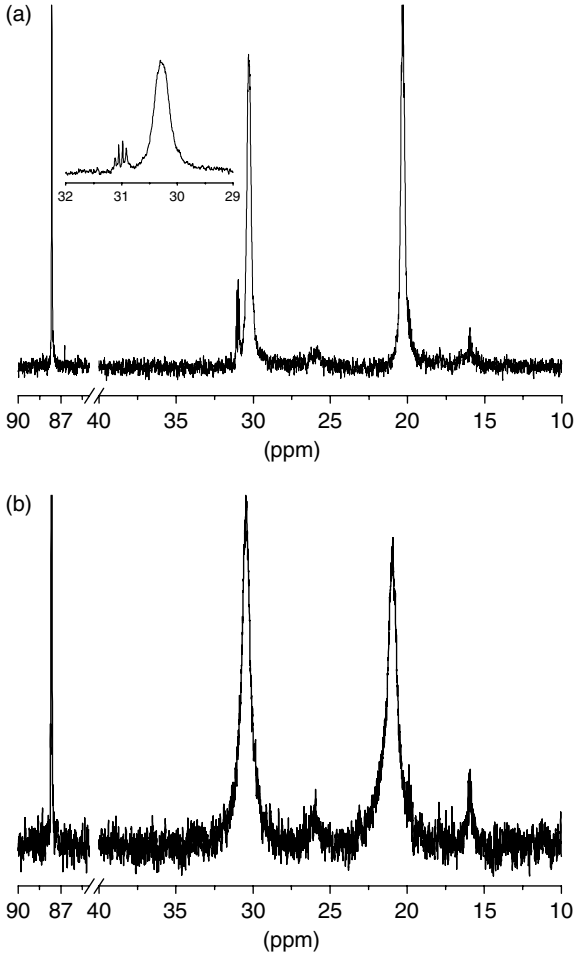

Figure 8. ^{19}F NMR spectra of BSSB solution measured after its addition to cellular media of erythrocytes (a), and blood plasma (b) from wistar rats (*Experimental*). 500 μl of 5-mM solution of BSSB in 0.1 M phosphate buffer, pH 7.0, 1.25 mM CF_3COOH, was mixed with 100 μl of D_2O and 400 μl of the cellular media (a) or blood plasma (b). Number of scans was 64 for each spectrum. Signal at 87.65 ddm corresponds to anion of trifluoroacetic acid and was used as a reference.

blood plasma (5) these data can be used for the determination of ASH concentration.

Using all obtained data and taking into account that the initial linewidth of BSSB before addition of thiol is 11 Hz (data not shown), the concentration of GSH and albumin can be determined. Independently, it can be done from integral intensities of the signals and from an analysis of the broadening of BSSB NMR lines. The obtained results are listed in the Table 2.

Line broadening of BSSB due to the exchange reaction (8) (*Discussion*) with a measured rate constant, $k = 63 \times 10^3$ M^{-1} s^{-1}, was used for the determination of concentration of GSH. Data presented in Fig. 7 were used as calibration

Table 2. Concentration of ASH in blood plasma, and GSH in erythrocyte and whole rat blood determined by ^{19}F NMR approach using BSSB

	From integral intensities	From linewidth	Literature data
[GSH] in erythrocytes, mM	1.86 ± 0.26	1.74 ± 0.28	1.9 ± 0.3[11]
[GSH] in blood, mM[a]	0.93 ± 0.23	0.87 ± 0.14	0.95 ± 0.2[11]
[ASH] in blood plasma, mM	0.56 ± 0.14	0.5 ± 0.8	0.61 ± 0.15[5]

[a] Numbers were calculated taking into account experimentally determined fraction of erythrocytes in the blood samples.

curve for the determination of albumin concentration. Results presented in Table 2 are in good agreement with literature data. This demonstrates the applicability of the method for the determination of thiols *ex vivo*.

DISCUSSION

Reaction mechanism

Thiol–disulfide exchange reaction between the disulfide, BSSB, and the thiol, GSH, results in the formation of a monothiol, BSH, mixed disulfide, BSSG, and disulfide GSSG, according to the reactions:

$$\mathrm{BSSB} + \mathrm{GSH} \xleftrightarrow{K_1} \mathrm{BSSG} + \mathrm{BSH} \quad (1)$$

$$\mathrm{BSSG} + \mathrm{GSH} \xleftrightarrow{K_2} \mathrm{GSSG} + \mathrm{BSH} \quad (2)$$

where K_1 and K_2 are the equilibrium constants at pH 7.0. According to these reactions, the ^{19}F NMR multiplet signals of the product P_2 at 31.2 and 20.43 ppm (Fig. 3) should be assigned to the mixed disulfide BSSG.

The equilibrium concentrations of the compounds [BSSB], [BSH], [BSSG], [GSH] and [GSSG] are described by the following equations:

$$K_1 = \frac{[\mathrm{BSSG}][\mathrm{BSH}]}{[\mathrm{BSSB}][\mathrm{GSH}]} \quad (3)$$

$$K_2 = \frac{[\mathrm{GSSG}][\mathrm{BSH}]}{[\mathrm{BSSG}][\mathrm{GSH}]} \quad (4)$$

According to these equations, the equilibrium concentrations of BSSB and BSSG are inversely proportional to the concentration of glutathione, in qualitative agreement with the data shown in Fig. 3.

Note that the BSSB molecule has two fluorine-containing aryl groups while molecules of BSSG and BSH have only one aryl group. The corresponding balance equations are:

$$2[\mathrm{BSSB}]_0 = 2[\mathrm{BSSB}] + [\mathrm{BSSG}] + [\mathrm{BSH}] \quad (5)$$

$$[\mathrm{GSH}]_0 = [\mathrm{GSH}] + [\mathrm{BSSG}] + 2[\mathrm{GSSG}] \quad (6)$$

$$[\mathrm{BSSB}]_0 = [\mathrm{BSSB}] + [\mathrm{BSSG}] + [\mathrm{GSSG}] \quad (7)$$

where $[\mathrm{BSSB}]_0$ and $[\mathrm{GSH}]_0$ are the initial concentrations of BSSB and GSH, correspondingly. The system of Eqns (3–7) cannot be solved analytically. Fitting numerical calculations using Eqns (3–7) with varying parameters K_1 and K_2 to the experimental data (Fig. 4) yields $K_1/K_2 = 11.6$ and $K_1, K_2 > 10^3$. Note, that equimolarity of BSH and BSSG formed at low concentration of added GSH is explained by a ~10-fold higher value of K_1 in comparison with K_2. Large values of K_1 and K_2 are characteristic for thiol–disulfide exchange reactions of aryldisulfides with low-molecular-weight thiols (e.g. the corresponding rate constant for 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent, with GSH is 1×10^3)[7], and apparently are explained by the influence of aryl fragment.

Line broadening

Linear dependence of linewidth of the signals of BSSB and BSH on concentrations of added BSH and BSSB (Fig. 5) supports the existence of exchange reactions between these compounds.

$$\mathrm{BSSB} + \mathrm{BSH} \xleftrightarrow{k} \mathrm{BSH} + \mathrm{BSSB} \quad (8)$$

In the case of the intermediate exchange between several positions, the linewidth of ith NMR line, LW_i, can be calculated in accordance with equation:[15]

$$\mathrm{LW}_i = \frac{2}{T_{2obs}} = \frac{2}{T_2^0} + \frac{2}{\tau_i} \quad (9)$$

where τ_i – the average lifetime in the ith state, T_2^0 and T_{2obs} are intrinsic and observed time of spin–spin relaxation, correspondingly. Taking into account that one molecule of BSSB has two exchangeable fluorine-containing aryl groups while BSH has only one group, the following equations for the LWs of NMR signals of these compounds are obtained:

$$\mathrm{LW}_{\mathrm{BSSB}} = \mathrm{LW}_{0\mathrm{BSSB}} + k[\mathrm{BSH}] \quad (10)$$

$$\mathrm{LW}_{\mathrm{BSH}} = \mathrm{LW}_{0\mathrm{BSH}} + 2k[\mathrm{BSSB}] \quad (11)$$

where $\mathrm{LW}_{\mathrm{BSSB}}$, $\mathrm{LW}_{0\mathrm{BSSB}}$, and $\mathrm{LW}_{\mathrm{BSH}}$, $\mathrm{LW}_{0\mathrm{BSH}}$ are current and initial LWs of the NMR signal of BSSB and BSH compounds, correspondingly. These equations explain experimental linear dependencies for $\mathrm{LW}_{\mathrm{BSH}}$ and $\mathrm{LW}_{\mathrm{BSSB}}$ on [BSSB] and [BSH], correspondingly (Fig. 5) with the value of rate constant of exchange reaction (8) being equal to $k = (63 \pm 11) \times 10^3\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. Moreover, they explain the twofold larger slope in the dependence of linewidth of BSH compared to that of BSSB.

Linewidth of ^{19}F NMR lines of BSSG is comparatively small and does not depend on concentrations of BSSB and BSH (Fig. 3). It can be explained by the low values

of rate constants of thiol–disulfide exchange of BSSG with thiols. Indeed, it was found that addition of 20 mM of GSSG to 5 mM of BSH does not significantly change the shape of NMR ^{19}F spectrum during half an hour. Nevertheless, the appearance of a low signal of BSSG (5% from added BSH, data not shown) was observed after five hours of incubation. Therefore, the value of rate constant of the reverse reaction (2) can be estimated to be in the range 1.4×10^{-3}–1.4×10^{-4} M^{-1} c^{-1}. Taking into account the obtained estimations of the equilibrium constants K_1 and K_2, the value of rate constant of the direct reaction (2) should be about 1.4–14 M^{-1} c^{-1}. This latter value is in the range of literature data for the rate constants for the thiol–disulfide exchange reactions.[7] On the basis of the obtained data, it should be expected that the NMR lines of BSSB broaden after addition of GSH, due to exchange reaction (8) with BSH formed. The calculations of the dependence of the ^{19}F NMR linewidth of BSSB on GSH concentration using a rate constant of the reaction (8) $k = 63$ $M^{-1}c^{-1}$ are in excellent agreement with experimental data (Fig. 7).

Moreover, using the obtained value for the rate constant of the exchange reaction (8) and Eqn (10) it is easy to show that linewidth of the BSSB signals under a BSH concentration of 1.5 mM should be about 100 Hz. This value is 10 times higher than the linewidth of BSSB before addition of BSH and twice the width of the whole multiplet signal of BSSB (~50 Hz, Fig. 3). These estimations explain the experimentally observed linear dependence of the width of whole multiplet signal of BSSB on BSH concentration when [BSH] >1.5 mM (Fig. 5).

Initial broadening of NMR ^{19}F spectral lines of BSSB (LW_{0BSSB}) and BSH (LW_{0BSH}) can be explained by the presence of BSH and BSSB, respectively, in their solutions. The presence of BSSB in BSH solutions is easily explained by oxidation of BSH by oxygen in the environment. Slight concentrations of BSH in the solution of BSSB can originate from hydrolysis of the latter.

The method also allows the measurement of thiol groups within proteins or other macromolecules. However, an interpretation of the NMR data in this case may be more complex and less informative as was demonstrated for the case of hemoglobin and albumin. Bovine hemoglobin is a quaternary-structure protein containing two pairs of polypeptide chains and four groups of heme. This structure contains two cysteine residues easily available for thiol–disulfide exchange.[2–4] Therefore, at least three different products can be formed in the reaction of Hem with BSSB. However, in the case of complete modification of the SH groups of hemoglobin, the formation of a single double disulfide is expected.

$$Hem(SH)_2 + 2BSSB \longleftrightarrow Hem(SSB)_2 + 2BSH \qquad (12)$$

NMR ^{19}F lines of this double disulfide should overlap with broad lines of BSSB (Fig. 6b). While the signal at 18.00 ppm can be attributed to one of the NMR lines of the product, the exact analysis of the spectrum presented in Fig. 6b is complicated because of line broadening.

Albumin, ASH, is a protein with molecular weight about 65 kDa, which contains one available SH group.[5] Therefore, the formation of one additional product in the reaction of ASH with BSSB is expected:

$$BSSB + ASH \longleftrightarrow BSSA + BSH \qquad (13)$$

The formation of the disulfide of albumin as well as the disulfide of hemoglobin is sterically hindered because of the high molecular weights of the reagents.

NMR spectral lines of BSSA should overlap with broad lines of BSSB (Fig. 6a). Therefore the expected ratio of the integrated intensities of experimental NMR ^{19}F lines of BSSB and BSH for addition of 0.67 mM of ASH to 5 mM BSSB solution under complete modification of albumin (13) should be $(2 \times (5 - 0.67) + 0.67)/0.67 = 13.9$ which is in a good agreement with experimentally obtained value, 14.4 ± 1.5 (Fig. 6a).

From the number of available SH groups, the expected line broadening of ^{19}F spectral lines of BSSB due to the exchange reaction (8) after addition of ASH should be comparable with that observed after addition of GSH and about two times higher after addition of Hem. However, as is seen from Fig. 7, the real slopes in these cases are seven and four times larger, respectively. Moreover, the linewidth of formed BSH at concentrations of BSSB larger than 4 mM after addition of ASH to BSSB is equal to 75 Hz instead of expected 281 Hz (Eqn 11). These facts cannot be explained by an intermediate exchange reaction model and are the subjects of ongoing investigation.

The application of the approach for thiols detection in rat blood shows good agreement with literature data, and, therefore, demonstrates the applicability of the method for the determination of thiols *ex vivo*.

CONCLUSIONS

The technique developed for thiol determination described here is based on thiol–disulfide exchange between the newly synthesized disulfide compound, BSSB, and thiols. Sensitivity of linewidth of BSSB to the concentration of SH compound allows determination of 50 μmol l^{-1} of thiol (corresponding to a linewidth broadening of 3 Hz) which is slightly lower than the sensitivity of EPR-based technique using biradical disulfides.[10,11] However, the ^{19}F NMR spectrum allows for an unambiguous conclusion about the structure of the presented thiol (Fig. 8a). Stability of the formed diamagnetic compounds in the reducing cellular environment provides a significant advantage of NMR approach over EPR detection.[16] The NMR technique also can be performed in nontransparent samples, which is not possible using standard optical methods.[6–9] The rate constants for the thiol–disulfide exchange reactions of the BSSB with thiols studied are too high for the kinetics to be detected. Therefore an application of an excess of BSSB over the thiols is required for thiols detection using measurement of the equilibrium, BSSB/BSH. This significantly limits the number of biological applications of the proposed technique using this particular fluorinated disulfide probe. Measurement of the line broadening of BSSB for the thiol detection is an alternative approach that

has certain advantages in the case of line-overlapping and complexity of integration of the observed signals.

Synthesis of fluorinated aliphatic disulfides may overcome some of the mentioned problems. These compounds are characterized by the rate and equilibrium constants for thiol–disulfide exchange reaction lying in the range 0.3–1.0 M^{-1} s^{-1}, respectively.[7] It may allow the use of lower disulfide concentrations and registering the kinetics of the exchange reactions.

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