Fluorescence of sanguinarine: spectral changes on interaction with amino acids†

Marika Janovská,[a] Martin Kubala,*[a] Vilím Šimánek[b] and Jitka Ulrichová[b]

Received 8th December 2009, Accepted 3rd June 2010
DOI: 10.1039/b925828k

The quaternary isoquinoline alkaloid, sanguinarine (SG) exhibits a wide range of biological activities. This study examines spectral changes expected from SG binding to proteins. Fluorescence spectra of the cationic form of sanguinarine (SG^+) are sensitive to environment polarity. On the other hand, spectra of the neutral form of sanguinarine, pseudobase (SGOH) and dihydrosanguinarine (DHSG, the first metabolite of SG) exhibit higher sensitivity to the ability of solvent to form a solute-to-solvent hydrogen bonds. Interaction with cysteine has been the only mode of SG binding to enzymes that has been considered so far. In reality, our experiments have revealed spectral changes on specific interactions of SG^+ with Cys, Glu and Tyr in the protic environment and with Arg and Glu in the aprotic environment. We have also detected interactions of SGOH with Cys in the protic environment and with Cys, Glu and Lys in the aprotic environment. The DHSG spectra were only altered in the presence of the Cys analog in the protic environment. We have also demonstrated that spectral change analysis can aid investigation of SG/DHSG interactions with proteins and we were able to identify SG^+-binding site on Na^+/K^+-ATPase.

Introduction

Sanguinarine (SG), is a quaternary benzo[*c*]phenanthridine alkaloid (QBA) isolated from *Chelidonium majus*, *Macleaya cordata* and *Sanguinaria canadensis*. It displays a plethora of biological activities, *e.g.* antimicrobial, anti-inflammatory, adrenolytic and sympatholytic. It can also block proliferation and induce apoptosis in different mammalian normal and tumor cells.[1,2] SG is used in dental hygiene preparations, feed additives and veterinary drugs.[2] On the other hand, SG is suspected of being the toxic component of argemone oil, responsible for the epidemic dropsy syndrome in humans.[3]

However, the mechanism of SG action is not fully understood at a molecular level. A large number of studies demonstrate that SG can inhibit a wide range of enzymes *e.g.* aminotransferases[4] lipoxygenase,[5] cholinesterases,[6] Na^+/K^+-ATPase[7] and Ca^{2+}-ATPase in skeletal muscle,[8] and interact with albumin.[9] The iminium bond of SG is susceptible to nucleophilic attack, forming covalent bonds with SH groups and inhibiting SH-containing proteins,[10,11] and it has been proposed that the free –SH group of cysteinyl residues may be the site of SG interaction with enzymes.

In aqueous solution, sanguinarine is in equilibrium between its charged quaternary form (SG^+) and neutral form, pseudobase (SGOH) with $pK_A = 8.06$.[10,12,13] The reduced form, dihydrosanguinarine (DHSG) has been identified as a SG metabolite in plasma and liver (Fig. 1). The formation of DHSG might be the first step in SG detoxification in mammals.[14,15]

In a previous study,[12] we described the basic steady-state and time-resolved fluorescence characteristics of SG^+, SGOH and DHSG. At physiological pH, there is a dynamic equilibrium between SG^+ and SGOH, and in fact, these two forms can never be separated. However a knowledge of their spectral properties allows us to observe them separately. Thus, setting the excitation to 327 nm (or emission to 418 nm), we can observe SGOH alone, while SG^+ is "invisible", and *vice versa*, by setting the excitation to 475 nm (or emission to 590 nm), we can observe only SG^+, while SGOH remains "invisible".

In this study we monitored the fine changes in the fluorescence spectra of SG^+, SGOH and DHSG caused by interactions that are expected from QBA–enzyme complex formation. We

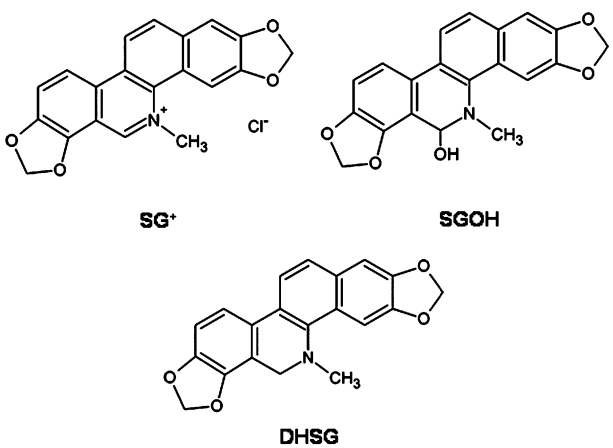

Fig. 1 Structures of sanguinarine chloride (SG^+), sanguinarine pseudobase (SGOH) and dihydrosanguinarine (DHSG).

[a] *Laboratory of Biophysics, Faculty of Sciences, Palacky University, tr. 17. listopadu 12, CZ-77146 Olomouc, Czech Republic. E-mail: mkubala@prfnw.upol.cz; Fax: +420-585634002; Tel: +420-585634179*
[b] *Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic*

† Electronic supplementary information (ESI) available: Excitation and emission spectra. See DOI: 10.1039/b925828k

evaluated (i) the general effect of polarity environment (which is predicted to decrease upon binding from aqueous solvent to protein) and (ii) the influence of amino acids reactive groups. We demonstrated that precise analysis of the fluorescence spectra can provide valuable structural information about the SG/enzyme interaction and we were able to identify the SG^+-binding site on the Na^+/K^+-ATPase.

Experimental

Chemicals

Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo-[4,5-*i*]phenanthridinium chloride) and NADH were purchased from Sigma-Aldrich (Prague, Czech Republic). Dihydrosanguinarine (13,14-dihydro-13-methyl[1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*]phenanthridine, DHSG), 99% purity, MP 189–191 °C was prepared from sanguinarine by reaction with $NaBH_4$ in methanol.[16]

Effect of polarity on the fluorescence spectra of SG^+, SGOH and DHSG was evaluated in chloroform, butanol, acetone, ethanol, methanol, DMSO, 2-butanethiol, 1,2-ethanedithiol, and aqueous Tris buffer solution, pH 7.5. All the solvents were from Sigma and were of spectrophotometric grade.

Neutral amino acid analogues Ac-Ser-OMe, Ac-Tyr-NH_2, Ac-Glu-NH_2, Ac-Gln-NH_2, Ac-Lys-NH_2·HCl, Ac-Arg-NH_2-acetate salt, where Ac stands for acetyl and Me for methyl, were supplied by Bachem and Ac-Cys-OMe was supplied by Fluka.

Excitation and emission spectra

The steady-state fluorescence emission spectra of 10 μM SG or 5 μM DHSG were measured on a spectrofluorimeter F4500 (Hitachi, Japan). Data were collected using excitation wavelength 327 nm or 475 nm for SG, and 327 nm for DHSG, with a scan-speed 240 nm min^{-1}. Slits were set to 10 nm for both the excitation and emission channel, respectively. Spectra were collected in 0.2 nm steps and measurements were performed at 295 K.

The spectra were measured in 100% solutions of chloroform, butanol, acetone, ethyl alcohol, methanol, DMSO, 1-butanethiol, 1,2-ethanedithiol and aqueous buffer solution (pH 7.5) and 10% solution of 1-butanethiol and 1,2-ethanedithiol in 20 mM Tris buffer, pH 7.5. Further, a 1 mM solution of the neutral analogs of amino acids Ser, Tyr, Glu, Gln, Lys, Arg and Cys in Tris buffer, pH 7.5 were used to test the influence of the functional groups found within proteins. The measurement was performed in freshly prepared solutions of amino acid analogs. The experiment with Cys analog was done in a buffer that was deoxygenized by nitrogen to avoid oxidation.

Solvent effects are usually evaluated in the wavenumber scale. Therefore, the spectra were converted into the wavenumber scale using:

$$I(\bar{\nu}) = I(\lambda)\cdot\lambda^2 \quad (1)$$

where λ is wavelength, $\bar{\nu}$ is corresponding wavenumber and I is fluorescence intensity. The maximum of the peak $\bar{\nu}_{max}$ was determined after smoothing by averaging over 10 adjacent points. The solvent effects were evaluated by plotting of $\bar{\nu}_{max}$ *vs.* ε_r (relative static permittivity), using the Lippert plot $(\bar{\nu}_{ex} - \bar{\nu}_{em})$ *vs.* Δf, where $\Delta f = (\varepsilon_r - 1)/(2\varepsilon_r + 1) - (n^2 - 1)/(2n^2 + 1)$,[17] or the Kamlet–Taft solvatochromic analysis:[18,19]

$$V = \bar{\nu}_0 + a\alpha + b\beta + p\pi^* \quad (2)$$

where V is the evaluated spectral characteristic (in our case $\bar{\nu}_{ex}$, $\bar{\nu}_{em}$ or $\bar{\nu}_{ex}$-$\bar{\nu}_{em}$), $\bar{\nu}_0$, a, b and p are the fitted parameters, the values of α (describing the ability of the solvent to donate a proton in a solvent-to-solute hydrogen bond), β (describing the ability of the solvent to accept a proton in a solute-to-solvent hydrogen bond), and π^* (which measures the ability of the solvent to stabilize a charge or a dipole by virtue of its dielectric effect) for individual solvents were taken from ref. 18. Chloroform as a chlorinated solvent was excluded from the Kamlet–Taft analyses.

After elimination of data revealing apparent specific reactivity between solvent and SGOH, SG^+ or DHSG, the remaining points in the graphs were fitted by linear function.

Emission spectra of 1.25 μM SG were also measured in the presence of 5 μM isolated porcine cerebral cortex Na^+/K^+-ATPase (Sigma), in the 10 mM Tris buffer, pH 7.5, containing 10 mM NaCl and 140 mM KCl and 20 mM sucrose.

Results

As we had shown before[12] SGOH in aqueous buffer at pH 7.5 exhibits maxima of excitation/emission spectra at 327/418 nm which corresponds to 30 510/23 810 cm^{-1} on the wavenumber scale (ESI, Fig. S1†), while SG^+ exhibits spectra with maxima at 475/590 nm which corresponds to 21 030/16 800 cm^{-1} on the wavenumber scale. Note that the SG^+ excitation spectrum is apparently composed of two strongly overlapping peaks (ESI, Fig. S2†). The DHSG spectra have maxima at 327/446 nm, which corresponds to 30 510/22 350 cm^{-1} (ESI, Fig. S3†). The spectra are essentially the same in distilled water, Tris or phosphate buffer (not shown). The spectra were reproducible with accuracy of ± 2 nm, which corresponds to approx. 200 cm^{-1} in the 327 nm region, 100 cm^{-1} in the 418 nm, in the 446 nm, and in the 475 nm regions, and 50 cm^{-1} in the 590 nm region.

Solvent effects

The spectral changes observed in various solvents can be attributed either to general changes in fluorophor environment polarity or to the specific interactions between the fluorophor and reactive groups of the solvent molecules.

For SGOH, we can see little red-shift (compared to the aqueous Tris buffer, pH 7.5) in the emission spectrum obtained in the 1-butanethiol (−870 cm^{-1}) and excitation spectrum in 1,2-ethanedithiol (−1110 cm^{-1}). Notably, these changes were not observed in the 10% solution of 1-butanethiol in the aqueous Tris buffer. Evaluation of the dependence of both excitation and emission peaks on the relative permittivity of the solution (Fig. 2), as well as a Lippert plot (Fig. 5), showed no dependence of the fluorescence properties on environment polarity (see Table 1). Detailed analysis using the Kamlet–Taft parameters that also

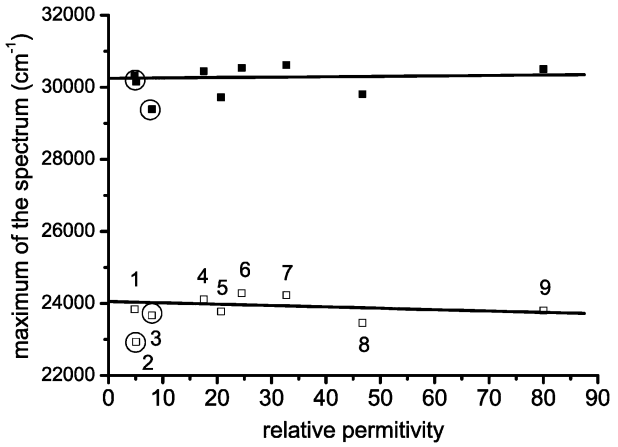

Fig. 2 Sensitivity of the SGOH excitation (full symbols) and emission (open) spectra to the solvent polarity. Solvents: (1) chloroform, (2) 2-butanethiol, (3) 1,2-ethanedithiol, (4) butanol, (5) acetone, (6) ethanol, (7) methanol, (8) DMSO, (9) aqueous buffer. The encircled points were excluded from the linear fitting.

involves the hydrogen bonding effects revealed that the spectral properties are mainly influenced by the ability of SGOH to donate proton to the solvent (Table 2, parameter *b*).

The situation is different for SG^+ (Fig. 3). In the excitation spectrum, there are apparent vibronic bands spaced by $\sim$400 cm^{-1} in all organic solvents (ESI, Fig. S4†). Concerning the spectral shifts, we can again see the anomalous behavior of the fluorescence spectra in the solutions of 1,2-ethanedithiol and 1-butanethiol. However, in this case, these specific effects are superimposed on the general sensitivity of the SG^+ spectra to the polarity of the solvent. This is most apparent in the dependence of the excitation peaks on the relative permittivity (correlation coefficient $r = 0.97$) and can also be traced in the emission spectra and Lippert plot (Fig. 5, Table 1). The Kamlet–Taft solvatochromic analysis showed high correlation coefficients for all excitation wavelength-, emission wavelength- and Stokes-shift solvent dependence, revealing that the polarity is the most important factor (Table 2, parameter *p*).

For DHSG, we can see large red-shift in excitation and blue-shift in emission spectra (-2780 cm^{-1} and $+3520$ cm^{-1}, respectively) only for 1,2-ethanedithiol (Fig. 4), and the peak in the emission spectrum is split into a doublet with energy difference of $\sim$1100 cm^{-1} (ESI, Fig. S5†). Again, this effect is not observed in the 10% solution in the aqueous buffer. Moreover, the excitation spectra show sensitivity to solvent polarity which is best demonstrated in the Lippert plot in this case (Fig. 5, Table 1) and also reflected in the Kamlet–Taft analysis (Table 2). The latter also shows the ability of DHSG to donate protons to the solvent.

Table 1 Sensitivity of the SGOH, SG^+ and DHSG fluorescence spectra to the solvent polarity

	Slope/cm^{-1} [a]	r [b]
SGOH, excitation sp., emission 418 nm	1.2 ± 6.6	0.08
SGOH, emission sp., excitation 327 nm	−3.8 ± 5.1	−0.32
SG^+, excitation sp., emission 590 nm	13.1 ± 1.5	0.97
SG^+, emission sp., excitation 475 nm	2.0 ± 1.3	0.55
DHSG, excitation sp., emission 446 nm	13.4 ± 12.0	0.41
DHSG, emission sp., excitation 327 nm	−3.0 ± 5.0	−0.23
Lippert plot, SGOH	−300 ± 1800	−0.08
Lippert plot, SG^+	3300 ± 1900	0.61
Lippert plot, DHSG	11200 ± 2500	0.87

[a] For the excitation and emission spectra, the value gives the slope of the dependence $\bar{\nu}_{max}$ *vs.* ε, for the Lippert plots the slope of the dependence $(\bar{\nu}_{ex} - \bar{\nu}_{em})$ *vs.* Δf, where $\Delta f = (\varepsilon - 1)/(2\varepsilon + 1) - (n^2 - 1)/(2n^2 + 1)$. [b] Correlation coefficient.

Table 2 Kamlet–Taft parameters for the best fits of the experimental data to the eqn (2)

Species	Parameter	$\bar{\nu}_0$/cm^{-1}	a/cm^{-1}	b/cm^{-1}	p/cm^{-1}	r [a]
SGOH	$\bar{\nu}_{ex}$	26 221	1102	3008	1911	0.85
	$\bar{\nu}_{em}$	13 867	1112	8374	5260	0.83
	$\bar{\nu}_{ex} - \bar{\nu}_{em}$	12 353	10	−5366	−3349	0.84
SG^+	$\bar{\nu}_{ex}$	19 829	157	−141	933	0.95
	$\bar{\nu}_{em}$	16 617	110	−167	54	0.94
	$\bar{\nu}_{ex} - \bar{\nu}_{em}$	3212	46	27	879	0.90
DHSG	$\bar{\nu}_{ex}$	27 770	1443	2129	732	0.93
	$\bar{\nu}_{em}$	27 442	145	−4569	−3367	0.90
	$\bar{\nu}_{ex} - \bar{\nu}_{em}$	328	1298	6698	4100	0.88

[a] Correlation coefficient.

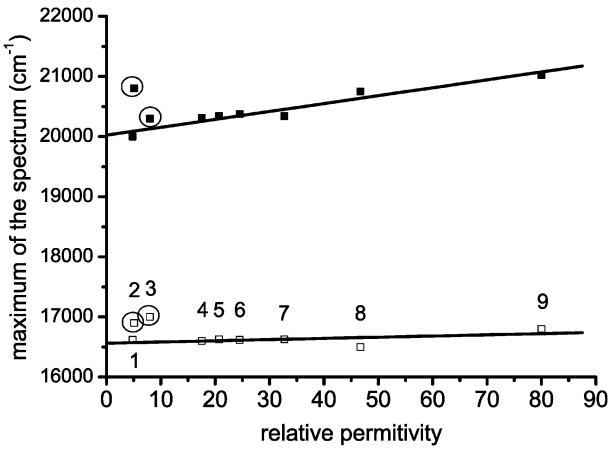

Fig. 3 Sensitivity of the SG^+ excitation (full symbols) and emission (open) spectra to the solvent polarity. Solvents: (1) chloroform, (2) 2-butanethiol, (3) 1,2-ethanedithiol, (4) butanol, (5) acetone, (6) ethanol, (7) methanol, (8) DMSO, (9) aqueous buffer. The encircled points were excluded from the linear fitting.

Specific interaction of SG and DHSG with amino acids residues

The neutral analogs of amino acids were used to test possible specific interactions of SGOH, SG^+ and DHSG. The selected analogs of Cys (–SH), Ser (–OH), Glu (–COOH), Gln ($-CONH_2$), Lys ($-NH_2$), Arg ($-NH-C-(NH_2)_2$) contain all reactive groups that are found within amino acids, Tyr was selected as a representative of aromatic residues. In the protein/ligand interaction, the ligand can be located on the protein surface, thus substantially experiencing the surrounding aqueous environment, or in some binding pocket, where it is well-protected from water. Therefore, the spectral changes were evaluated either in aqueous buffer (protic environment) or in acetone (aprotic environment).

In aqueous buffer, the observed changes upon interaction with amino acids are rather small. For SGOH, a small blue-shift ($+340$ cm^{-1}) in the excitation spectra, and red shift

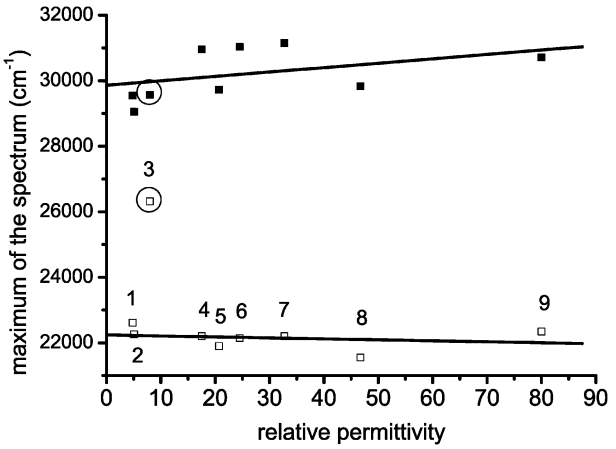

Fig. 4 Sensitivity of the DHSG excitation (full symbols) and emission (open) spectra to the solvent polarity. Solvents: (1) chloroform, (2) 2-butanethiol, (3) 1,2-ethanedithiol, (4) butanol, (5) acetone, (6) ethanol, (7) methanol, (8) DMSO, (9) aqueous buffer. The encircled points were excluded from the linear fitting.

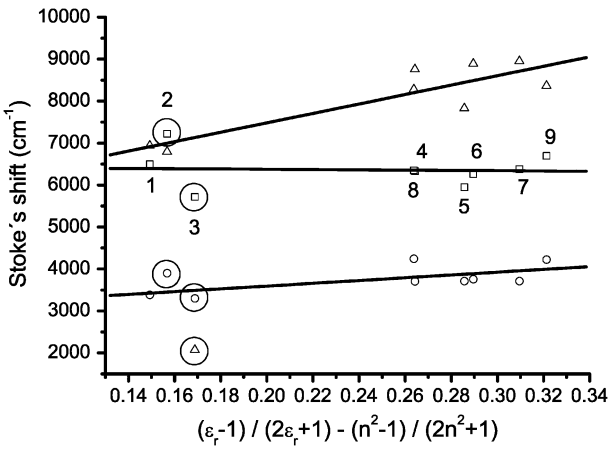

Fig. 5 Lippert plots for SGOH (squares), SG^+ (circles) and DHSG (tringles). Solvents: (1) chloroform, (2) 2-butanethiol, (3) 1,2-ethanedithiol, (4) butanol, (5) acetone, (6) ethanol, (7) methanol, (8) DMSO, (9) aqueous buffer. The encircled points were excluded from the linear fitting.

(-580 cm^{-1}) in the emission spectrum, were observed only in the presence of the Cys analog (not shown). These spectral shifts were accompanied by a $\sim$4-fold decrease in fluorescence intensity. The presence of the other amino acid analogs had no influence on the spectra. For SG^+, both the excitation and emission spectra are substantially blue-shifted ($+740$ cm^{-1}, and $+470$ cm^{-1}, respectively) only in the presence of Cys analog, which is again accompanied by a decrease in fluorescence intensity ($\sim$7-fold). Further, we could detect a small red shift in the emission spectra in the presence of Glu and Tyr analogs (-110 cm^{-1} and -170 cm^{-1}, respectively) (Fig. 7). In the presence of Tyr, the minor peak of the doublet in the SG^+ excitation spectrum is strongly suppressed. For DHSG, the excitation spectrum was blue-shifted (630 cm^{-1}) only in the presence of the Cys analog (not shown). However in emission spectra, no alteration in the presence of the amino acid analogs can be seen.

The effects are substantially different in the aprotic environment (acetone). We can observe a blue shift in the excitation spectrum of SGOH (Fig. 6) in the presence of Cys or Glu analogs ($+680$ cm^{-1} or $+360$ cm^{-1}, respectively), while in the emission spectrum, a red shift for Cys, Glu and Lys analogs (-600 cm^{-1}, -650 cm^{-1} and -430 cm^{-1}, respectively) can be observed. Both the Cys and Glu analogs decrease the SG^+ fluorescence intensity $\sim$10-fold. In the case of the Lys analog, the decrease is only 2-fold. For SG^+ (Fig. 8), we can detect large changes in the presence of the Arg analog in both excitation (red shift -1970 cm^{-1}) and emission (blue shift $+1450$ cm^{-1}) spectra, again accompanied by a huge decrease in fluorescence intensity. In contrast, in the presence of the Glu analog we observed a $\sim$15-fold increase in intensity but no spectral shifts in this case. The presence of other amino acid analogs had no effect on the spectra of SG^+. No amino acid analog altered the excitation and emission spectra of DHSG in acetone (not shown).

Changes in SG spectra induced by the interaction with Na^+/K^+-ATPase

Changes in the SG spectra were observed also upon incubation with the Na^+/K^+-ATPase. In the enzyme presence, the maxima in the SG^+ excitation- and emission spectra were located at 20 470 cm^{-1} and 17 720 cm^{-1}, respectively, and the maxima for SGOH were located at 30 400 cm^{-1} and 24 510 cm^{-1}, respectively.

Discussion

Interaction with various cellular proteins may be an important step in the mechanism of SG biological action. However, the details of these putative interactions are poorly understood. Investigation is also complicated by the fact that under physiological pH, SG is in equilibrium between the cationic (SG^+) and neutral (SGOH) forms and, *in vivo* it is metabolized to DHSG. Given the intrinsic fluorescence of all forms of SG, the great sensitivity and variability of fluorescence spectroscopy makes it a promising tool in SG analyses. Spectral and time-resolved analysis has already proven to be able to distinguish the individual forms and their possible

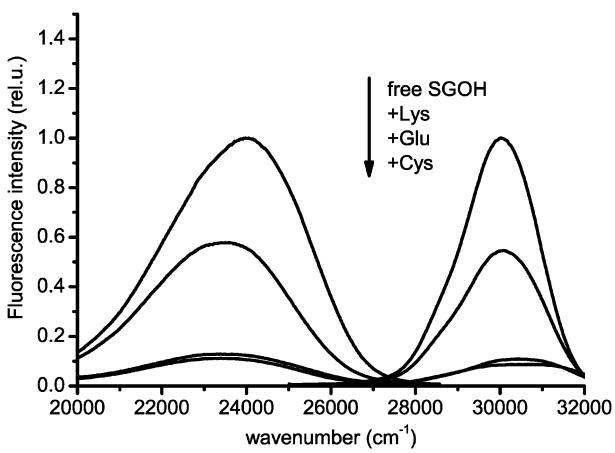

Fig. 6 Sensitivity of the SGOH excitation (right) and emission (left) spectra to the presence of amino acid reactive groups in acetone.

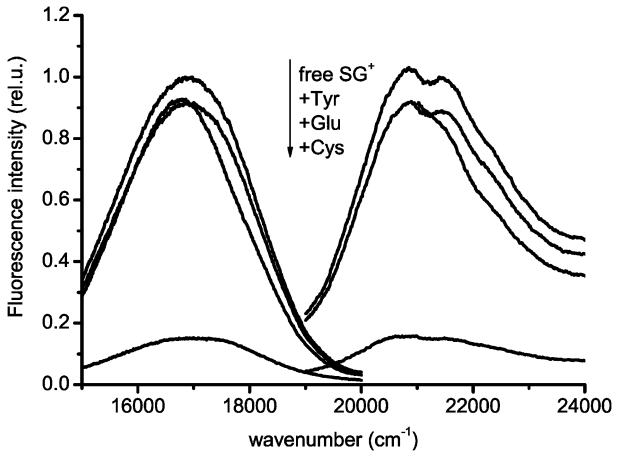

Fig. 7 Sensitivity of the SG^+ excitation (right) and emission (left) spectra to the presence of amino acid reactive groups in aqueous buffer. The red-part of the SG^+ excitation spectrum in the presence of Tyr perfectly overlaps the excitation spectrum in the presence of Glu.

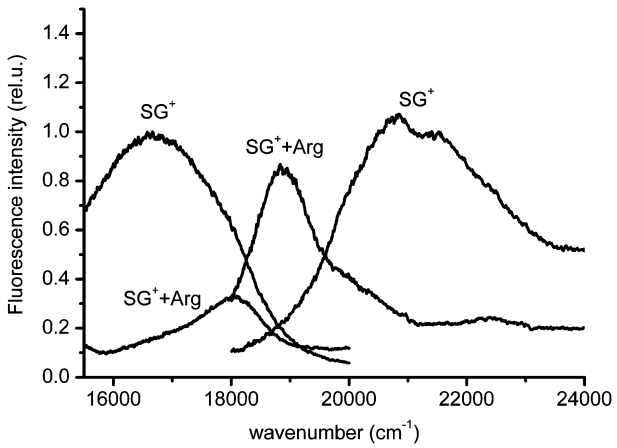

Fig. 8 Sensitivity of the SG^+ excitation (right) and emission (left) spectra to the presence of arginine in acetone.

interconversions.[12] Hence, a knowledge of the fluorescence properties enables separate observation of individual SG forms, even in situations when they cannot be separated physically. In this study, we analyzed the finer spectral changes that could be expected from interaction of SG with proteins, with the aim of acquiring information about the structural details of the SG/protein interaction.

From the viewpoint of physical chemistry, two kinds of effects should be considered. First, the polarity of the SG microenvironment is expected to decrease when it is transferred from aqueous environment (free form) to protein (bound form). Second, there may be a specific interaction between SG and a specific amino acid reactive group. Further, the nature of these specific interactions can differ, depending on the accessibility of the interaction site to water (protein surface or hydrophobic cavity). This study revealed that SG intrinsic fluorescence characteristics are sensitive to all these effects.

The effect of environment polarity

A large number of polarity scales have been introduced to evaluate the effect of environment polarity on the fluorescence spectra.[20,21] In our study, we included the solvent-dependence of the fluorescence spectra on permittivity as well, which is commonly used in other experimental techniques, such as *e.g.* potentiometry. In fluorescence spectroscopy, the most popular way of evaluating the polarity effects is the Lippert plot. However, substantial deviations from Lippert dependence are found for molecules prone to hydrogen-bonding interactions.[20] A more general approach is represented by the Kamlet–Taft analysis, which also involves parameters accounting for the fluorophore's ability to donate or accept protons from the solvent. Using this approach, we were able to fit the data with $r \geq 0.83$ in all cases, and they revealed the dominating contribution of the polarity term (parameter p) for SG^+, while the ability to donate a proton to the solvent was the most important in the spectra of SGOH and DHSG (parameter b). For both SG^+ and DHSG, the maxima were red-shifted with increasing solvent polarity, which is characteristic for the $\pi \rightarrow \pi^*$ transitions in the planar nitrogen-containing heterocycles.[20] This is in agreement with the theoretical calculations for SG^+ (for DHSG, the calculation has not been performed) which predict a nearly planar shape of SG^+, including the N^+–CH_3 bond. This calculation also predicts that involvement of the –OH group in SGOH causes significant distortion of the planarity of the aromatic system and both the –CH_3 and –OH groups deviate from the plane of the rings (49° and 59°, respectively, in opposite directions).[22] Hence, $n \rightarrow \pi^*$ transitions may be involved in some solvents, and this complicates the influence of polarity on the spectra, and for the SGOH spectra, we were unable to find any significant dependence on environment polarity.

Specific interaction with reactive groups of amino acids and effect of aqueous solvent accessibility

The fluorescence spectra can also be altered on specific interaction with amino acid reactive groups. In principle, this can influence the excitation spectrum, emission spectrum, or both, and can be manifested as a spectral shift and/or a fluorescence intensity change. We also found that the effect can be different in an aqueous environment (which simulates interaction on the protein surface) or in the aprotic solvent (which simulates interaction in the protein binding pocket).

It has been shown that the iminium bond of SG interacts with the free SH group of cysteinyl residues on enzymes.[10,11,23] This is in accord with our observations, and we were able to detect shifts in SGOH spectra in both aqueous buffer and acetone, while Cys analog shifted only SG^+ emission spectrum in the aqueous buffer. In all cases, the spectral shifts were accompanied by a massive quenching of SG fluorescence. They are likely a consequence of the intermolecular excited-state electron transfer (ESET) from the –SH group to the –N–CH_3 group. ESET is one of common mechanisms responsible for fluorescence quenching.[20] The evidence for interaction with the thio group is also supported by the observation that all solvents containing –SH group(s) exhibited specific spectral effects. Interestingly, the largest effects were observed for the interaction of SGOH and SG^+ with 1,2-ethanedithiol in the aprotic environment. This was the only specific interaction that was also manifested in the

DHSG spectra and it is possible that the mode of interaction is different from compounds containing only a single –SH group. The emission spectrum of DHSG in the presence of 1,2-ethanedithiol showed a strong vibrational pattern, which reflects a strong coupling of the electronic transitions to the particular vibration mode. In the *ab initio* calculations of SG, the energy of $\sim$1106 cm^{-1} (which best match the energy difference of the bands) was attributed to the vibrations of dioxolane rings.[22]

The binding to cysteine has been the only mode of interaction with enzymes that has been considered for SG up to now. Our experiments revealed very clearly that both SGOH and SG^+ can also interact with other amino acids. SGOH can interact with Glu and Lys in the aprotic environment, while SG^+ can interact with Glu and Tyr in the protic environment, and with Arg and Glu in the aprotic environment. The interaction of SG^+ with tyrosine results in the loss of vibronic structure of excitation spectrum. Although the resolution of fluorescence spectra is insufficient to identify unambiguously the precise mode of vibration coupled to the electronic transition, the energy of $\sim$400 cm^{-1} is typical for the skeletal vibrations of the aromatic system. Tyrosine probably interacts with the alkaloid through stacking interaction, thus, disturbing the coupling of the vibronic mode to the electronic transition. Deviations from the planarity of the aromatic system in the SG neutral form[22] explain, why this kind of interaction is observed only for SG^+, and not for SGOH. The charged amino acids can interact with SGOH or SG^+ only in aprotic environment. They can effectively form hydrogen bonds either to the nitrogen atom (Glu), to the SGOH hydroxy group (Lys), or to the oxygens within the dioxolane rings (Arg). Hydrogen bonding can alter both the spectra and quantum yield of the fluorophore, which was observed also in our experiments. The excitation- as well as emission spectra of DHSG were rather insensitive to the presence of amino acid analogs (other than Cys).

Based on a knowledge of the above spectral analyses we were able to elucidate the structural details of SG interactions with Na^+/K^+-ATPase. Na^+/K^+-ATPase is one of the most important enzymes in the metabolism of all animal cells, maintaining the membrane potential and steep gradient of sodium within the plasma membrane.[24] From mechanistic studies, it has been suggested as a possible molecular target in SG biological action.[7] The high concentration of potassium forces the enzyme to adopt the so-called E2 conformation (in contrast to E1 conformation, which is obtained in high concentration of sodium), which has been recently successfully determined by X-ray crystallography.[25,26] The fluorescence spectra of SG changed upon interaction with Na^+/K^+-ATPase. While the changes for the SGOH form were rather small, in the case of SG^+ we observed substantial red-shift in the excitation spectrum and large blue-shift in the emission spectrum together with a fluorescence intensity decrease. Such behavior corresponds well to the observations obtained for the interaction of SG^+ with the Arg analog in the aprotic environment. Inspection of the Na^+/K^+-ATPase high resolution structure reveals that most of its arginine residues are located on the surface of the molecule. The only exception is the Arg979 on the α-subunit, which seems to be protected by the extracellular part of the β-subunit from the solvent (Fig. 9). Hence, the Arg979 seems to be a hot candidate for being the interaction site of SG^+ on the Na^+/K^+-ATPase. However, this hypothesis must be verified by further experiments.

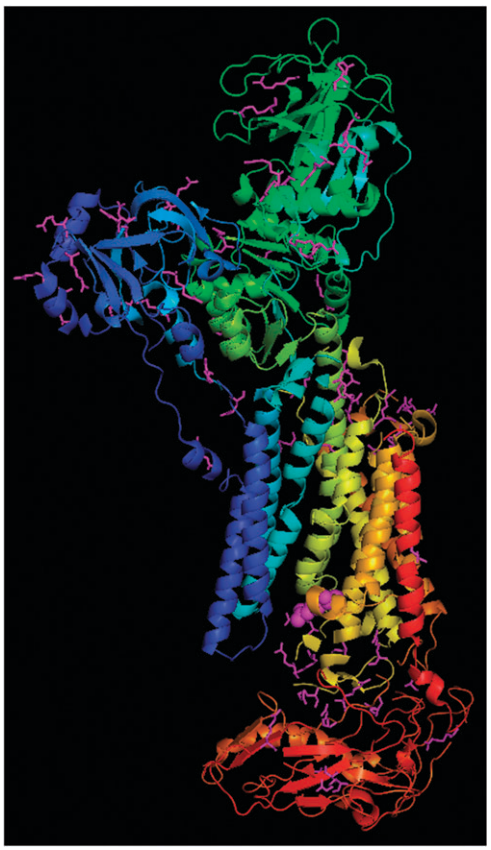

Fig. 9 Localization of arginine residues within the Na^+/K^+-ATPase in the E2 conformation.

In conclusion, the fluorescence spectra of both forms of SG and to a lesser extent DHSG proved sensitive to interactions predicted from binding to proteins. Our experiments revealed that there are also other modes of SG interaction with proteins than binding to cysteins and we have demonstrated that one such example is SG^+ binding to Na^+/K^+-ATPase. The fluorescence method is generally useful for other enzymes too, and can be used both for detecting interactions and elucidating the structural details of the interaction. Recalling that fluorescence methods can also be applied to analysis of living cells (including microscopy applications), detailed analysis of SG fluorescence has very considerable potential for monitoring SG molecular interactions.

Acknowledgements

This work was supported by the grants of the Czech Science Foundation GACR 203/07/0564, 525/07/0871 and 522/08/H003, and the research projects of Czech Ministry of Education, Youth and Sports MSM 6198959215 and MSM 6198959216.

References

1 V. Simanek, R. Vespalec, A. Sedo, J. Ulrichova and J. Vicar, in *Chemical Probes in Biology*, ed. M. Schneider, 2003, pp. 245–254.
2 J. Malikova, A. Zdarilova and A. Hlobilkova, *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.*, 2006, **150**, 5–12.
3 M. Das and S. K. Khanna, *Crit. Rev. Toxicol.*, 1997, **27**, 273–297.
4 L. Zajoncova, P. Kosina, J. Vicar, J. Ulrichova and P. Pec, *J. Enzyme Inhib. Med. Chem.*, 2005, **20**, 261–267.
5 C. Vavreckova, I. Gawlik and K. Muller, *Planta Med.*, 1996, **62**, 397–401.
6 L. P. Kuznetsova, E. B. Nikol'skaya, E. E. Sochilina and M. D. Faddeeva, *J. Evol. Biochem. Physiol.*, 2002, **38**, 35–39.
7 E. Seifen, R. J. Adams and R. K. Riemer, *Eur. J. Pharmacol.*, 1979, **60**, 373–377.
8 M. Fadeeva and T. Beliaeva, *Tsitologia*, 1997, **37**, 181–208.
9 H. Paulova and J. Slavik, *Pharmazie*, 1993, **48**, 555–556.
10 D. Walterova, V. Preininger, F. Grambal, V. Simanek and F. Santavy, *Heterocycles*, 1980, **14**, 597–600.
11 R. Vespalec, P. Bartak, V. Simanek and M. Vlckova, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2003, **797**, 357–366.
12 M. Janovska, M. Kubala, V. Simanek and J. Ulrichova, *Anal. Bioanal. Chem.*, 2009, **395**, 235–240.
13 M. Vlckova, P. Bartak and V. Kuban, *J. Chromatogr., A*, 2004, **1040**, 141–145.
14 J. Psotova, B. Klejdus, R. Vecera, P. Kosina, V. Kuban, J. Vicar, V. Simanek and J. Ulrichova, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2006, **830**, 165–172.
15 A. Deroussent, M. Ré, H. Hoellinger and T. Cresteil, *J. Pharm. Biomed. Anal.*, 2010, **52**, 391–397.
16 J. Vicar, M. Soural and J. Hlavac, *Chem. Listy*, **104**, 51–53.
17 N. Mataga, Y. Kaifu and M. Koizumi, *Bull. Chem. Soc. Jpn.*, 1956, **29**, 465–470.
18 M. J. Kamlet, J. L. M. Abboud, M. H. Abraham and R. W. Taft, *J. Org. Chem.*, 1983, **48**, 2877–2887.
19 M. S. Diaz, M. L. Freile and M. I. Gutierrez, *Photochem. Photobiol. Sci.*, 2009, **8**, 970–974.
20 B. Valeur, *Molecular Fluorescence*, Wiley, Weinheim, 2006.
21 D. M. Sena, H. O. Pastore and F. B. T. Pessine, *Phys. Chem. Chem. Phys.*, 2009, **11**, 7219–7224.
22 I. Motewich, N. Strekal, J. Nowicky and S. Maskewich, *J. Appl. Spectrosc.*, 2007, **74**, 666–672.
23 P. Bartak, V. Simanek, M. Vlckova, J. Ulrichova and R. Vespalec, *J. Phys. Org. Chem.*, 2003, **16**, 803–810.
24 M. Kubala, *Proteins: Struct., Funct., Bioinf.*, 2006, **64**, 1–12.
25 J. P. Morth, B. P. Pedersen, M. S. Toustrup-Jensen, T. L. M. Sorensen, J. Petersen, J. P. Andersen, B. Vilsen and P. Nissen, *Nature*, 2007, **450**, 1043–U1046.
26 T. Shinoda, H. Ogawa, F. Cornelius and C. Toyoshima, *Nature*, 2009, **459**, 446–U167.