Molecular organization of the human serotonin transporter at the air/water interface

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Abstract The serotonin transporter (SERT) is the target of several important antidepressant and psychostimulant drugs. It has been shown that under defined conditions, the transporter spread at the air/water interface was able to bind its specific ligands. In this paper, the interfacial organization of the protein has been assessed from dynamic surface pressure and ellipsometric measurements. For areas comprising between 10 400 and 7100 Å^2/molecule, ellipsometric measurements reveal an important change in the thickness of the SERT film. This change was attributed to the reorientation of the transporter molecules from a horizontal to their natural predictive transmembrane orientation. The thickness of the SERT film at 7100 Å^2/molecule was found to be approximately equal to 84 Å and coincided well with the theoretical value estimated from the calculations based on the dimensions of α-helices containing membrane proteins. These data suggest that the three-dimensional arrangement of the SERT may be represented as a box with lengths d_z = 83–85 Å and d_y or d_x = 41–47 Å.

Key words: Serotonin; Transporter; Surface pressure; Ellipsometry; Protein monolayer

1. Introduction

It is well known that the shape and structure of cells as well as their recognition functions are primarily dependent on the nature and specificity of proteins present in their membranes. If, theoretically, proteins may adopt an almost unlimited number of conformations, the side chains of their amino acids which associate with one another and with the surrounding water molecules by non-bonded interactions make them always fold into a limited number of conformations, in equilibrium with a native three-dimensional (3D) structure. Under non-physiological conditions proteins may denature and lose their native conformation. However, when appropriate conditions are restored they may refold into their original conformation. Usually, proteins are adsorbed when in contact with surfaces. The process of protein adsorption at both solid/liquid and air/liquid interfaces has been extensively studied [1–7]. Protein adsorption from solutions onto the air/water interface was described as a three-step process consisting of (i) protein diffusion from the subphase toward the interface, (ii) adsorption–penetration of protein molecules into the interface and (iii) rearrangement of their segments at the interface [5–7]. For proteins deposited at the air/water interface there is no diffusion and the kinetics of surface tension variation may be considered as that due only to protein rearrangement at the interface which results in denaturation and eventually leads to its refolding into its native conformation [2]. An intermediary molecular organization of a deposited protein after its conformational stabilization would necessarily involve exposure of its hydrophobic amino acids toward the air phase and of its hydrophilic amino acids toward the aqueous subphase. A protein under such conditions will maintain its secondary structure but will lose its original 3D conformation [8]. The degree to which protein monolayers are compressed was found to considerably affect the orientation of molecules at the interfaces [9–11].

The preservation of the native conformation of a spread protein can be confirmed either by size calculations or by biological assays which generally show that the protein activity remains intact. Thus, among others, rhodopsin [9], cytochrome P450 scc [11] and myelin basic protein [12] have been shown to maintain their original 3D size and were not denatured upon spreading at the air/water interface. In their pioneering work, Korenbrot and Pramik [13] have suggested that the conformation of rhodopsin at the interface is similar to that in its native environment. Furthermore, the spectroscopic and functional behavior of bacteriorhodopsin has been shown to be preserved when a protein was spread in a monolayer [14].

Recently we have described the interaction, at the air/water interface, between the purified human serotonin transporter (SERT) spread in a monolayer and serotonin analogs (5-hydroxyindoleacetic acid, 5-hydroxytryptophan (5-HT), etc.) [15] or its pharmacological ligands (tricyclics, serotonin-selective reuptake inhibitors, etc.) [16] dissolved in the subphase. SERT is a 68 kDa protein constituted of 630 amino acids distributed in 12 transmembrane predicted domains consisting of approximately 20 amino acids, five intracellular and six extracellular loops, with N- and C-terminal intracellular domains [17–19]. The interaction of SERT with its ligands at the

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Abbreviations: SERT, serotonin transporter; 5-HT, 5-hydroxytryptophan

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air/water interface [15,16] appeared to be dependent on its surface density. Indeed, at a surface density of 1.5 μg/cm^2, SERT molecules were brought into a favorable conformation which enabled the binding of ligand molecules. Below this surface density, binding could not occur. This observation led us to consider the influence of the extent of compression on the organization of the protein in the monolayer.

Hence, the purpose of the present study was to investigate molecular rearrangements within a SERT monolayer, especially those controlled by altering protein surface density. With this aim in mind, we have coupled dynamic surface pressure data on a Langmuir-type film balance and ellipsometric measurements. Whereas the first would provide information on the surface area occupied by a film-forming SERT, ellipsometry would make it possible to evaluate changes in its thickness as it has previously been used for the evaluation of the thickness of protein, lipid or polymer adsorbed layers [20–22]. The elucidation of the molecular architecture of SERT films would shed light on its arrangements in biological environments.

2. Materials and methods

2.1. Materials

Ultrapure water was obtained by osmosis from a MilliRO6 Plus Millipore apparatus and then double-distilled from permanganate solution in an all-Pyrex apparatus. Its pH was 5.50 and its surface tension was higher than 71.8 mN/m at 25°C. Sodium chloride (150 mmol/l) and sodium phosphate (10 mmol/l) used to prepare buffer solutions (pH adjusted to 7.40) were Normapur from Prolabo (Paris, France). All glassware was cleaned with a sulfochromic mixture and then rinsed thoroughly with the distilled water.

2.2. SERT purification

SERT was purified from human blood platelets, a classical cell model for the study of 5-HT uptake. As previously described in detail [23], after treatment of the platelet membrane fractions by sulfhydryl-dependent bacterial protein toxins, SERT purification was obtained by one-step affinity chromatography using columns with 6-fluorotryptamine as ligand and elution with a Na^+-free buffer. The purified SERT migrated on 2D SDS–PAGE as a single band with an apparent mass of 68 kDa and exhibited an apparent isoelectric point of 5.6–6.2. This procedure led to the purification of SERT to homogeneity, allowing determination of its amino acid composition (not shown) and of some microsequences: M(1)ETTPLNSQKQLSACEDGED, A(181)WALYYLISSFTDQLPWTSC and T(301)LPGAWRGVLFY LKPNWQKL. Two sialic acid residues were detected per molecule of purified material. The purified protein bound [^{3}H]paroxetine (K_d = 0.23 nM) and [^{3}H]serotonin (K_d = 86 nM). These results are similar to previous purifications with platelet [16,23] or brain [24] material. The protein concentration was assayed using the Micro BCA protein assay kit (Pierce) with bovine serum albumin as standard.

2.3. Surface pressure measurements

The autorecording Langmuir-type film balance (MCN Lauda, Germany) was used to record compression isotherms of SERT monolayers (surface pressure versus area per quantity of protein or per number of molecules at the interface). The film balance was enclosed in a Plexiglas box and thermostated at 22°C. In order to use as little protein as possible, the surface of the trough was reduced to 270 cm^2. After sweeping off the surface with a capillary pipet connected to a vacuum pump, aliquots of a SERT aqueous solution were spread onto the buffer subphase. The rate of compression was 2 cm/min.

2.4. Ellipsometry measurements

The refractive index of different diluted SERT solutions was determined with an Abbe refractometer (room temperature, white light). The highest concentration of protein that could be obtained was 0.13 g/ml. Ellipsometry was performed using a Plasmos SD2300 ellipsometer (Germany). The instrument was automated by computer-controlled stepping motors on the polarizer and the analyzer. The light source was a 632.8 nm HeNe laser and two different angles of incidence were used: 52° and 45°. SERT was spread on a home-made microtrough and the surface tension was measured with a Wilhelmy platinum plate attached by a thread to a force transducer (Krüss, digital tensiometer K10ST). To minimize evaporation and possible long-term drift due to sample contamination, the microtrough was enclosed in a Plexiglas box with a small groove to allow the passage of the laser beam. The amount of SERT spread was 0.075 μg/cm^2.

The ellipsometric angle change ($\delta\Delta$), resulting from the presence of a monomolecular SERT layer spread at the air/water interface, is related to the refractive index (n) and the thickness (d) of the film, as described elsewhere [9,25]. The equation of De Feijter et al. [26] has been used to correlate the surface density (Γ) and d. From ellipsometric measurements, the thickness of the SERT monolayer was determined as a function of the extent of compression of the film.

2.5. Compression work calculation

To determine the surface area per molecule at each compressional pressure, we have calculated the compression work of SERT monolayers at the air/water interface. This work, W_c, can be estimated from the π–A isotherm [27]:

$$W_c = \int_{A_{initial}}^{A_{final}} \pi \, dA \quad (1)$$

The integral represents the area under the curve of a π–A isotherm, where $A_{initial}$ is large (π = 10 mN/m) and A_{final} is taken on collapse of the monolayer. Throughout the text which follows, the abbreviations d_x and d_y will denote the size of the protein along its x- and y-axes, in the plane perpendicular to the α-helices, while d_z will correspond to the length of the protein along the z-axis, parallel to the α-helices.

3. Results

3.1. Surface pressure measurements

The interfacial behavior of the SERT was assessed by dynamic compression of its monolayers after 1 h following protein deposition. The recorded π–A isotherms are illustrated in Fig. 1. At low amounts of spread protein ($\leq$ 20 μg), the isotherm profiles appeared to be similar to another. The change in the surface area per mg of SERT was insignificant, and the same three inflection points at 2 ± 1, 17 ± 0.9 and 25 ± 1 mN/m (A, B and C, respectively) were observed at all isotherms. The compression work (W_c) for amounts of protein less than 20 μg was found to increase linearly from 0 to 1.3×10^{-4} J (Fig.

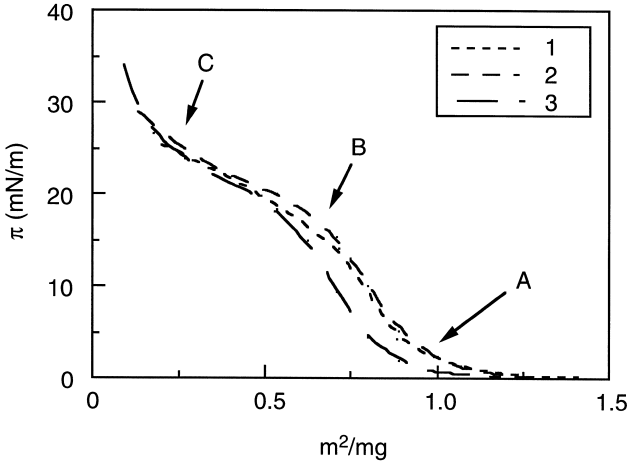

Fig. 1. SERT compression isotherms obtained 1 h after protein spreading and for different amounts of spread protein: (1) 9.5 μg, (2) 13.5 μg, (3) 20.3 μg.

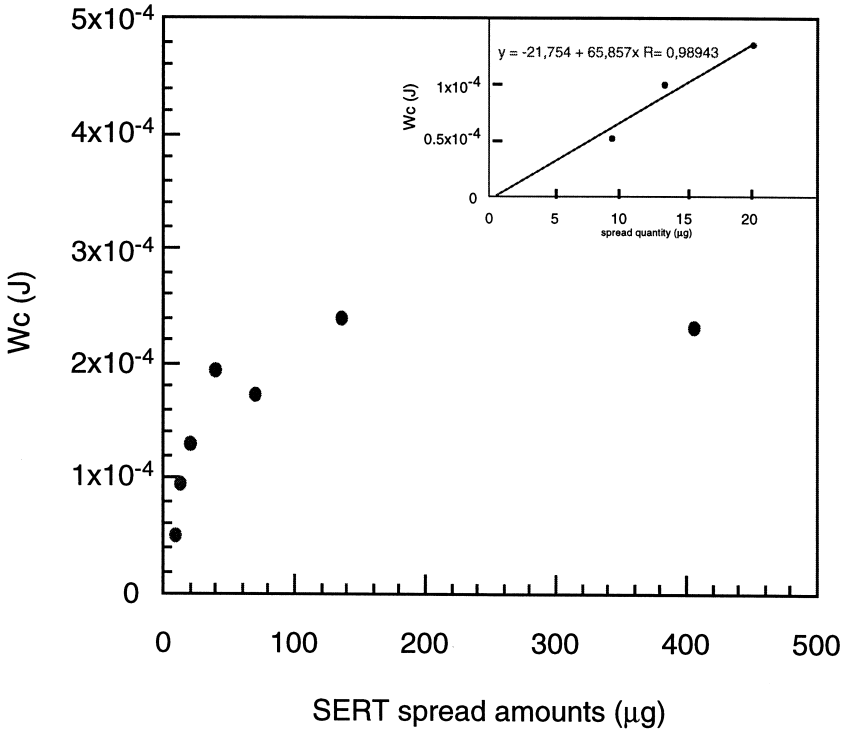

Fig. 2. SERT compression work, 1 h following protein deposition. Inset represents compressional work, W_c, versus deposited protein amounts.

2). The failure of the plot to cross the 0.0 coordinate indicates that a fraction of the initially deposited protein was lost into the subphase during the spreading step [12,28]. Our calculations yielded a W_c zero value for a spread amount of 0.33 μg (Fig. 2, inset). However, the protein loss for spread amounts ranging from 9 to 20 μg did not exceed 3.7%. Above 20 μg, the increase in W_c was less significant, indicating that an important loss due to exclusion and solubilization of the protein in the subphase took place during the spreading step. As inferred from the value of W_c, the actual molecular areas of the adsorbed protein at the surface pressures of 2, 17 and 25 mN/m amounted to 10 400 ± 680, 7100 ± 420 and 1844 ± 321 Å^2, respectively (Fig. 3).

3.2. Ellipsometry measurements

The refractive index, n, of SERT solutions was found to be a linear function of the protein concentration up to the highest concentration used (0.13 g/ml) (Fig. 4). The dn/dc slope, i.e. the so-called refractive index increment, was calculated using a least-square method. It yielded the value of 0.189 (R = 0.998). This value being similar to those found for globular proteins (0.18–0.20), we have assumed, as for lysozyme or other globular proteins [26], that the dn/dc ratio remains constant up to a SERT concentration as high as 0.4 g/ml.

As shown in Table 1, the ellipsometric angle change (δΔ) altered during the SERT compression. At 10 400 Å^2 (point A), δΔ was equal to 4.2047° and 0.4857° for light incidences of 52° and 45° respectively. An ellipsometric jump appeared when the available area per molecule decreased. At 7100 Å^2 (point B), δΔ was equal to 7.22° and 0.9827° depending on the light incidence (Table 1).

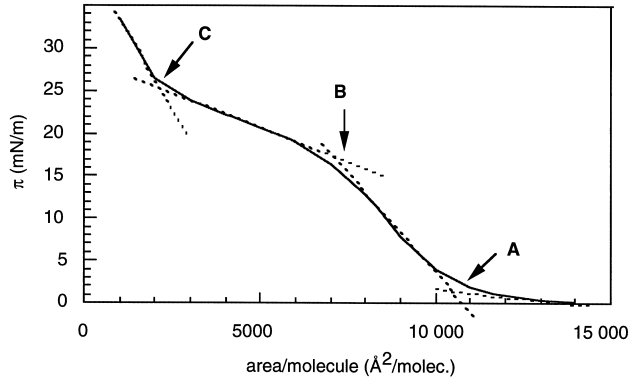

Fig. 3. π–A isotherm of the SERT at the air/water interface.

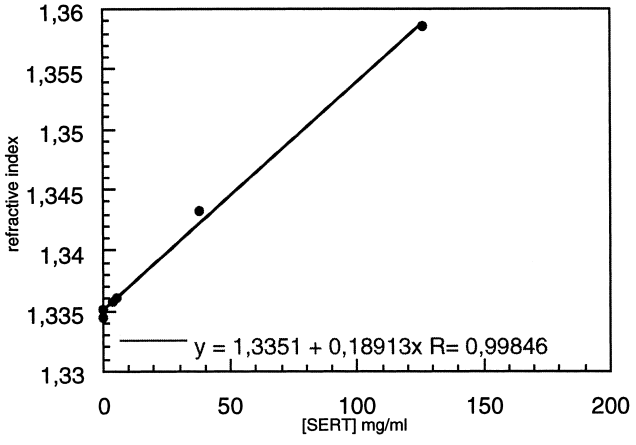

Fig. 4. Refractive index of SERT solutions.

Table 1
Ellipsometric changes ($\delta\Delta$) and thickness of SERT monolayer at inflexion points A and B for two incidence angles

Area (Å^2)	$\delta\Delta$ (°)		Thickness (Å)	
	52°	45°	52°	45°
A: 10 400 ± 680	4.205 ± 0.296	0.486 ± 0.024	47 ± 3	42 ± 2
B: 7100 ± 420	7.22 ± 0.8298	0.983 ± 0.018	83 ± 10	85 ± 2

4. Discussion

In a previous paper [16] we have studied the interfacial behavior of the transporter, at constant area, using the Wilhelmy plate method. The conformational rearrangements of SERT molecules as a function of time in the presence and in the absence of a ligand in the subphase were assessed from the magnitude of surface tension changes. Two-step kinetics were evidenced in which a short equilibrium step (1 or 2 h) was followed by a slow pressure increase which lasted several hours (around 20 h). Since the protein was able to strongly interact with some of its ligands (imipramine, paroxetine or indalpine) during the first 2 h following its deposition [16], it was considered that SERT preserved its binding activity during this period and maintained a conformation similar to that of the native SERT. For this reason, all the compression experiments described in the present report were performed 1 h following protein deposition.

The three threshold surface pressures at 2, 17 and 25 mN/m may be considered as rather low values compared to the surface pressure displayed by lipid monolayers. However, these pressures are in good agreement with the values generated by most peptide and protein macromolecules spread alone. Moreover, a three-step π–A profile has often been described in the literature for α-helix-containing membrane proteins. For example, the π–A isotherms of monolayers of gramicidin A [29], gramicidin B and C [30], melittin [31] or human hepatitis B virus (amino acids 120–145), a peptide containing one α-helix [32], exhibited inflexion points at 2, 15 and 20–23 mN/m. Under the surface pressure corresponding to the first inflexion point (2 mN/m), the helices are parallel to the air/water interface. Above 23 mN/m, a dramatic rise in the surface pressure was observed for all these systems. At a surface pressure around 20–23 mN/m, prior to the substantial rise in π, the helices were described as packed and perpendicular to the plane of the interface.

In our experiments (Fig. 3), the appearance of point C, at 25 mN/m, occurred at a molecular area of 1844 ± 321 Å^2 which corresponded to the surface available per predictive transmembrane domain ranging from 130 to 180 Å^2. This value is in good agreement with that reported for the area per helix (160–170 Å^2) obtained from calculations [33,34] or deduced from surface pressure experiments at the air/water interface [31,32]. Therefore, whereas point C seems to correspond to the arrangement of closely packed helices, point B (Fig. 3), at 7100 ± 420 Å^2, may be considered as representative of the perpendicular orientation of the protein with respect to the interface plane. This orientation is generally attributed to the SERT orientation in membranes [35]. Compared to the area occupied per monomer (1844 Å^2), the area available at point B is much larger. It is reasonable to think that the reorientation of helix bundles creates voids between individual molecules and that these voids cannot immediately be filled up to form a closely packed layer at the air/water interface [9,36]. An important, calculated [37] or observed [38] tilt angle, around 20° for 12-helix bundles, may result in a large spacing between molecules. Finally, point A at 10 400 Å^2 (Fig. 3) may be attributed to the parallel orientation of the SERT with respect to the interface. These orientational changes would account both for the ability of SERT molecules to bind their ligands at surface areas lower than 10 000 Å^2/molecule and for their failure to bind these ligands above the threshold A value, as previously noted for pinoline [16]. Salesse et al. [9] had described such an interfacial reorientation for rhodopsin, a seven-helix transmembrane protein, in either the presence or the absence of lipids.

Ellipsometric measurements allowed us to confirm these results. Despite unchanged ψ, an increase in Δ during compression (data not shown) was observed when the area available per molecule decreased (Table 1). This would indicate that changes in d and/or in n took place within the film. Thus compression experiments and compression work (W_c) calculations allowed us to evaluate surface densities of a spread SERT monolayer, and if the refractive index increments could also be determined, the thickness and the refractive index of the monolayer still remained unknown. However, an insight into the magnitude of these values may be obtained from general information available on the dimensions of α-helices containing membrane proteins [39,40]. From these data it is known that whereas the area of a protein ($d_x \times d_y$) in a membrane plane depends on how these α-helices are arranged, the magnitude of d_z is essentially related to the length of its hydrophilic chains and loops. Indeed, it seems likely that the more rigid helices anchored in a hydrophobic bilayer core do not produce any important variations in protein length along the z-axis. However, the angle between α-helices and the bilayer normal, the tilt angle, and the number of amino acids are probably amongst the most important factors influencing the thickness of the transmembrane domain bundles [41].

The α-helices of the SERT protein are formed of 18–26 amino acids with an average of 20 [18]. Considering one α-helix with 3.6 residues per turn and a 1.5 Å rise for each residue along the z-axis, the mean thickness of a transmembrane domain would be equal to 30 Å. At the surface coverage corresponding to 7100 Å^2/molecule, from the combination of experimental results (molecular area, index increment) and structural data (thickness and molecular weight of the predictive helix bundle [18]), the refractive index for the membrane part of the protein was found to be equal to 1.374 as calculated from the De Feijter model. Extrapolated to the whole molecule (transmembrane domains together with hydrophilic loops) this refractive index gives a thickness of 79.4 Å. Using data reported in the literature [42–44], the same approach was used to calculate the refractive index for rhodopsin. In this case, the refractive index was 1.407, a value comparable both to that used by Salesse et al. [9] (1.405–1.410) and to those

experimentally determined by Sidman [45] (1.4106, 1.4056 and 1.4076, depending on the origin of the rod outer segments). Moreover, taking this value of the refractive index for calculations, the length of rhodopsin along the z-axis was found to be 73.05 Å, a value in good agreement with that found by Salesse et al. [9] and by Corless et al. [46], who reported values of 73 Å [9] and 70 Å [46].

From the calculated SERT refractive index and the results of ellipsometric measurements, it was possible to determine the thickness of the monolayer at points A and B. At 7100 Å^2 (point B), the SERT thickness was found to be equal to about 83–85 Å (Table 1). That the thickness of SERT was higher compared to that of rhodopsin or bacteriorhodopsin [47] results from its higher content of amino acids in hydrophilic loops. Indeed, whereas for rhodopsin the longest loop contains 40 amino acids and the mean number of residues is around 23 amino acids per loop, for SERT the longest hydrophilic loop contains 70 amino acids and the mean number of amino acids per loop is around 30.

At point A, where we suppose that the transporter is oriented parallel to the interface, the thickness of the monomolecular film and thus the dimension of the SERT according to x- and/or y-axis is around 42–47 Å (Table 1). These lengths, in the plane perpendicular to α-helices, are very close to the expected values. Notwithstanding these data, information on the dimensions of membrane transporters is scarce. However, it is worthwhile to remember the value reported for NhaA. NhaA is the first and so far the only ion-coupled transport protein containing 12 transmembrane domains that could be crystallized [38,48]. The transporter belongs to the same family to which SERT belongs. In its 2D crystal structure, NhaA appears to be a dimer (48×181 or 191 Å) composed of two small monomers with the dimensions in the plane perpendicular to the helices equal to 48×38 Å. At point C (where α-helices of SERT are supposed to be perpendicular to the plane of the interface) the molecular area is 1844 Å^2 (Fig. 3), and is very close to that attributed to the NhaA monomer ($d_x \times d_y = 48 \times 38 = 1824$ Å^2). This would strongly suggest that reorientation of the SERT takes place during compression. This also accounts for the role of the surface density in SERT arrangements at constant area and its binding capacity [16].

In conclusion, using biophysical methods, the dimensions of a biogenic amine transporter have been determined for the first time. From a biological point of view, the similarity of helix bundle arrangements between NhaA and SERT would mean that the serotonin transporter is capable of organizing itself as an oligomer. The possible existence of such quaternary structures attributed to SERT was also suggested in recent pharmacological studies of this protein [49,50].

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