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Ligand binding induces a sharp decrease in hydrophobicity of folate binding protein assessed by 1-anilinonaphthalene-8-sulphonate which suppresses self-association of the hydrophobic apo-protein

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ABSTRACT

High affinity folate binding protein (FBP) regulates as a soluble protein and as a cellular receptor intracellular trafficking of folic acid, a vitamin of great importance to cell growth and division. We addressed two issues of potential importance to the biological function of FBP, a possible decrease of the surface hydrophobicity associated with the ligand-induced conformation change of FBP, and protein-inter-protein interactions involved in self-association of hydrophobic apo-FBP.

The extrinsic fluorescent apolar dye 1-anilinonaphthalene-8-sulphonate (ANS) exhibited enhanced fluorescence intensity and a blueshift of emission maximum from 510–520 nm to 460–470 nm upon addition of apo-FBP indicating binding to a strongly hydrophobic environment. Neither enhancement of fluorescence nor blueshift of ANS emission maximum occurred when folate-ligated holo-FBP replaced apo-FBP. The drastic decrease in surface hydrophobicity of holo-FBP could have bearings on the biological function of FBP since changes in surface hydrophobicity have critical effects on the biological function of receptors and transport proteins.

ANS interacts with exposed hydrophobic surfaces on proteins and may thereby block and prevent aggregation of proteins (chaperone-like effect). Hence, hydrophobic interactions seemed to participate in the concentration-dependent self-association of apo-FBP which was suppressed by high ANS concentrations in light scatter measurements

1. Introduction

The high-affinity folate binding protein (FBP) present in cells and body fluids of several mammals regulates homeostasis and intracellular trafficking of the vitamin folic acid [1,2]. Anchored to the outer cell surface by glycosyl phosphatidyl inositol (GPI), FBP serves as a folate receptor (FR) internalizing folates, which play a critical role in cellular DNA synthesis and repair [1,2]. Enzymatic cleavage of the GPI anchor leads to shedding of soluble FBP from the cell surface into the surrounding body fluids at concentrations of 1–2 nM except in milk which contains more than 100-fold higher concentrations [1–3].

A method combining cation exchange and ligand (methotrexate) affinity chromatography has made a large scale purification of soluble FBP from cow's whey powder feasible [4,5], and enabled us to analyze the structure of the native protein. FBP has a molecular size of 28.9 kDa in mass spectrometry, and its primary structure consists of 222 amino acid residues including 11 tryptophans [4–6]. The protein is basic with multiple pIs in the pH range 7–8 likely due to variations in the carbohydrate content [4,5].

We have previously reported two interesting structural characteristics of FBP.

1) Ligand (folate) binding induced a conformation change of FBP which involved the secondary structure and resulted in a transfer of tryptophan residues from the surface to the hydrophobic core of FBP [7–11]. The weak affinity of holo (folate-bound)-FBP to hydrophobic gels as compared to apo-FBP [7] suggested a decrease of hydrophobicity subsequent to folate binding. The latter finding seems interesting since it has received attention that the biological function of important transport and receptor proteins is critically affected by ligand-induced changes of their surface hydrophobicity [12,13].

Abbreviations: FBP, folate binding protein; FR, folate receptor; ANS, 1-anilinonaphthalene-8-sulphonate; PBS, phosphate buffered saline; GPI, glycosyl phosphatidyl inositol.

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2) Apo-FBP exhibits a marked propensity to reversible concentration-dependent self-association [6,14–16]. The nature of this protein-inter-protein reaction is yet unresolved but hydrophobic patches on protein interfaces involved are suspected to play a critical role [6,17].

The above issues were addressed in the present study. The surface hydrophobicity of FBP and changes thereof subsequent to ligand (folate) binding was thus assessed through fluorescence spectroscopic measurements of the interaction between the extrinsic fluorescent apolar dye, 1-anilinonaphthalene-8-sulfonate (ANS) and apo and holo-FBP. Fluorescent probes like ANS interact noncovalently with proteins via hydrophobic or even electrostatic interactions, and become highly fluorescent in apolar organic solvents or upon binding to hydrophobic patches on protein surfaces [18–20]. Binding of ANS to hydrophobic surfaces is associated with enhanced fluorescence (increase of quantum yield) and blueshift of emission maximum [18–20].

The second issue addressed was a possible participation of hydrophobic bindings/interactions in self-association of apo-FBP. Through its interaction with hydrophobic interfaces/surfaces on proteins ANS may block and prevent aggregation of native proteins (chaperone-like effect) [21,22]. The study therefore includes light scattering measurements to disclose a possible effect of ANS on self-association of FBP.

2. Materials and methods

Folate (pteroyl glutamate) and 1-anilinonaphtalene-8-sulphonate (ANS) ammonium salt were supplied by Sigma (Aldrich).

FBP was purified from cow's whey powder as described above [4,5], and dialyzed against 0.2 M acetate buffer, pH 3.5 to remove endogenous folate.

2.1. Sample preparation for spectroscopic measurements

A stock solution of FBP was prepared in PBS of pH 7.4, and working solutions prepared as dilutions of the stock solution in PBS buffer. Stock and working solutions of ANS and folate were likewise prepared in PBS buffer of pH 7.4. At neutral pH FBP carries no net charge (pI 7–8) which should minimize electrostatic interactions between positive charges on FBP and the negatively charged sulfonate group of ANS.

2.2. Fluorescence measurements

Fluorescence spectra were acquired on a FS920 spectrofluorometer (Edinburgh Instruments) equipped with an external temperature controller set at 20 °C. Emission spectra of various mixtures of ANS, FBP, folate and PBS buffer were measured with excitation wavelengths 280 and 350 nm, and with emission from 300 to 550 nm and from 400 to 650 nm, respectively. Samples were measured in a right angle setup, using a 10×2 mm quartz cuvette, with the 2 mm light way in the excitation direction.

In all fluorescence measurements the monochromator slit widths were set at 5 nm for both excitation and emission, and an integration time of 0.1 s/nm was used.

Fluorescence data were recorded in photon counts/second and were corrected for the wavelength dependent excitation intensity by an internal reference detector. All spectra are used to compare inter-sample variation and therefore it is not necessary to correct the spectra.

2.3. Light scattering measurements

Light scattering measurements were performed as fluorescence synchronous scans, with an offset between excitation and emission of 0 nm, thus we were measuring exactly the maximum of the Rayleigh scatter peak. The experimental procedure is apart from minor modifications similar to that recently described [21,22].

2.4. Inner filter effects

At low concentrations of fluorophores, fluorescence spectroscopy follows Lambert Beers law of a direct linear connection between concentration and signal intensity. At higher concentrations self-absorption of emitted light can occur. This phenomenon is known as concentration quenching or inner filter effect. In this study we found a perfect linear relation between fluorescence intensity and ANS (0–30 μM) and FBP (0–1.6 μM). An apparent inner filter effect of approximately 20% was only observed at the high (3.33 μM) concentration of FBP.

3. Results and discussion

3.1. Excitation and emission spectra of fluorophores

The excitation and emission spectra of ANS, folate and apo-FBP were recorded at pH 7.4 (data not shown).

The most surprising finding - to our knowledge reported for the first time - was that ANS in addition to its well-known excitation maximum at 350 nm had a second one at 270 nm, though still only one emission maximum at 510–520 nm. We preferred excitation of ANS at 280 nm and not at 270 nm where emission maximum coincided with the second order Rayleigh scatter peak at 520 nm. Excitation of ANS at 280 nm instead of at 350 nm results in a drastic increase of the emission intensity consistent with the much higher peak of absorbance at 280 nm.

The two excitation maxima of folate at 280 and 340 nm resulted in a broad two-peak emission maximum at 430–470 nm overlapping the blueshifted emission spectrum of the ANS–FBP complex.

The excitation and emission maxima of apo-FBP were 280 and 350 nm, respectively.

3.2. Effect of ANS on intrinsic fluorescence and self-association of apo-FBP at pI

The purpose of the experiments in Fig. 1 was to establish whether ANS binds to exposed hydrophobic patches on the surface of apo-FBP and if binding of ANS to hydrophobic interfaces involved in protein-inter-protein interactions could suppress self-association of apo-FBP. Fig. 1 (left) shows that intrinsic fluorescence of apo-FBP is gradually diminished (quenched) in the presence of increasing concentrations of ANS. This energy transfer from tryptophan residues to ANS molecules indicates that ANS binds to hydrophobic patches on the surface of apo-FBP located in the proximity of tryptophan groups. The quenching effect achieved its maximum at 10–12 μM ANS.

A series of light scatter experiments (Fig. 1, right) showed that ANS suppresses self-association of 0.83 and 3.33 μM apo-FBP. The higher intensity at 3.3 μM FBP as compared to 0.83 μM is consistent with the well-established propensity of apo-FBP to concentration-dependent self-association [6,14–16]. The light scatter intensity decreased in the presence of a high (16 μM) ANS concentration indicating a reduced tendency to self-association of apo-FBP. The effect of ANS was naturally more pronounced at the high FBP concentration where higher orders of multimers are formed

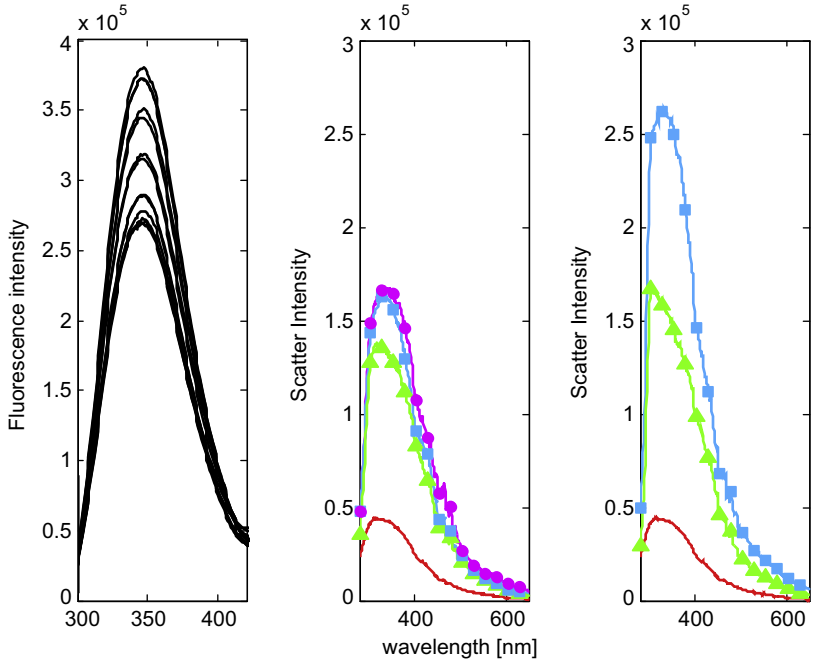

Fig. 1. (left): Effect of increasing concentrations of ANS (from top to bottom, 2,4,6,8,10,12,14,16,20,30 μM) on intrinsic fluorescence of 0.83 μM apo-FBP (excitation at 280 nm, emission maximum at 350 nm). (right): Effect of ANS on self-association of apo-FBP (0.83 and 3.33 μM). Light scatter plot by synchronous scans of the Rayleigh scatter peak with 0 nm offsett. FBP, light blue curves (squares); FBP + ANS 16 μM, light green curves (triangles); FBP + 2 μM ANS, purple curve (circles). PBS buffer of pH 7.4, red curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[6,14–16]. No effect on the light scatter intensity was seen in the presence of a low (2 μM) concentration of ANS.

Hydrophobic patches on protein surfaces are often involved in protein-inter-protein interactions such as self-association, which seem to implicate tryptophan groups [6,17]. A decrease of intrinsic tryptophan fluorescence subsequent to protein aggregation [21] accords herewith since it could be due to shielding of tryptophan groups on hydrophobic interfaces between aggregated protein molecules. The concentration-dependent ability of ANS to prevent self-association of native proteins [21,22] is related to its interaction with hydrophobic interfaces participating in protein–protein interactions. Hence, our data indicate that hydrophobic interactions play a decisive role in the self-association process of apo-FBP, and offer a reasonable explanation to the apparently weak effect of high ANS concentrations on intrinsic fluorescence of apo-FBP. Fluorescence is thus decreased due to energy transfer from tryptophan groups but at the same time increased due to solvent exposure of shielded tryptophan groups after suppression of apo-FBP self-association.

3.3. Enhanced fluorescence intensity and blueshift of ANS emission maximum in the presence of apo-FBP

Two different experiments were performed to analyze binding of ANS to a hydrophobic environment on the surface of apo-FBP.

In the first experiment concentration of apo-FBP (0.83 μM) was kept constant and increasing concentrations of ANS added (Fig. 2). In the second one ANS was kept at a constant concentration (10 and 2 uM) and concentration of apo-FBP increased (Fig. 3).

Fig. 2 shows increasing emission at 510–520 nm with raising concentrations of ANS after excitation at 280 nm (upper figure) and at 350 nm (lower figure). Addition of apo-FBP enhanced fluorescence intensity (several hundred per cent) and blueshifted the emission maximum of ANS to 480 nm (excitation at 280 nm) while a less pronounced (35%) enhancement of fluorescence and blueshift to a wavelength just below 500 nm was observed after excitation at 350 nm.

Delta enhancement of ANS fluorescence seemed to exhibit a linear increase with raising concentrations of ANS at both excitation wavelengths in the presence of a constant concentration of apo-FBP (0.83 μM). This suggests that the ability of apo-FBP to bind ANS is not exceeded even at high concentrations of ANS, where suppression of apo-FBP self-association (Fig. 1) could expose additional hydrophobic surfaces available for ANS binding.

In the second experiment we studied the effect of increasing concentrations of apo-FBP on fluorescence intensity and emission maximum of ANS (Fig. 3). A rise in concentration of apo-FBP from nil to 3.33 μM increased fluorescence intensity of 10 μM ANS at the current emission maximum from 4000 to 18000 and from 8000 to 180000 after excitation at 350 and 280 nm, respectively. A similar although less pronounced effect was seen with 2 μM ANS (data not shown). Delta enhancement of ANS (10 and 2 μM) fluorescence seemed to exhibit a linear increase with raising concentrations of apo-FBP at both excitation wavelengths (data not shown).

The emission maximum of ANS (10 μM) exhibited a blueshift to shorter wavelengths with increasing concentrations of apo-FBP. The blueshift approached a maximum delta value of 45–50 nm at 3.3 μM apo-FBP at both wavelengths (Fig. 3, right figure). The blueshift curve was less steep at 2 μM ANS especially after excitation at 350 nm.

All together these observations indicate binding of ANS to a highly hydrophobic microenvironment on the surface of apo-FBP since the binding is associated with increased quantum yield (enhanced fluorescence intensity at a given ANS concentration with apo-FBP added) and a blueshift in the wavelength of peak emission (Figs. 2 and 3). The emission intensity of ANS after excitation at 280 nm was several fold higher than after excitation at 350 nm, and so was the absolute and relative increase in quantum yield of ANS in the presence of apo-FBP (Figs. 2 and 3). The dependence of delta blueshift on the apo-FBP concentration was virtually the same after excitation at 280 and 350 nm at a high ANS concentra-

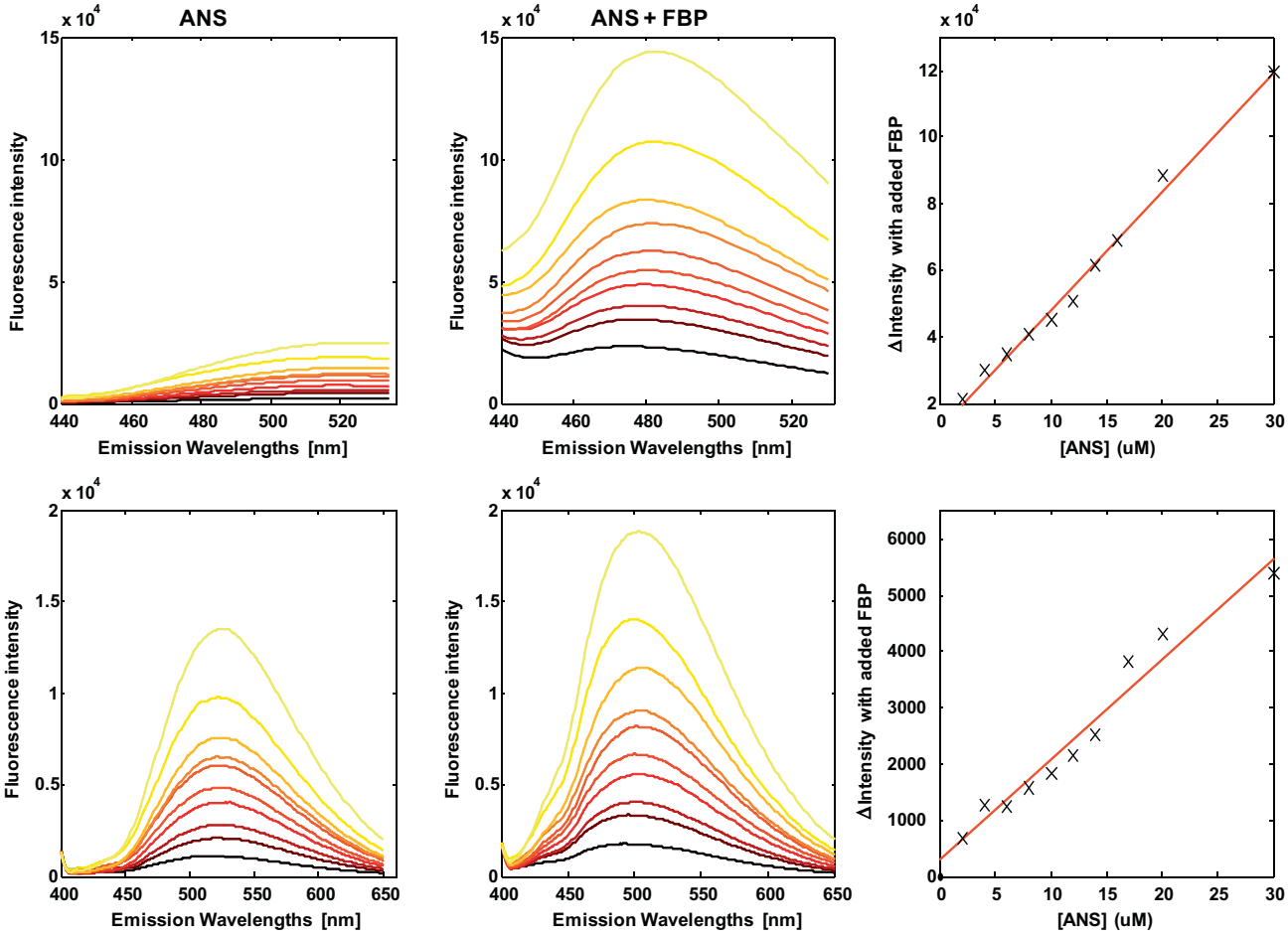

Fig. 2. Effect of apo-FBP (0.83 μM) on the emission spectrum of ANS (2–30 μM). Upper figure, ANS excitation at 280 nm. Lower figure, ANS excitation at 350 nm. Note the different ordinate scales in upper and lower figure. Emission spectra recorded at increasing concentrations of ANS (left plots), ANS with 0.83 μM apo-FBP added (middle plots) and the linear relation between concentration of ANS and increase (delta fluorescence) of ANS fluorescence with apo-FBP added (right plots). Colors indicate increasing ANS concentration from black to yellow (bottom to top). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tion (Fig. 3, right) but consistently lower values were obtained after excitation at 350 nm at a low ANS concentration, particularly at apo-FBP concentrations < 1 μM (Figs. 2 and 3, right).

3.4. Weak affinity of ANS to folate-bound holo-FBP

We have previously reported how ligand (folate) binding to FBP induces conformational changes involving a reorientation of tryptophan groups from the solvent surface to the inner hydrophobic core of FBP as evidenced by a diminished intrinsic fluorescence and blueshift of tryptophan emission maximum [10]. This event was associated with a reduced affinity to hydrophobic gels making us suspect a decrease in the surface hydrophobicity of holo-FBP [7]. To test this hypothesis we compared the affinity of ANS for apo and holo-FBP (Fig. 4).

Ligation of FBP (0.83 uM) with excess of folate reduced intrinsic fluorescence (tryptophan) of FBP by more than 80 per cent (Fig. 4, left plot), and blueshifted the emission maximum of tryptophan from 350 nm to 330–335 nm. Holo-FBP exhibited a weak binding affinity to ANS (10 μM), since ANS caused only a small decrease in intrinsic fluorescence of holo-FBP, and neither a blueshift nor enhancement of ANS emission was observed. The latter data sharply contrast the high affinity of ANS to apo-FBP evidenced by a markedly diminished intrinsic fluorescence of apo-FBP, a blueshift of emission maximum and a five-fold enhancement of the fluorescence intensity of ANS (Fig. 4, left plot). Similar albeit less marked effects were observed after excitation of ANS at 350 nm with apo-FBP added (data not shown). A series of experiments with 2 μM instead of 10 μM ANS gave similar results (data not shown).

We performed additional experiments with a high concentration (3.33 μM) of apo/holo-FBP combined with a high (20 μM) and a low (2 μM) ANS concentration. Addition of the high concentration of holo-FBP neither enhanced nor blueshifted the fluorescence of ANS after excitation at 280 nm (Fig. 4, middle plot). The nearly six-fold enhancement of ANS fluorescence seen after excitation at 350 nm (Fig. 4, right plot) was reduced (80%) when apo-FBP was replaced by equimolar holo-FBP.

Experiments with 2 μM ANS gave similar results (data not shown), however, with the noticeable exception that while no decrease of intrinsic fluorescence of apo-FBP occurred at 20 μM ANS (Fig. 4, left plot) this was actually the case with 2 μM ANS. This observation is explainable considering contributory fluorescence from tryptophan groups which become solvent exposed when multimers formed at high concentrations of apo-FBP deaggregate in the presence of high ANS concentrations (cf Fig. 1, right).

In our previous studies changes in the conformational structure of FBP occurred upon folate binding as evidenced by circular dichroism, infrared and near-infrared spectroscopy, fluorescence spectroscopy and stopped- flow fluorescence studies [7–11]. These structural alterations lead to a sharp decrease in the surface hydrophobicity of holo-FBP as shown herein, and reorientation of hydrophobic tryptophan residues from the solvent exposed surface of

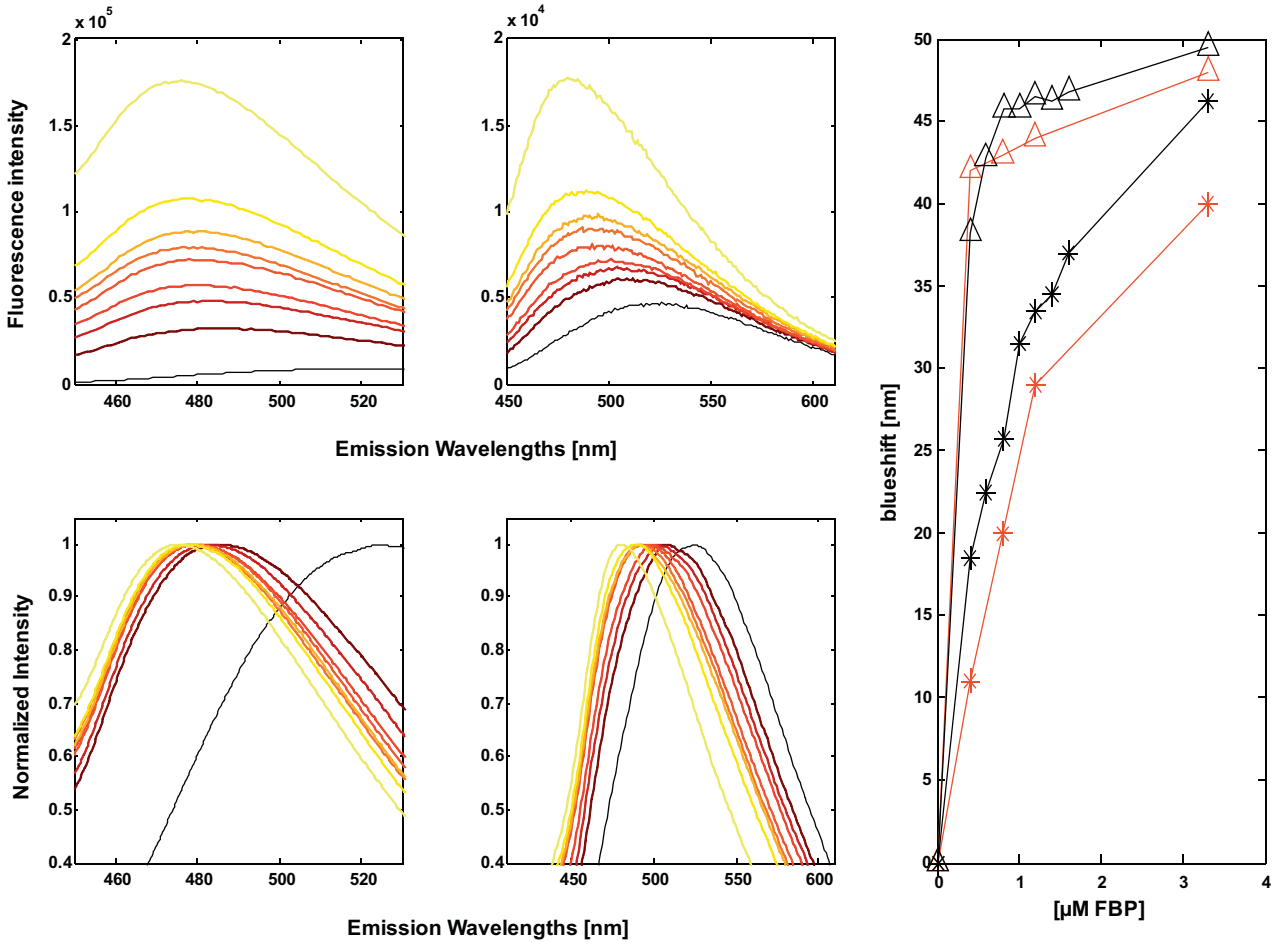

Fig. 3. Effect of increasing concentrations of apo-FBP on fluorescence intensity and maximum emission wavelength of ANS (10 μM). Upper figure Emission spectra of ANS after excitation at 280 nm (left) and at 350 nm (right) in the presence of 0.41, 0.62, 0.83, 1.0, 1.2, 1.4, 1.66 and 3.33 μM apo-FBP, from bottom (brown) to top (yellow). Black, no apo-FBP added. Note the different ordinate scales in the left and right plot. Lower figure Matching normalized emission spectra of ANS after excitation at 280 and 350 nm as above. Right figure Relationship between concentration of added apo-FBP (abscissa) and delta blueshift (nm) of the ANS peaks compared to no apo-FBP addition (ordinate). Excitation of 2 μM ANS at 280 nm red triangles, and at 350 nm red asterisks (lower curves). Excitation of 10 μM ANS at 280 nm black triangles, and at 350 nm black asterisks (upper curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apo-FBP to the inner apolar hydrophobic core of holo-FBP [10] could at least to some extent contribute to this effect.

Changes in the surface hydrophobicity of receptors and transport proteins subsequent to ligand binding and the important biological implications thereof have been reported in the literature.

The calcium binding protein, calmodulin (CaM), which stimulates the Ca^{++}-sensitive phosphodiesterase is an interesting example [12]. Binding of calcium induces conformational changes in CaM with exposure of a hydrophobic domain on the protein surface which binds hydrophobic fluorescent probes, e.g. ANS. This hydrophobic domain may serve as the protein–protein interface for the calcium-dependent complexation between CaM and phosphodiesterase in view of the well-established involvement of hydrophobic interactions in protein associations [12].

Another example is the human sodium/D-glucose cotransporter1 (hSGL T1). Changes in its surface hydrophobicity associated with different conformational states may affect trans-membrane passage of sugars and sodium ions [13]. Binding of the substrates D-glucose and sodium reduced hydrophobicity, whereas binding of phlorizin, an inhibitor of glucose transport, induced a different more rigid conformational state of hSGLT1 being more hydrophobic and less suitable for trans-membrane transport [13].

Ligand-induced changes of surface hydrophobicity had thus a decisive impact on the biological function of the above-described receptor or transport proteins. The biological significance of a drastic decrease in surface hydrophobicity of holo-FBP has yet to be resolved. The hydrophobic surface of apo-FBP (a soluble apoform of the folate receptor) should be suitable for binding of the aromatic pteridine ring, the moiety in the folate molecule shown to play the most decisive role in the interaction with apo-FBP [10]. A molecular model based on the sequence homology between the riboflavin-binding protein and FBP [23–25] predicts binding of folate to a hydrophobic pocket on FBP. Reasoning by analogy from the aforementioned studies on glucose transport across cell membranes, it is tempting to suggest that a decrease in surface hydrophobicity may facilitate translocation of holo folate receptors into the cell interior?

The self-association behavior of apo and holo forms of FBP seems to be markedly affected by the ligand -induced changes in surface hydrophobicity. The present data are thus consistent with the participation of hydrophobic interfaces in the concentration-dependent and reversible self-association of apo-FBP at pI [6,14–16]. Nevertheless, holo-FBP has despite its hydrophilic nature an even greater propensity to self-association into higher orders of very stable and water soluble multimers [6,14–16] suggesting involvement of hydrophilic rather than hydrophobic interfaces.

To conclude. The fluorescent probe ANS has been utilized to disclose a sharp decrease of FBP surface hydrophobicity upon folate binding and define the hydrophobic nature of interactions involved in self-association of apo-FBP. It seems reasonable to assume that

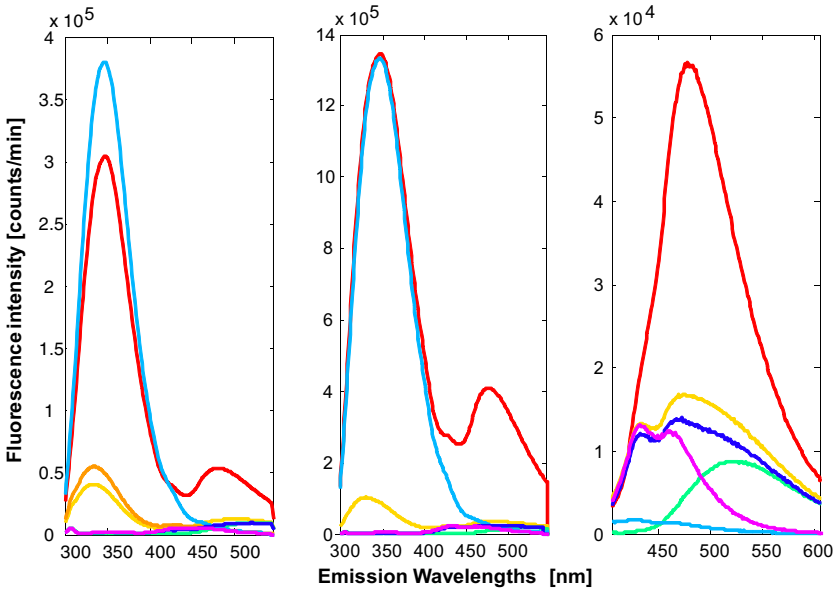

Fig. 4. Effect of ligand (folate) binding on the interaction between ANS and FBP. Left plot, Excitation at 280 nm with emission spectra of apo-FBP (0.83 μM), light blue; apo-FBP plus ANS (10 μM), red; holo-FBP (apo-FBP plus 2 μM folate), orange; holo-FBP plus ANS, yellow; ANS, light green; folate, magenta; ANS plus folate, dark blue. Middle and right plot, Excitation at 280 (middle plot) and 350 nm (right plot). Note the different ordinate scales in the middle and right plot. Emission spectra of apo-FBP (3.33 μM), light blue; apo-FBP plus ANS (20 μM), red; holo-FBP (apo-FBP plus10 μM folate) plus ANS, yellow; ANS, light green; folate, magenta; ANS plus folate, dark blue.

this information on important aspects of the structure of soluble FBP is applicable to membrane-bound folate receptors at the molecular level. As proposed, a markedly reduced surface hydrophobicity may facilitate intracellular passage of holo-receptors. Another possibility is that holo and apo receptors are allocated/sorted to different cellular compartments/structures according to surface hydrophobicity. This is an interesting aspect since apo and holo forms of FBP are prone to associate into firm asymmetric complexes leading to removal of apo-FBP from reaction with folate [6]. Natural and synthetic detergents normalized folate binding kinetics, and as shown herein probably due to a hydrophobicity/hydrophilicity phase separation of apo-FBP and holo-FBP [6].

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