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Partly folded states of bovine carbonic anhydrase interact with zwitterionic and anionic lipid membranes

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Abstract

The acidic, partly folded states of bovine carbonic anhydrase II (BCAII) were used as an experimental system to study the interactions of partly denatured proteins with lipid membranes. The pH dependence of their interactions with palmitoyloleoyl phosphatidylcholine (POPC) and palmitoyloleoyl phosphatidylglycerol (POPG) membranes was studied. A filtration binding assay shows that acidic partly folded states of BCAII bind to POPC membranes. Fluorescence emission spectra from Trp residues of the bound protein are slightly shifted to shorter wavelength and can be quenched by a water-soluble quencher of fluorescence, indicating that the binding occurs without deep penetration of Trp residues into the membrane. The content of β-structures of the protein in solution, as revealed by FT-IR spectroscopy, decreases in the partly folded states and the binding to POPC membrane occurs without further changes of secondary structure. In the presence of 0.1 M NaCl, a partly folded state self-aggregates and does not bind to POPC membrane. At acidic pH, BCAII binds to POPG membranes both at high and low ionic strength. The binding to the anionic lipid occurs with protein self-aggregation within the lipid–protein complexes and with changes in the secondary structure; large blue shifts in the fluorescence emission spectra and the decrease in the exposure to water-soluble acrylamide quencher of Trp fluorescence strongly suggest that BCAII penetrates the hydrocarbon domain in the POPG–protein complexes.

Keywords: Carbonic anhydrase; Partly folded protein; Lipid–protein interaction; Fluorescence; FT-IR

1. Introduction

The translocation of proteins across biological membranes in export and import processes requires the partial unfolding of the polypeptide chain [1–6]. It has been proposed that the partly folded state competent for membrane translocation is the molten globule [7]. It is also recognized that the insertion and the translocation of bacterial toxins into the host membrane require partial unfolding [8,9]. These bacterial toxins are soluble globular proteins and the conformational change that leads to membrane insertion is triggered by acidification of the protein environment. Partly folded intermediates are also relevant for the interactions of integral membrane proteins: the insertion of OmpA of *Escherichia coli* is coupled to the refolding process which occurs through a partly folded intermediate at the membrane surface [10,11]. The lipid membrane can also

Abbreviations: BCAII, bovine carbonic anhydrase II; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; FT-IR, Fourier transform infrared; LUV, large unilamellar vesicle

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play an important role in the unfolding reaction as demonstrated by the membrane-promoted transition to the molten globule of acetylcholinesterase [12]. Finally, several soluble globular proteins whose physiological roles are not directly related to membrane processes can also interact with lipid membranes under conditions of partial unfolding: the membrane interaction of the soluble protein α-lactalbumin, for example, occurs under conditions in which the aqueous medium induces the transition to a partly unfolded, compact protein [13–16].

Taken together, these examples suggest that the capacity of interaction with lipid membranes might be a distinctive property of partly folded states. In the present work, the interactions of the native and acidic unfolded compact states of BCAII with lipid membranes were studied. BCAII is a soluble globular protein located in the cytosol of erythrocytes. It is not expected that BCAII interacts physiologically with lipid membranes. The transition from the native to the unfolded state does not occur in a simple two-state process. Wong and Hamlin [17] have described two equilibrium folding intermediates at acidic pH for BCAII: at pH 3.6 appears a compact, partly folded intermediate with native secondary structure, exposed hydrophobic domains and missing tertiary contacts. At pH 2.5, a second transition occurs: more hydrophobic residues are exposed to the aqueous solvent and a large increase in the viscosity occurs while a compact structure is conserved.

Fluorescence and FT-IR spectroscopy of BCAII were used to study the conformation state of the protein and the interactions with membranes of the zwitterionic lipid POPC and the anionic lipid POPG within the pH range 7.4 to 2 at low and high ionic strength. Filtration through Centricon membranes and gel-filtration was also used to measure directly the binding of BCAII to the membranes. It was found that, depending on the pH, ionic strength and membrane lipid composition, BCAII establishes different kind of interactions with lipid membranes.

2. Materials and methods

2.1. Reagents

Carbonic anhydrase II from bovine erythrocytes (isoelectric point 5.9, formerly designated as carbonic anhydrase B), D_2O 99+%, NaOD, DCl, NaCl, Tris (tris(hydroxymethyl)aminomethane) glycine and Sephadex G-200 were from Sigma (St. Louis, MO) POPC and POPG were from Avanti Polar Lipids (Alabaster, AL).

2.2. Sample preparation for fluorescence spectroscopy and binding assays

To prepare large unilamellar vesicles (LUVs), the desired amount of lipid in chloroform solution was dried by a gentle stream of nitrogen. Remaining solvent was removed under high vacuum for several hours. The lipids were hydrated with bidistilled water containing 0.01 or 0.1 M NaCl. The aqueous suspensions were frozen and thawed five times and extruded through a polycarbonate filter, 100 nm pore diameter, in an extrusion device from Avestin (Ottawa, Canada). BCAII in the lyophilized form was solubilized in 10 mM Tris pH 7.4 or 10 mM Tris, 0.1 M NaCl pH 7.4 and centrifuged 30 min at $100\,000 \times g$ to eliminate any possible non-solubilized material. LUVs were mixed with the protein in 20–50 μl. A buffer at the desired pH and salt concentration was added to reach a final volume of 150 μl for fluorescence, 700 μl for the Centricon binding assay or 100 μl for gel-filtration. The samples were incubated overnight at 25°C and the pH values were measured immediately before the filtration assay or the spectroscopic measurements. No protein degradation was observed by SDS–PAGE. The buffer solutions between pH 2 and 4 contained 10 mM glycine, between pH 4 and 6.5 contained 10 mM acetic acid/sodium acetate, and at pH 7–7.4 contained 10 mM Tris. The pH was measured with a glass combination microelectrode MI-410 from Microelectrodes (Bedford, NH, USA).

2.3. Binding assays

2.3.1. Centricon-filtration

Samples containing pure protein and lipid–protein mixtures were loaded in the upper chamber of Centricon 100 concentrators (Amicon, Beverly, MA) and spun down at 3000 rpm in a Sorvall SM-24 rotor at room temperature until 50–60% of the initial volume was eluted. The protein concentration in the initial

sample and in the eluted fraction was measured by absorbance at 280 nm. The turbidity due to the LUVs remains in the upper chamber.

2.3.2. Gel-filtration

A binding assay was performed according to the method of Hummel and Dreyer [18]. A Sephadex G-200 column, 5 ml bed volume, was equilibrated with a buffer at the desired pH containing 0.7 μM BCAII. Once the column was equilibrated with the protein, 100 μl of a sample containing LUVs and BCAII at the same concentration than in the elution buffer was loaded. The flow rate was set at 0.24 ml/min. The UV absorbance at the output of the column was continuously monitored.

2.4. Fluorescence spectroscopy

The fluorescence spectra were recorded in a SLM 4800C spectrofluorometer. A quartz cell of 3 mm path length in a holder thermostatized at 25°C was used. All slits were set at 4 nm. Trp residues were selectively excited at 295 nm. The Raman contribution from water was subtracted from all spectra. To correct for the contribution of scattered light at the emission wavelength of Trp in the lipid-containing samples, the scattered light of samples containing pure lipid was recorded, scaled up to exactly match the spectra of the protein-containing samples in the region where only scatter is observed (about 260–80 nm) and subtracted from the spectra of the protein-containing samples. For the quenching experiment, samples of 1 ml initial volume were used. Small aliquots of a 6 M acrylamide solution were added stepwise. Fluorescence intensities were measured from the area of the spectra between 315 and 450 nm.

2.5. FT-IR spectroscopy

Five hundred micrograms of BCAII were solubilized in 50 μl of pure D_2O or D_2O containing 0.1 M NaCl. The p*D* was read with the glass microelectrode and adjusted with DCl or NaOD. Lipid-containing samples were prepared by adding the protein solution to 2 mg of dried lipid, incubating at room temperature for 2 h to allow lipid hydration, and adjusting the p*D*. All samples were incubated for 24 h at room temperature to allow deuterium exchange of amide protons. p*D* values were read before the spectroscopic measurement and are informed without correction for the difference in H^+ and D^+ activity [19]. FT-IR spectra were recorded at room temperature (about 25°C) on a Bruker IFS-28 spectrometer purged with dry air. A resolution of 2 cm^{-1} was used. Samples were placed in a cell with CaF_2 windows and 100-μm Teflon spacers. Signal from water vapor was digitally subtracted. Spectral resolution was enhanced by self-deconvolution with a program written by Dr. H.H. Mantsch [20] using a full bandwidth at half height (FWHH) of 17 cm^{-1} and an enhancement resolution factor (*k* parameter) of 1.75. The frequency position and the area of the individual components were determined by second derivative and curve fitting.

3. Results

3.1. Binding of BCAII to POPC and POPG LUVs

The binding of BCAII to lipid membranes as a function of the pH was measured by filtration through Centricon membranes. A protein of 30 kDa as BCAII passes through the 100-kDa cut-off membrane filter used in this assay while the LUVs are retained (see Section 2). The fraction of protein found in the eluant, when loaded in the presence or in the absence of lipid membranes, is shown in Fig. 1. The fraction of membrane-bound protein is obtained by comparing the amount of protein eluted in the absence with the amount eluted in the presence of lipids. As described below, the elution of the pure protein itself depends on the pH and the ionic strength. Further retention in the upper chamber, when loaded in the presence of lipid membranes, is due to binding to the lipids. At low ionic strength (Fig. 1b), the pure protein self-aggregates at pH between 3 and 4, as suggested by the small decrease in the fraction of eluted protein. A larger amount of protein is retained in the presence of POPC LUVs, indicating that BCAII binds to the zwitterionic lipid within this pH range [17]. It can be suspected that further retention of the protein in the upper chamber at acidic pH is due to clogging of the Centricon filter by LUVs. At least one possible cause of filter clogging is discarded: dynamic light-scattering measure-

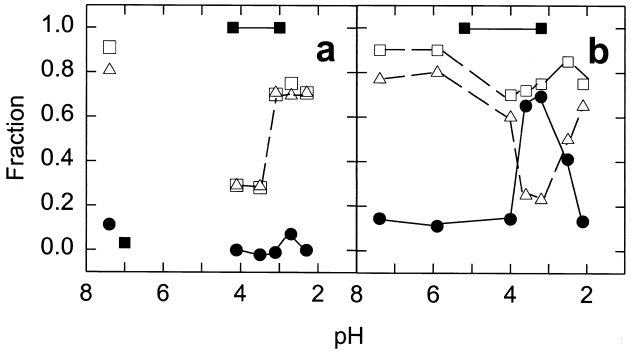

Fig. 1. Binding of BCAII to lipid membranes as a function of pH. (a) The aqueous medium contained 10 mM buffer plus 0.1 M NaCl. (b) The aqueous medium contained 10 mM buffer. Open symbols represent the fraction of the loaded protein that was found in the eluate: pure protein (□), protein in the presence of POPC LUVs (△). Filled symbols represent the fraction of protein bound to POPC lipid membranes (●) and to POPG lipid membranes (■). Initial protein and lipid concentrations were 2 μM, and 1.9 mM, respectively.

ments show that there is no vesicle aggregation. At pH 3 and pH 7, whether in the absence or in the presence of protein, similar Gaussian distributions of particle sizes with a maximum at 90 nm are observed (results not shown).

At pH 2, low ionic strength, the binding to the lipid is drastically reduced. In the presence of high ionic strength (Fig. 1a), a larger protein self-aggregation within the pH range corresponding to a partly folded state is observed. This makes it difficult to evaluate the binding to POPC membranes at high ionic strength. Nevertheless, the presence of POPC does not increase the retention in the upper chamber indicating a lack of binding in this condition. At acidic pHs, 100% of the protein is bound to POPG membranes whether at high or low ionic strength (see Fig. 1).

Because denatured, aggregated protein, could be retained as well as the lipid-bound protein, it must be considered that this methodology only provides a qualitative measurement of binding.

Binding to lipid vesicles was also demonstrated by gel filtration. The method of Hummel and Dreyer [18] was used. A Sephadex column is equilibrated with a buffer containing the ligand (BCAII in this case). A sample containing the macromolecule (LUVs, in this case) and the ligand at the same total concentration than in the equilibrating buffer is loaded. The binding is evidenced by the appearance of a trough in the elution profile of the ligand after the elution of the excluded particles. The area of the trough is a measure of the amount of ligand bound to the macromolecule. When POPG vesicles (lipid concentration 2 mM) are loaded at pH 5.5 and low ionic strength a deep and extended trough in the elution of BCAII is observed after the elution of the vesicles. The integrated area of the trough indicates that 0.012 mol of protein are bound per mol of lipid. It must be noticed that the amount of BCAII bound to POPG is larger than the amount available in the loaded sample (3.5×10^{-4} mol/mol). When POPC vesicles are loaded under conditions that the filtration assay reveals protein binding (i.e. pH 3 and low ionic strength) a weaker trough is observed. The integrated area indicates that 1×10^{-3} mol of protein are bound per mol of lipid. Again, this ratio is larger than the ratio of protein/lipid in the loaded sample (1×10^{-4} mol/mol). When POPC vesicles are loaded at pH 7 and low ionic strength (no binding, according to the Centricon assay), no depletion in the protein elution profile is observed after the elution of the vesicles. It is concluded that the gel filtration assay confirms the results observed with the Centricon assay: BCAII strongly binds to POPG vesicles at acidic pH; the acidic partly folded intermediate binds to POPC at low ionic strength, although more weakly than to POPG.

3.2. Fluorescence spectroscopy

The fluorescence emission of Trp residues was used to monitor the conformational changes and the interactions of pure BCAII or BCAII in the presence of lipid membranes as a function of the pH. Blue and red shifts of the emission bands can be interpreted as the transfer of Trp residues to an environment of lower or higher polarity, respectively [21,22]. The crystal structure [23] and the contribution of individual Trp residues to the fluorescence emission of human carbonic anhydrase [24] are known. Although this information is not directly available for BCAII, it can be assumed that the structure and Trp fluorescence pattern of both proteins are similar because of the high homology between them. Mårtenson et al. [24] have shown that the fluorescence of native human carbonic anhydrase mainly arises from the

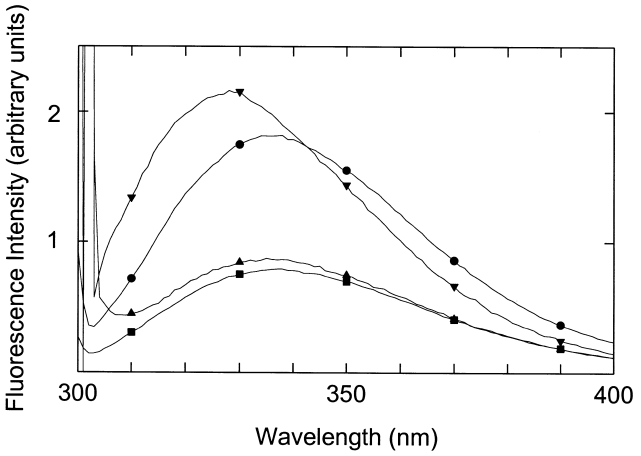

Fig. 2. Fluorescence emission spectra of BCAII. All samples contained 2 μM BCAII. Protein in the absence of lipid, no added salt, pH 7.2 (●), pH 2.8 (■); in the presence of 2 mM POPC, no added salt, pH 2.8 (▲); and in the presence of 2 mM POPG, no added salt, pH 3.1 (▼).

buried Trp-97 and Trp-245, while the fluorescence from the other five Trp residues is partly quenched due to intramolecular interactions.

The spectra of BCAII from representative samples are shown in Fig. 2. The ratio between the fluorescence intensity at 335 nm and the intensity at 355 nm (FI_{335}/FI_{355}) was used as a more sensitive parameter to detect changes in the band position than the wavelength of maximum emission, λ_{max} [25,26]; an increase in FI_{335}/FI_{355} reflects a blue shift of the spectrum. Under native conditions, i.e. pH 7, the spectra show a λ_{max} of 335 nm and a value for FI_{335}/FI_{355} of 1.3. To obtain information about the interaction with the membranes, the position of the emission band of the pure protein was compared with that observed in the presence of membranes at different pH values. The experiments were done at high and low ionic strength.

3.2.1. High ionic strength, pure protein

In the absence of membranes and in the presence of 0.1 M NaCl, the transition to the partly unfolded intermediate described by Wong and Hamling [17] within the pH range 3.8–3.2 occurs with a blue shift (Fig. 3a). Further decrease of the pH produces the second unfolded state identified as the acid-denatured state [17]. Spectra from this intermediate, within the pH range 3–2, are red shifted with respect to the first intermediate, but still blue shifted with respect to the native protein. It is expected that protein unfolding, even in a partial degree, leads to red shifted spectra; considering that the protein self-aggregates, the opposite spectral behavior observed here can be due to the transfer of Trp residues to an environment of low polarity within the protein aggregates.

3.2.2. High ionic strength, protein in the presence of membranes

The same profile of FI_{335}/FI_{355} as a function of the pH is observed in the presence of POPC membranes indicating that, neither the native form of BCAII nor the partly unfolded states interact with the zwitterionic lipid in the presence of high ionic strength, as already suggested by the filtration assays. In the presence of membranes composed of the anionic lipid POPG, a slightly blue-shifted spectrum is already observed at pH 7.4–7 (Fig. 3a). As the pH decreases, a large blue shift is observed between pH 6.0 and 5.0 with a mid-point for the transition at approximately pH 5.4. A second transition with smaller amplitude, toward longer wavelengths, is observed between pH 5 and 2 with a midpoint at pH 3.7. The spectral changes observed at acidic pH indicate the transfer of Trp residues to an environment of lower polarity in the lipid–protein complexes. Most probably, this apolar environment is provided by the lipid hydrocarbon chains, although protein–protein interactions can also contribute to the blue shift as described

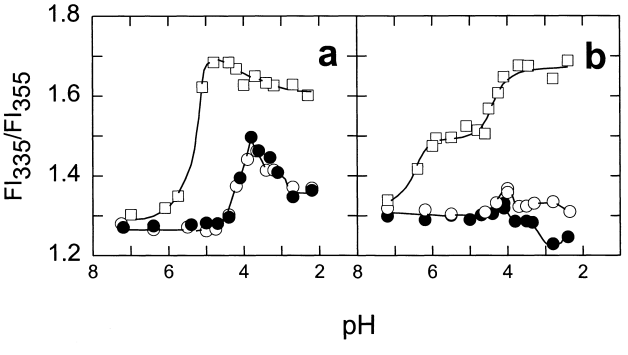

Fig. 3. Dependence of the position of the fluorescence emission band of BCAII with the pH. The band position was monitored by the ratio of the fluorescence intensity at 335 nm to that at 355 nm. (a) Samples containing 0.1 M NaCl. (b) Samples in the absence of NaCl. Samples contained 2 μM BCAII; in the absence of lipid (●); in the presence of 1.9 mM POPC (○); in the presence of 1.9 mM POPG (□). Temperature, 25°C.

below. It must be considered that the H^+ ion concentration near a negatively charged surface is higher than in the bulk solution: the electrostatic surface potential of phosphatidylglycerol membranes in contact with an aqueous medium containing 0.1 M NaCl is −60 mV [27]; this surface potential produces an increase in the surface H^+ ion concentration by a factor of $\exp(-z38.96\varphi_0)$, where z is the electric charge on the ion and φ_0 is the electric surface potential expressed in volts [28]. The result of this calculation is that the pH on the surface of POPG membranes is about one pH unit lower than in the bulk solution. Because the fluorescence changes in the presence of POPG vesicles also occur at one pH unit below the pH for the transition to the partly folded state in solution, it can be proposed that the interaction with POPG is triggered by the same transition that leads to self-aggregation in the absence of anionic membranes.

3.2.3. Low ionic strength, pure protein

At low ionic strength and in the absence of membranes, a small and reproducible transition to shorter wavelengths is also observed at pH about 4.1, although with a smaller amplitude than the transition observed in the presence of high ionic strength. As described above for the filtration assay, less aggregation is observed in this case than in the presence of 0.1 M NaCl, supporting the proposal that blue shifts in the absence of lipids are a consequence of protein–protein interactions. Below pH 4.0, the spectra display FI_{335}/FI_{355} values similar to the native protein and the band position remains constant within the pH range 3.8–3.3. Below pH 3.3, another transition occurs and the spectra are red-shifted.

3.2.4. Low ionic strength, protein in the presence of membranes

In the presence of POPC membranes at pH below 4.1, a transition similar to that observed in the absence of membranes occurs (Fig. 3b). Nevertheless, the spectra are blue shifted with respect to the pure protein, indicating an interaction with the zwitterionic lipid. A larger blue shift is observed at pH 2.7. In the presence of POPG, a rather weak interaction of BCAII with the anionic lipid is observed already at pH 7.4. As the H^+ ion concentration increases, a stronger interaction with the anionic membranes is evidenced by a large blue shift. This transition occurs in two well-separated steps with midpoints at pH 6.4 and 4.3. In the presence of POPG, BCAII is not detected free in solution between pH 5 and 2 (see Fig. 1); it can be concluded that the second transition at pH 4.3 is not due to further binding of the protein, but to different characteristics of the lipid–protein complex. As expected, the interaction with the membrane revealed by the spectral blue-shift, is triggered at a bulk pH even higher than in the presence of 0.1 M NaCl: the electric surface potential φ_0, and consequently the surface H^+ ion concentration of the POPG membranes, are larger at low ionic strength.

In summary, the partly unfolded state of BCAII at pH 4–3.8 presents blue-shifted spectra with respect to the native state, most probably due to protein self-aggregation; the effect is less noticeable at low ionic strength. The spectra of the acidic intermediate at pH 2.5–3 are red-shifted at low ionic strength and blue-shifted in the presence of 0.1 M NaCl with respect to the native state. According to the spectral band position, only the partly folded intermediates at low ionic strength interact with POPC membranes. Large blue-shifts are observed in the presence of POPG membranes as the pH decreases. The first transition as the pH decreases reveals the binding of BCAII to the POPG membranes, while the blue shift observed at lower pH indicates rearrangements of a membrane-bound protein.

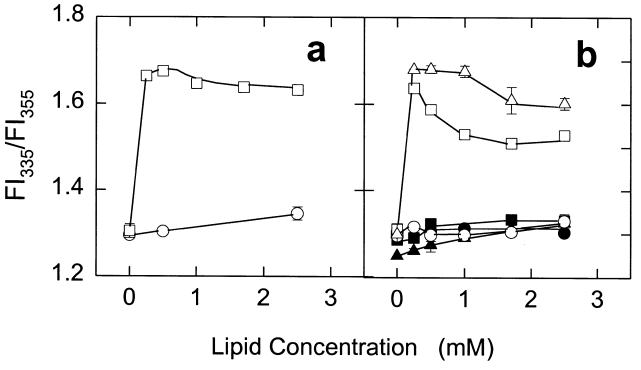

Fig. 4. Dependence of the position of the fluorescence emission spectra of BCAII with the lipid concentration. Protein concentration: 2 μM. (a) Samples containing 0.1 M NaCl; the lipid used is POPG; pH 7 (○), pH 4.3 (□). (b) Samples in the absence of NaCl; the lipids and pH are POPG, pH 7 (○); POPG, pH 5.0 (□); POPG, pH 3.0 (△); POPC, pH 7.2 (●); POPC, pH 3.6 (■); POPC pH 2.6 (▲). Temperature, 25°C.

3.3. Dependence of the fluorescence with the lipid concentration

At low ionic strength, pH 3.6 and 2.6 (Fig. 4b), the spectra from BCAII shift to shorter wavelength when the concentration of POPC increases. The dependence of FI_{335}/FI_{355} with the POPC concentration can be described considering a simple equilibrium between membrane-bound and free protein: there is a unique form of the membrane-bound protein whose FI_{335}/FI_{355} value is obtained when 100% of the protein is bound to the membrane, i.e. in the presence of a large excess of lipid. Intermediate values of the spectroscopic parameter contain proportional contributions of the bound and free protein. POPG membranes instead, already induce a large and steep blue shift at low lipid concentrations. The spectra are then red shifted as the lipid concentration increases. This effect is observed whether in the presence (Fig. 4a) or in the absence (Fig. 4b) of 0.1 M NaCl. This result strongly suggests that BCAII self-aggregates in lipid–protein complexes of high protein/lipid ratio displaying a blue shifted spectrum. As more lipid becomes available, the protein is diluted within the complex and displays a spectrum that is red-shifted as compared to the aggregated species. It must be noticed that the value of FI_{335}/FI_{355} in POPC-containing samples extrapolates to a maximal value well below that obtained with POPG. This indicates that the spectroscopic differences are due to intrinsic differences of conformation and interactions and not simply to different amounts of bound protein.

3.4. Dependence of the fluorescence with the protein concentration

In view that the filtration assay indicates that BCAII self-aggregates in 0.1 M NaCl pH 4, it can be proposed that the large blue shift observed under these conditions (Fig. 1a) is due to protein–protein interactions. In order to study this possibility, the spectral shifts as a function of protein concentration were measured. Fig. 5 shows that at pH 4 and 0.1 M NaCl the band position is blue shifted as the concentration of BCAII increases. Under experimental conditions in which self-aggregation is expected to be less important, no significant variation of the band position with the protein concentration is observed.

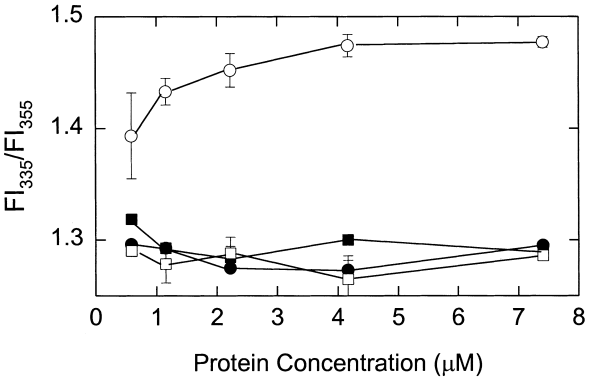

Fig. 5. Dependence of the position of the fluorescence emission spectra of BCAII with the protein concentration. Open symbols: samples prepared in the presence of 0.1 M NaCl; closed symbols: samples in the absence of NaCl. pH 3.5 (○); pH 7 (□); pH 7 (■); pH 3.9 (●).

3.5. Fluorescence quenching

The fluorescence from Trp residues was quenched with acrylamide, a water-soluble quencher, in order to investigate the shielding of the fluorophores from the aqueous solvent due to the interaction with lipid membranes. The data were analyzed according to the classic Stern–Volmer equation: $FI_0/FI = 1+K_{SV}[Q]$. FI_0 and FI are the fluorescence intensities in the absence and in the presence of acrylamide respectively, $[Q]$ is the molar concentration of quencher and K_{SV} is the Stern–Volmer constant. The plots of FI_0/FI as a function of $[Q]$ yield straight lines within

Table 1
K_{SV} values (M^{-1}) for the acrylamide quenching of the Trp fluorescence

pH	Pure protein	POPG
5.0	7.5	2.9
3.5	8.8	2.5
5.0	6.8[a]	3.3[a]
		POPC
3.7	8.8	7.9
2.6	9.6	6.6
3.5	5.5[a]	5.5[a]

Samples contained 10 μM of protein pure in solution or in the presence of 2 mM of the indicated lipid.
[a]Samples containing 0.1 M NaCl.

the concentration range 0–0.2 M. The static contribution to the quenching [29] was not considered. K_{SV} is proportional to the fluorescence lifetime and to the frequency of collisions between quencher and fluorophore. If the fluorescence lifetime remains constant, a decrease in the value of K_{SV} can be interpreted as a decrease in the exposure of fluorophores to the aqueous solvent. Table 1 shows the values of K_{SV} for BCAII pure in solution and in the presence of lipid membranes at several pH values. At low ionic strength, in the absence of lipids and pH values at which the protein is partly unfolded (pHs 3.5, 3.7, and 2.6 in Table 1) K_{SV} slightly increases as compared with the value observed for the compact state (pH 5 in Table 1). In the presence of NaCl 0.1 M and pH 3.5, the quenching decreases as compared with pH 5, this could be due to the shielding of Trp residues in the aggregated protein, although changes in the fluorescence lifetime can also be involved. In the presence of POPC membranes and low ionic strength, a decrease in K_{SV} is observed at pH 2.6, indicating some extent of Trp shielding from the aqueous medium. Again, this change could also be influenced by a change in the fluorescence lifetime. A clear decrease in the exposure of Trp to the solvent is observed when the protein is bound to POPG.

3.6. FT-IR spectroscopy

It can be expected that the binding of the partly folded states to the lipid membrane results in conformational changes of the protein. To get insight into this point, the FT-IR spectra of BCAII in the presence of lipid membranes were compared with the spectra in solution without lipids.

At p*D* 6.8, the integrated area of the components assigned to β-structures (bands at 1625, 1636, and 1678 cm^{-1}) yields the 51% of total area of the amide I′ band while 18% corresponds to helix structures (band at 1657 cm^{-1}). Components at 1647 cm^{-1} assigned to unordered structures, and at 1668 and 1690 cm^{-1} which can be assigned to turn and bends are also observed. The same results are obtained whether at high or low ionic strength and are in excellent agreement with the results form Byler and Susi [30]. The presence of POPC (not shown) or POPG does not produce significative changes in the spectra as expected from the lack of binding to the lipids at this p*D* value. At p*D* 3.5 (Fig. 6) in the absence of lipids and in the presence of salt, components at 1618 and 1684 cm^{-1} appear. They can be assigned to protein–protein contacts between unfolded chains [31–33]. The components assigned to β-structures are greatly diminished. These observations indicate that changes in the content or in the arrangement of β-structures occur at p*D* 3.5. A band that can be assigned to α-helix at 1658 cm^{-1} remains present. At p*D* 2.4, in the absence of lipid, and at high ionic strength, the bands at low and high frequencies due to protein aggregation are not present. Instead of the band at 1657 cm^{-1} (assigned to α-helix in the native conformation) a band at 1650 cm^{-1} appears, although it cannot be decided whether it is due to a helix structure or to a shifted component due to unordered chains. The binding to POPG membranes at p*D* 3.5 and 2.4 produces a large change as compared with the spectra in solution. The main feature is a large increase in the intensity of the band due to protein–protein contacts. This is in agreement with the proposal that BCAII further aggregates in the POPG–protein complexes. The presence of POPC membranes does not produce any noticeably change in the spectra (not shown).

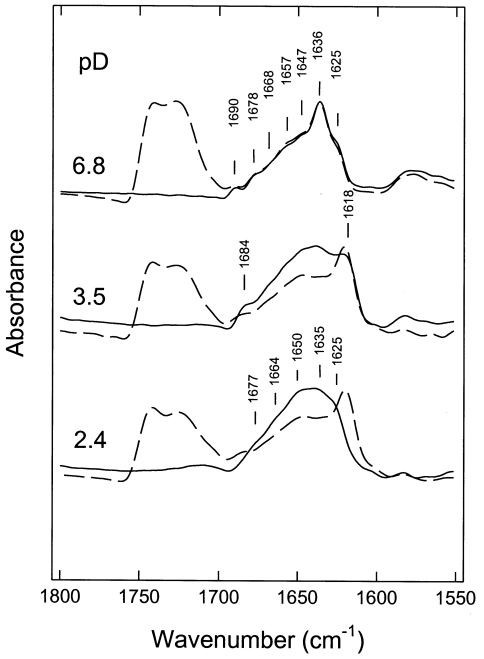

Fig. 6. Self-deconvolved FT-IR spectra of BCAII in the absence (continuous line) or in the presence (dotted line) of POPG. p*D*s are as indicated in the figure. Samples contained 0.1 M NaCl.

In the absence of added salt, the components at 1636 cm^{-1} also decrease at acidic p*D*s, although other secondary structure components are still present. The band at low frequencies due to protein aggregation is not observed at any p*D* value in the absence of salt for the protein in solution. Similar effects as described above in the presence of salt are observed at low ionic strength when POPG membranes are present. The presence of POPC does not produce major changes in the spectra at the p*D* studied whether at high or low ionic strength.

It is concluded that the partly unfolded states at acidic p*D*s, as revealed by FT-IR spectroscopy, retain a certain amount of secondary structure, although different from the native one. This is in agreement with the changes in CD spectra described by Wong and Hamlin [17]. The present results show that the changes in secondary structure are mainly due to loss or rearrangement of β-sheet components. The binding to the zwitterionic lipid does not produce noticeable changes in the secondary structure which is in agreement with a peripheral interaction. The binding to POPG instead, occurs with increase in the protein–protein interaction and further decrease in the content of β-structures.

4. Discussion

The main conclusions from the present study are: (1) the acidic partly folded states of BCAII have an increased capacity to bind to POPC membranes as compared to the native and to the unfolded state; (2) the binding to the zwitterionic lipid occurs without deep penetration of Trp residues into the membrane suggesting a peripheral interaction of the whole protein, although penetration of other segments cannot be ruled out; and (3) the binding to anionic lipid membrane is also triggered by acidic conditions, but in this case, the interaction occurs with penetration into the hydrocarbon core, protein self-aggregation, and changes in the protein secondary structure.

These results can improve the understanding of the interactions of partly folded proteins with lipid membranes and the role of anionic lipids in translocation process. The interaction with the zwitterionic membrane results in the transfer of Trp residues to a less polar environment. The changes in the fluorescence spectra are relatively small, suggesting that the Trp residues do not penetrate the hydrocarbon region of the membrane. The changes in the Trp environment observed upon binding can also reflect a conformational change induced by the interaction with the surface. In any case, these changes do not involve large rearrangements of secondary structure as can be concluded from the comparison of infrared spectra in solution and in the presence of POPC membranes. The exposure of non-polar residues to the aqueous solvent is a characteristic of the partly folded states [34]; the transfer of these residues from the aqueous to the membrane environment can be the driving force for the binding to the lipid membranes. A peripheral interaction without deep penetration of BCAII into the hydrocarbon membrane core could be driven by hydrophobic residues: Jakobs and White [35] have shown that the removal of a small fraction of hydrophobic surface from the contact with the solvent can account for the binding of small peptides to lipid membranes. In the more unfolded state at pH 2, even more hydrophobic residues are exposed to the solvent [17], nevertheless the interaction of BCAII with the membrane decreases: binding is reduced and fluorescence spectra tend to be similar in the presence and in the absence of POPC membrane. Apparently, hydrophobic domains or patches, which are disrupted at pH 2, are necessary to trigger the interaction with the lipid membrane. Other explanations can be proposed to explain the reduced binding at pH 2. One could be that the phosphate groups in POPC are partly protonated [36], and this can favor an electrostatic repulsion between the membrane surface and the positively charged protein. Another reason could be an unfavorable loss of entropy upon binding: the conformational entropy of an unfolded polypeptide chain is increased because of the large internal degree of freedom. [37,38]; if the binding to the membranes occurs with restrictions in the peptide internal motions, the unfavorable loss of conformational entropy could be the reason for the reduced binding under these conditions.

In the presence of high ionic strength (0.1 M NaCl) no compact state of BCAII binds to POPC membrane. Infrared and fluorescence spectroscopy and the filtration assay indicate that in the absence

of membranes BCAII self-aggregates at pH between 4.1–3.5, most probably due to the decrease in intermolecular electrostatic repulsion. It can be proposed that the lack of binding at high ionic strength is due to the competition with the self-aggregation process. Ionic strength could also influence directly on the interaction between the zwitterionic membrane and the protein: the dipole organization of the zwitterionic membranes results in the negative end of the general dipole pointing towards the aqueous phase [39] helping the binding of positively charged molecules; the increase in ionic strength should decrease the electrostatic attraction between the charged protein and lipid dipoles decreasing the binding to the membrane.

The binding to the anionic lipid is also dependent on the pH and occurs at acidic pHs both at high and low ionic strength. The large changes in the fluorescence spectra of the protein bound to POPG membranes indicate that Trp residues are transferred to an environment of low polarity; together with the decrease of fluorescence quenching by the water-soluble acrylamide, it strongly suggests that the Trp residues are inserted into a hydrocarbon environment within the lipid–protein complexes. The dependence of fluorescence spectral shifts with POPG concentration (Fig. 4) indicates that protein–protein interactions also contribute to these spectral changes.

The anionic lipids can participate in the protein binding in several ways. Because the H^+ ion concentration on the surface of the anionic interface is higher than in the bulk solution (see Section 3), the pH-dependent membrane interaction should be observed at higher bulk pH than in zwitterionic vesicles: the same profile of fluorescence intensity versus pH, although shifted towards pH values larger than in the presence of POPC LUVs, should be observed in the presence of POPG LUVs. The concentration of positively charged protein within the Gouy–Chapman double layer is also greater than in the bulk of the solution or at the surface of zwitterionic lipid membranes. This should produce an increase in the apparent affinity for POPG membranes as compared with POPC. If these were the only ways by which the anionic lipid influence the binding, similar spectroscopic changes should be observed in the presence of saturating amounts of membranes, whether anionic or zwitterionic. Clearly, other effects besides the increase in protein and H^+ ion surface concentration are involved in the binding to the anionic lipid: fluorescence changes not only occur at higher pH, but also the extent of the changes and the shape of the curves are different in the presence of POPG as compared to POPC membranes (Fig. 4a and b), indicating that the interaction with the anionic lipid is intrinsically different. The shift to shorter wavelengths, observed as the pH decreases, occurs together with the binding to POPG; this blue shift must contain contributions due both to the transfer from the aqueous medium to the membrane, and to protein–protein interactions within the lipid–protein complexes. Once the protein is bound, at low ionic strength, a second blue shift is observed as the pH decreases. Because the general exposure of Trp residues to the fluorescence quencher (Table 1) are not significantly different at pH 5.0 and 3.5, it can be suggested that this second blue shift contains more contributions from structural rearrangements than from further insertion into the membrane.

The molten globule state of α-lactalbumin, a globular, soluble protein [40], and its interactions with lipid membranes have been extensively studied [13–15,41]. Hanssens et al. [42] have shown that at acidic pH, α-lactalbumin has a surfactant-like effect on phosphatidylcholine membranes inducing the formation of mixed micelles of protein and lipid. A remarkable restriction in the mobility of spin probes attached to the C-12 position of PC have also been observed, indicating interactions with the lipid hydrocarbon chain of the zwitterionic lipid (Montich and Marsh, unpublished results). There is a clear difference between the nature of the interactions, triggered by H^+ ion binding, that α-lactalbumin and BCAII establish with zwitterionic lipids; while α-lactalbumin behaves like an integral membrane protein, BCAII binds peripherally without deep penetration into the membrane core. Apparently, the only presence of solvent-exposed hydrophobic domains in partly folded proteins does not determine the nature of the interaction with lipid membranes. Other features, such as the particular characteristics of the remaining secondary structure and the relative stability of the membrane-inserted protein, are also relevant.

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