ROLE OF TYROSINE RESIDUES IN THE BINDING OF COLIPASE TO TAURODEOXYCHOLATE MICELLES

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1. Introduction

Colipase, a small protein synthesized by the pancreas, counteracts the inhibitory effect of bile salts upon the hydrolysis of emulsified triglycerides by pancreatic lipase [1–4]. It is now accepted that colipase helps the anchoring of lipase to the interface in the presence of bile salts [5–8]. However colipase has been shown to form a 1 : 1 complex with a micelle of pure bile salt [9–11]. The formation of this complex is probably not related to any physiological role but rather results from the property of the protein to adsorb at polar interfaces [6]. The type of interaction in the micelle–colipase complex could thus constitute an attractive model to study the structure–function relationship of this protein in homogeneous phase by spectrophotometric methods.

In this work, the role of the tyrosine residues in the binding process has been investigated. The three tyrosine residues of the molecule, which does not contain any tryptophan residues, are very close in the primary structure [12] and are located in an especially hydrophobic region: Leu–Tyr–Gly–Val–Tyr–Tyr–Lys. It has been shown that the ultraviolet spectrum of these residues is strongly perturbed in the presence of taurodeoxycholate micelles [11], suggesting that some of them are part of the binding site of colipase. This study has been extended by solvent perturbation of the ultraviolet spectrum [13] and by fluorescence quenching of the tyrosine residues with small ions [14].

The results presented here strongly suggest that two tyrosine residues of colipase interact with the hydrophobic core of the taurodeoxycholate micelle and therefore are involved in colipase binding to interfaces.

2. Materials and methods

2.1. *Materials*

Pancreatic colipase I, kindly provided by Dr M. Charles, was prepared as in [10]. This form contains 84 residues [12] with glycine as N-terminal residue. Longer colipase, prepared by E. Fogglizzo and C. Chapus (unpublished), containing 105–110 residues with valine as N-terminal residue, has also been used in some studies. No significant differences between these two colipases could be noticed in those assays. The colipase concentration was calculated by using a molar absorption coefficient at 280 nm of 3920.

The solvents (R.P. grade from Prolabo) were redistilled before use. KI and CsCl were obtained from Merck. Sodium taurodeoxycholic acid (Sigma) was purified to remove a fluorescent impurity by treating an ethanolic solution with charcoal and then recrystallized twice from absolute ethanol. Deuterium oxide (99.8% D_2O) was purchased from CEA, Saclay.

2.2 *Methods*

Ultraviolet difference spectra were made with a Cary 14 spectrophotometer in double compartment cuvettes thermostated at 20°C. Fluorescence spectra were recorded at 20°C with the aid of a FICA 55 absolute differential spectrofluorimeter. Quenching measurements by I^- and Cs^+ (0–0.2 M) were performed

at constant ionic strength adjusted by the addition of NaCl. A small amount of $S_2O_3^{2-}$ (10^{-4} M) was added to the iodide solution to prevent I_3^- formation. The excitation and emission wavelengths were 280 nm and 303 nm, respectively. In these conditions no correction was necessary for the iodide absorption. The quantum yield of colipase was calculated by comparing the integrated fluorescence intensity of colipase and that of a solution of L-tyrosine in water corrected for the same absorption at the exciting wavelength and using the value of 0.21 as the quantum yield of L-tyrosine [15].

3. Results

3.1. *Accessibility of tyrosine residues by solvent perturbation of adsorption spectra*

Accessibility of the tyrosine residues to different solvent molecules of increasing size has been measured in native colipase (table 1). In each case the ultraviolet difference spectrum showed two maxima and the differential molar absorptions were compared to that given in [13] for a model compound, *N*-acetyl tyrosine ethyl ester. Assuming for each tyrosine residue the same $\Delta\epsilon_M$ taken as equal to that of the model compound, it can be calculated that at least two residues were accessible to methanol. The accessibility of tyrosines then slightly decreases when the size of the perturbing solvent molecule increases.

3.2. *Accessibility of tyrosine residues by fluorescence quenchers and D_2O in the absence or in the presence of micelles*

Two ions of opposite charges (I^- and Cs^+) were chosen to account for an eventual electrostatic repulsion due to the emitter surroundings. The modified Stern-Volmer plot (fig.1) proposed [14] was used since in some case only a fraction of the total fluorescence could be quenched. The plots in fig.1 are apparently linear both for I^- and Cs^+ and give a similar value for the Stern-Volmer (K_Q = 4.6 and 5 M^{-1}) constant, which correspond to a diffusion controlled reaction between the quencher and the chromophores, assuming an identical quantum yield for the three tyrosine residues (table 2).

However, whereas the total tyrosine fluorescence could be quenched by iodide, only a third of it was accessible to Cs^+.

Surprisingly, and by contrast with the ultraviolet spectrum, the intensity of fluorescence of colipase was not sensibly modified upon binding with taurode-

Table 1
Accessibility of tyrosine residues to different solute molecules in native colipase

Perturbent	Mean diam.	Δ_{max} (nm)	$\Delta\epsilon_M$ for *N*-acetyl tyrosine ethyl ester	$\Delta\epsilon_M$ for colipase	No. exposed tyrosine/mol colipase
Methanol 20%	2.8 Å	285.5	75.5	151.6	2.0
		278.5	41.4	109.3	2.6
Ethylene glycol 20%	4.4 Å	285.5	92.1	133.0	1.4
		278.5	54.9	75.3	1.4
Glycerol 20%	5.2 Å	286	84.1	100.0	1.2
		278.5	48.5	79.1	1.6
Glucose 20%	7.2 Å	286	41.5	55.3	1.3
		279	26.2	40.0	1.5

$\Delta\epsilon_M$ values for colipase have been determined in a 10 mM potassium phosphate buffer, at pH 6.8, containing 0.1 M KCl. The $\Delta\epsilon_M$ values for *N*-acetyl tyrosine ethyl ester have been taken from [13]

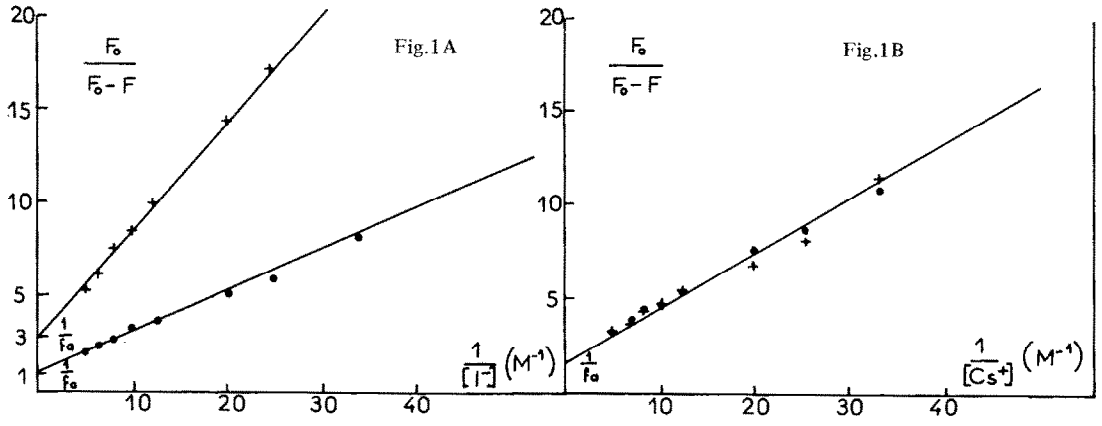

Fig.1. Modified Stern-Volmer plot for the quenching of colipase fluorescence by I^- (A) and Cs^+ (B). The experiments were carried out in a 10 mM Tris–HCl buffer, at pH 8.0. The total ionic strength was adjusted to 0.4 by adding adequate concentration of NaCl. The excitation and emission wavelengths were 280 nm and 303 nm, respectively. (•——•) In the absence of taurodeoxycholate; (+——+) in the presence of 8 mM sodium taurodeoxycholate. Colipase was 10 μM. Symbols as in [14].

oxycholate micelles. In the colipase–micelle complex the accessibility of tyrosine residues to Cs^+ remained the same as in the native colipase, whereas only one third of the fluorescence could be quenched by I^- (fig.1). This result suggests that some tyrosine residues were buried by the interaction with the micelle.

It has been shown that the quantum yield of tyrosine in aqueous solution could be enhanced by replacing H_2O by D_2O [16]. This property was used to investigate the accessibility to water of tyrosine residues in colipase, in a similar way as in the solvent perturbation method. The fluorescence intensity of tyrosine in aqueous solution was found to be multiplied by 1.2 in 100% D_2O whereas colipase fluorescence was increased 1.175-fold. The presence of taurodeoxycholate micelles did not noticeably change the accessibility to D_2O. By contrast with the results found with lecithin micelles [17] only a very small decrease in accessibility of 1,8-anilinonaphtalene sulfonate was observed when the probe was bound to the taurodeoxycholate micelle. These micelles might be more hydrated than the lecithin micelles and thus less sensitive to the solvent isotope effect. They are therefore not suitable to seek for a difference in accessibility of colipase tyrosine residues when the protein is bound to the micelle.

Table 2
Quenching of colipase fluorescence by I^- and Cs^+

	Quencher	K_Q (M^{-1})	f_a	Fluorescence lifetime in absence of quencher	Bimolecular rate constant of collisional quenching (k_q) (M^{-1} s^{-1})
Colipase	I^-	4.55	1	2.6×10^{-9} s	1.7×10^9
	Cs^+	5.20	0.3	2.6×10^{-9} s	2.9×10^9
Colipase + 8 mM taurodeoxycholate	I^-	5.3	0.3	2.6×10^{-9} s	2.04×10^9
	Cs^+	5.2	0.3	2.6×10^{-9} s	2.0×10^9

The Stern-Volmer constant K_Q and f_a were calculated from fig.1 as in [14]. The fluorescence lifetime was calculated as in section 2. k_q was calculated from $K_Q = k_q \tau$

3.3. *Origin of the spectral modification observed with colipase in the presence of taurodeoxycholate micelles*

The ultraviolet spectrum change resulting from the binding of colipase to micelles could be due either to the inclusion of the tyrosine residues into the hydrophobic core of the micelle or to a conformational change of the protein burying the tyrosine residues in a hydrophobic region of the molecule itself. Two models have been used to test these two possibilities:

(i) *N*-Acetyl tyrosine ethyl ester included into a micelle.

(ii) *N*-Acetyl tyrosine ethyl ester in ethanol which mimics the hydrophobic interior of the protein [13].

As shown in fig.2, increasing concentrations of taurodeoxycholate indeed perturbed the ultraviolet spectrum of the tyrosine analogue although with a much lower efficiency than in the case of colipase and the maximum difference was at 286 nm. In table 3, are reported the maximum wavelengths of the different spectra and the corresponding $\Delta\epsilon_M$ at 286 nm at infinite taurodeoxycholate concentration. The $\Delta\epsilon_M$ for colipase was exactly twice that of *N*-acetyl tyrosine ethyl ester in the same conditions. On the other hand, the wavelength of the second maximum of the difference spectrum was very close to that found for the model compound.

By contrast, table 3 shows that even if the perturbation by the micelle concerned only one tyrosine residue of the colipase, its $\Delta\epsilon_M$ would be too small to fit the model *N*-acetyl tyrosine ethyl ester in ethanol.

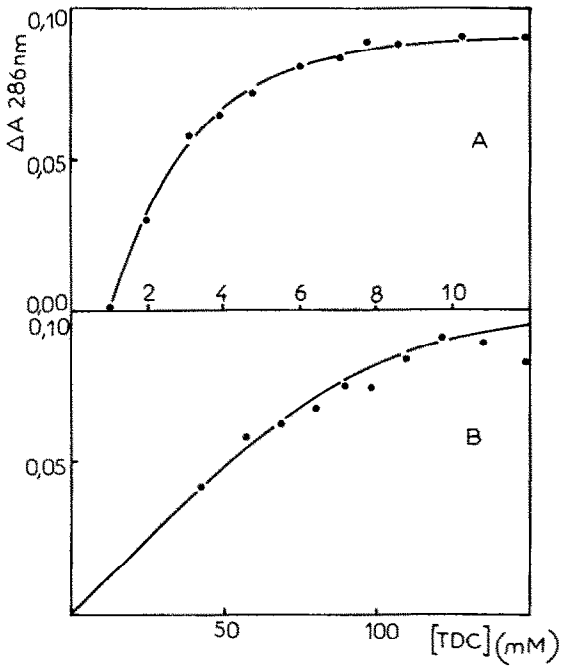

Fig.2. Binding of colipase (A) and *N*-acetyl tyrosine ethyl ester (B) to taurodeoxycholate micelles. Colipase and *N*-acetyl tyrosine ethyl ester were 0.17 mM and 0.56 mM, respectively, in the same buffer containing 0.1 M Tris–HCl, at pH 8.0, 0.1 M NaCl and 1 mM $CaCl_2$ at 20°C. The interaction was followed by measuring ΔA_{286} in the presence or in the absence of taurodeoxycholate.

Moreover, the position of the second maximum wavelength of the difference spectrum does not correspond to that of the model.

Table 3
Comparison of the spectral differences between colipase in the presence of taurodeoxycholate micelles and *N*-acetyl ethyl ester in the presence of taurodeoxycholate micelles or in 97% ethanol

	Colipase + taurodeoxycholate	*N*-Acetyl tyrosine ethyl ester + taurodeoxycholate	*N*-Acetyl tyrosine ethyl ester + ethanol
$\Delta\epsilon_M$ at 268 nm	590	289	735
Other λ_{max} (nm)	278.4	278.5	279.4

Same experimental conditions as in fig.2. The $\Delta\epsilon_M$ values were calculated from the asymptote of the saturation curve of the chromophores by taurodeoxycholate

4. Discussion

The results presented here have shown that in the native colipase the tyrosine residues are exposed to small ions or neutral molecules and therefore probably to water, as further suggested by the enhancement of colipase fluorescence in D_2O. The slight difference in accessibility to iodide and to methanol could be due to the smaller dimensions of the former (2.19 Å). Many arguments favour however the idea that the environment around the three tyrosine residues is different, as it has already been suggested by NMR data [18,19]. The results obtained by solvent perturbation of the absorption spectrum indicate that one tyrosine residue is not accessible to molecules larger than 2.8 Å whereas at least one tyrosine residue is still accessible to large molecules such as glucose (7.2 Å). On the other hand the fluorescence quenching experiments show that some tyrosine residues are surrounded by positively-charged groups, which do not allow the Cs^+ to reach them. From the accessible fraction to the quenchers (f_a) it is very tempting to assume that the tyrosine residues can be divided into two groups: one residue being accessible to all kind of molecules and very exposed at the surface of the protein, the two other being relatively more buried and surrounded by positively-charged groups.

The ultraviolet spectrum of tyrosine residues in colipase is strongly modified upon binding with taurodeoxycholate micelles [11]. Comparison with model compounds and studies of accessibility to ions strongly suggest that two tyrosine residues are involved in the binding of colipase to taurodeoxycholate micelles. It is likely that these two residues are, in the native protein, those which are surrounded by positively-charged groups since the accessibility to Cs^+ is not modified by the binding to the micelle. The comparison with model compounds favours an inclusion of the tyrosine residues inside the hydrophobic core of the micelle more than a colipase conformational change burying them inside a more hydrophobic region. These results of course do not exclude an eventual conformational change occurring in this region or any other part of the molecule during the binding process.

This interaction, however, is not believed to be the only one responsible for the affinity of colipase for micelles, as suggested by the much lower affinity of *N*-acetyl tyrosine ethyl ester for the micelle. The inclusion of the tyrosine residues inside the micelle could be favoured by positively-charged groups surrounding them in the protein which could interact with the negatively charged surface of the micelle. This is in agreement with [20]. By contrast, it is suggested that the tyrosine residues are not involved in binding [20]. It is likely, however, that chemical modifications of these residues with tetranitromethane for example are not able to change enough their polarity to sensibly affect their faculty of binding the interface. This type of approach to investigate a contribution of tyrosine residues to the binding process is certainly less promising than the one which has been presented in this paper. Indeed, the chemical structure of the essential residues is probably less compelling for the interactions of a protein with a micelle than for an enzyme with its substrate for example.

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