INTERACTIONS OF PANCREATIC COLIPASE WITH TAURODEOXYCHOLATE–OLEATE MIXTURES ABOVE THE CRITICAL MICELLE CONCENTRATION

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1. Introduction

The hydrolysis of insoluble triglycerides by pancreatic lipase requires pre-adsorption of the enzyme to the interface of emulsified particles [1]. This adsorption had been shown to be hindered by physiological concentrations of bile salts and restored by a pancreatic protein cofactor called colipase [2–4]. Colipase is supposed to anchor lipase during fat digestion at interfaces coated with bile salts and other amphiphiles [5], thus necessitating specific interactions between the cofactor, the enzyme and the interface.

The interactions of colipase with interfaces may be conveniently investigated using isotropic micellar dispersions of amphiphilic lipids which are more readily accessible to physical techniques than turbid emulsions. When applied to one of these systems, ultracentrifugation and UV spectrophotometry demonstrated that colipase formed complexes with pure NaTDC above the CMC and that, for the relatively low cofactor concentrations existing in vivo, the number of bile salt monomers bound to each cofactor molecule was approximately that present in a micelle under the same conditions [6,7]. In this type of complex called in what follows 'the binary complex', bile salts monomers were shown by small angle neutron scattering not to be distributed randomly over the surface of the protein but to form a micelle-like cluster distinct from and adjacent to colipase [8]. The site in colipase serving for micelle recognition may be assumed to be that insuring binding of the cofactor to mixed interfaces under physiological conditions [5].

However, the association constant related to the above binary complex with pure NaTDC micelles was shown by UV spectrophotometry not to exceed $1 \times 10^4\ M^{-1}$. A plausible explanation for this relatively low affinity is that the interfaces normally recognized by colipase during fat digestion are coated, not only with bile salts but also with other amphiphiles such as phospholipids and the fatty acids liberated in large amounts by sublingual [9,10] and pancreatic lipases. In fact, oleate has been qualitatively shown by Sephadex filtration to strengthen the colipase–bile salt association [11].

A more quantitative approach to the same problem is reported below. NaTDC–oleate mixtures were shown to form strictly homogeneous mixed micelle dispersions. The presence of oleate in these micelles increased the affinity constant with colipase by $\geqslant$100-fold.

2. Materials and methods

All assays were performed using a porcine colipase sample with an N-terminal valine (long form) kindly prepared by Dr M. Charles in this laboratory. The C-terminal sequence of the protein was not investigated. Commercial oleic acid (Fluka, puriss) dissolved in absolute methanol was adjusted to pH 9.0 with 1 M methanolic KOH and dried under vacuum. Other chemicals were as in [6,7].

Ultracentrifugations were performed at 20°C in a Spinco-Beckman ultracentrifuge, model E, with speed and temperature controls. Sedimentation coefficients

Abbreviations: NaTDC, sodium taurodeoxycholate; CMC, critical micelle concentration

were measured using a double sector capillary cell with synthetic boundary and Schlieren optics. The Yphantis [12] and Baldwin [13] methods were used for the determination of colipase and micelle weights. The partial specific volume of colipase and micelles was experimentally measured in a Parr microdensimeter. That of the complex ($\bar{\nu}_{1,2}$) was calculated from the values found for the two components ($\bar{\nu}_1$ and $\bar{\nu}_2$) using eq. (1):

$$(d_L + 1)\,\bar{\nu}_{1,2} = d_L\,\bar{\nu}_2 + \bar{\nu}_1 \qquad (1)$$

where d_L is the weight of component 1/g component 2 [14,15]. Micelle and complex concentrations were evaluated with the interference optics of the ultracentrifuge, using equation:

$$J_r = (dn/dc)ec/\lambda$$

where J_r is the number of displaced fringes, dn/dc the variation of the refraction index, e the thickness of the double sector central piece, c the solute concentration and λ the wavelength of the monochromatic light [16].

UV spectrophotometry assays were realized with an Acta III Beckman differential spectrophotometer and 1 cm quartz cuvettes with two compartments. Complex formation was derived from the height of the main tyrosine perturbation peak (λ = 288 nm) in difference spectra.

CMC were measured by spectrophotometry in presence of rhodamine 6G. As shown by fig.1, two straight lines with different slopes probably related to the successive formation of primary and secondary micelles [17] were obtained with pure NaTDC. The NaTDC concentration corresponding to the transition (~1.0 mM) is generally taken as the CMC of the salt. No sign of primary micelle formation was observed with NaTDC–oleate mixtures, thus allowing a better definition of the CMC. As expected, the CMC decreased as more oleate was incorporated into the micelles.

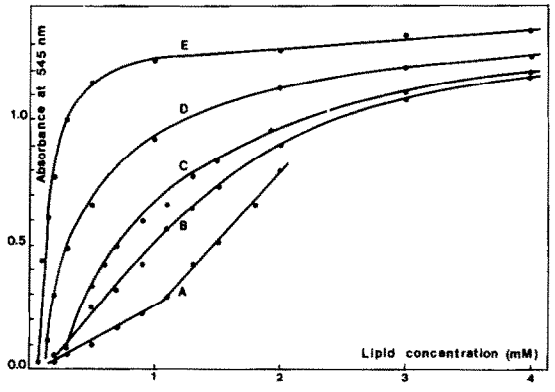

Fig.1. CMC of NaTDC–oleate mixtures as measured by spectrophotometry at 545 nm in presence of rhodamine 6 G. Solutions were made in a 50 mM Tris–HCl buffer (pH 8.0), 0.1 M KCl. (A) NaTDC alone; (B–E) NaTDC–oleate molar ratios of, respectively, 20:1, 10:1, 5:1 and 1:1.

3. Results and conclusions

3.1. *Ultracentrifugation*

Assays related to bile salt–oleate mixed micelle formation were done at pH 8.0 instead of pH 10 as in [17] in order to improve colipase binding and better to mimic physiological conditions. Turbid oleate solutions at pH 8.0 cleared up immediately upon addition of bile salt at the same pH. As shown by the typical sedimentation patterns reproduced in fig.2b, mixtures of equal molar quantities of NaTDC and oleate above the CMC (1:1 mixtures) yielded ⩾32 min centrifugation at 60 000 g sharp and symmetrical peaks indicating substantial homogeneity of the resulting micelle dispersions. Homogeneity was even better than with NaTDC alone (fig.2a) thus giving good evidence in favor of the formation of mixed NaTDC–oleate micelles at pH 8.0. Homogeneous micelle dispersions leading to significant sedimentation coefficient and micellar weight values at the

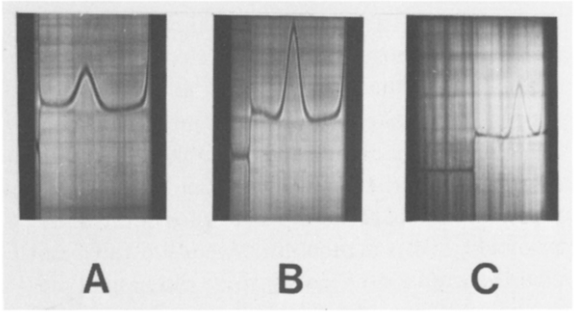

Fig.2. Sedimentation patterns yielded by: (A) NaTDC alone (12 mM); (B) 1:1 NaTDC–oleate mixtures (12 mM each); (C) mixtures of colipase (1 mM), NaTDC and oleate (6 mM each). Only pictures taken after 32 min at 60 000 × g are reproduced but the peaks remained symmetrical for longer time periods. All assays were performed at 20°C in 0.5 M Tris–HCl (pH 8.0) 0.1 M KCl.

Table 1
Ultracentrifugation of NaTDC-oleate mixed micelles

NaTDC–oleate molar ratio	Apparent $s_{20,w}$	$\bar{v}$ (g/ml)	Micellar M_r	Number of monomers/micelle		
				Total	NaTDC	Oleate
NaTDC alone	1.28	0.76_0	10 000[a]	19	19	0
10:1	1.17	0.78_6	11 000	22	20	2
2:1	1.08	0.80_0	22 000	48	32	16
1:1	1.06	0.83_5	22 300	53	26	26

[a] This value slightly differs from that reported in [5] because of the absence of $CaCl_2$ in the buffer

same pH were also obtained with 10:1 and 2:1 NaTDC–oleate mixtures (table 1). In good agreement with the known properties of pure oleate, inclusion of increasing proportions of this component into the micelles led to lower sedimentation coefficients but higher partial specific volumes, weights and aggregation numbers (no. monomers/micelle).

Moreover, the patterns illustrated in fig.2c showed that 1:1 NaTDC–oleate mixtures, like NaTDC alone, yielded upon addition of colipase a well defined complex (1.7 S). The binary nature of the complex was demonstrated by weight determination, using for the $\bar{v}$ of this complex a value (0.797) calculated by eq. (1) from those of colipase (0.710; [6]) and 1:1 micelles (0.835; table 1). The estimated M_r was 32 000, i.e., exactly the sum of the M_r of a colipase molecule (~10 000) and that of a 1:1 mixed micelle under the same conditions (22 000; table 1). A colipase excess over the micelles led, as in the case of NaTDC [6], to larger complexes with higher sedimentation coefficients.

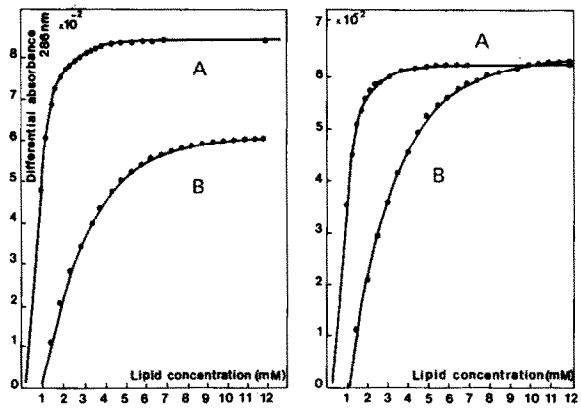

Fig.3. Formation of the binary complex as followed by spectrophotometry. Experimental points correspond to the height of the main tyrosine perturbation peak (288 nm) in difference colipase spectra in presence and absence of lipids at various concentrations. Same experimental conditions as in [5]. Left: saturation curve with a 1:1 NaTDC–oleate mixture (A) and with NaTDC alone (B). Right: curves (A) and (B) normalized to the same tyrosine signal.

3.2. *Spectrophotometry*

Fig.3 shows that the formation of the above complex was also associated with a strong perturbation of the cofactor tyrosine spectrum. The perturbation started exactly at the CMC and it increased with lipid concentration until saturation was attained. The maximal tyrosine signal obtained with 1:1 NaTDC–oleate mixtures was ~30% higher than with NaTDC alone (left). The saturation curves normalized to the same signal (right) strongly suggested a marked preference of colipase for the mixed lipid. The corresponding association constant could not be accurately calculated because of:

(i) The uncertainties associated with CMC determination;

(ii) The relatively narrow concentration range of the two components leading to binary complex formation;

(iii) The short interval between CMC and the saturating lipid concentration.

However, a fair estimate derived from Scatchard plots was 1×10^6–1×10^7 M^{-1}. This value is ⩾100-fold higher than with NaTDC [6,7]. The constant was already increased 10-fold with 10:1 NaTDC–oleate mixtures (2 oleate only for 20 NaTDC monomers in the micelles (table 1)).

In conclusion, the presence of aliphatic oleate chains besides the polycyclic structures of bile salts appears necessary for a good fit with the binding site of colipase. This effect may be due either to a

different charge distribution over the micelle surface and/or a modification of the geometry of the hydrophobic lipid core. The mechanism of complex formation (binding of colipase to pre-formed micelles or self association of lipid monomers on the cofactor site for concentrations similar to those inducing micelle formation) is still unknown. However, the obtention of a stable colipase–micelle complex will undoubtedly facilitate future investigations on its properties and on the chemical identification of the lipid recognizing site in colipase.

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