Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 114-119

THE HISTIDINE RESIDUES IN PIG AND HORSE COLIPASES.

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Received January 16,1981

SUMMARY

The single histidine of horse colipase B was shown to be at position 29, thus definitely proving that His_{86} present in the pig cofactor is not conserved in the horse. Carbethoxylation of histidines did not appreciably affect the capacity of the porcine cofactor to bind to hydrophobic interfaces and to anchor lipase in presence of bile salts, but it induced in the aromatic region of the molecule a significant transconformation detectable by spectrofluorimetry.

INTRODUCTION

The major function of colipase, a small protein cofactor secreted by pancreas, is to anchor lipase (EC 3.1.1.3) at triglyceride interfaces coated with bile salts, thus allowing lipolysis to proceed under physiological conditions (1-4). This function requires specific colipase-interface interactions which can be studied using isotropic micellar dispersions of bile salts and other polar lipids. When applied to mixtures of colipase and bile salts above the critical micelle concentration, ultracentrifugation (5) and UV differential spectrophotometry (6) have given evidence for the formation of a complex in which the number of lipid monomers attached to each colipase molecule is approximately that present in a micelle under the same conditions. As shown by small angle neutron scattering (7), the monomers in the complex are not distributed randomly over the protein surface. They form a micelle-like cluster distinct from and adjacent to colipase, thus demonstrating the existence in this latter protein of a topographically well defined site for micelle and interface recognition.

Porcine colipase contains three tyrosines at position 53, 56 and 57 (8). The properties of the adjacent Tyr_{56} and Tyr_{57} has been shown by UV spectrophotometry (6), fluorescence quenching (9), high resolution proton NMR spectroscopy and laser photo-CIDNP (10-12) to be strongly modified upon micelle fixation. Results obtained by the latter techniques were also consistent

Abbreviations used : CNBr, cyanogen bromide. DEPC, diethyl pyrocarbonate.

0006-291X/81/050114-06$01.00/0

with the existence of an interaction between the tyrosines and an histidine tentatively identified to His_{86} in the C-terminal part of the porcine cofactor. The interest of this latter assumption is to suggest that the above residues are brought close to each others in space by a special folding of the chain which, due account being taken of the nature of the neighbouring residues, should generate at the surface of the cofactor molecule a large hydrophobic-aromatic site for lipid binding (10).

However, it is noteworthy that the single histidine of horse colipase B is not present in the C-terminal peptide split off by CNBr fragmentation, but in the intermediary peptide extending from Met_{18} to Met_{n-16} (or Met_{n-17}) (13). This unexpected result prompted us to definitely ascertain the position of this histidine and more generally to evaluate the role of this type of residue in the function of colipase.

MATERIALS AND METHODS

MATERIALS

Pig and horse colipases and pig lipase were purified as described, respectively, by Chapus *et al.* (13) and Rovery *et al.* (14). Diethylpyrocarbonate was purchased from Fluka (Buchs, Switzerland).

METHODS

Carbethoxylation of histidine was carried out by incubation at room temperature of 0.1-0.2 mM solutions of the cofactor in 0.1 M phosphate pH 6.0 with a 60 fold molar excess of ethanolic diethylpyrocarbonate added in several portions (15). The reaction was followed by spectrophotometry at 242 nm. The number of modified histidines was calculated using a molar extinction coefficient of 3 200 at 242 nm for pure N-carbethoxy histidine. Low molecular weight products were removed from the preparations by filtration at 0° C through a Sephadex G 25 column (1 cm x 20 cm) equilibrated and eluted with 0.1 M Tris-HCl at pH 7.5.

Decarbethoxylation of modified colipases was achieved by a 24 h incubation in 0.2 M hydroxylamine at pH 7.0 and room temperature (15) followed by Sephadex filtration. The removal of carbethoxyl groups from the proteins was estimated spectrophotometrically at 242 nm.

Colipase activity and adsorption to siliconized glass beads were followed using the techniques described in ref. 13 and 4, respectively. The amount of unadsorbed colipase was measured by determination of the activity remaining in solution after glass beads removal by sedimentation.

Spectrofluorimetric assays. The intrinsic fluorescence of 2-10 µM colipase solutions in 20 mM Tris-HCl pH 8.0 NaCl 0.15 M was measured at 20° C in a Fica 55 absolute differential spectrofluorimeter. The fluorescence spectra were recorded between 250 and 450 nm.

Purification and automatic sequencing of the intermediary cyanogen bromide peptide from horse colipase B were carried out as described in ref. 13.

RESULTS AND DISCUSSION

Position of the histidine in horse colipase B.

The 53 first residues of the intermediary CNBr peptide isolated from horse colipase B (13) could unambiguously be sequenced by the automatic technique with the following results :

Met-Asn-Ser-Ala-Gln-Cys-Lys-Ser-Glu-Cys-Cys-His-Arg-Glu-Ser-Ser-Leu-Ser-Leu-Ala-
20 25 30 35

Arg-Cys-Ala-Ala-Lys-Ala-Ser-Glu-Asn-Ser-Glu-Cys-Ser-Ala-Trp-Thr-Leu-Tyr-Gly-Val-
40 45 50 55

Tyr-Tyr-Lys-Cys-Pro-Cys-Glu-Arg-Gly-Leu-Thr-Cys-Gln.
60 65 70

In this sequence, the residues are numbered assuming that the first methionine of the chain is at position 18 (16, 13). When compared to the sequence starting from the N-terminal valine proposed by Julien et al. (16) for the same protein, the main difference is that position 29 is occupied by an histidine in place of a threonine. This finding definitely proves that the histidine in the N-terminal part of pig colipase is conserved in horse colipase B while His_{86} is not conserved. Other differences are a glutamine instead of a glutamic acid at position 22 and the identification of residues 44, 47 and 50 as serine. Moreover, the known sequence of horse colipase B is now extended to the first 70 residues, thus leaving only a short interval with about 9 residues before the already sequenced C-terminal CNBr peptide (13).

Modification of the histidines by DEPC.

Pig colipase and horse colipases A and B treated with DEPC showed a UV difference spectrum with a maximum at 242 nm characteristic of carbethoxy histidine (15). The absorbance at 280 nm was unaffected suggesting that the tyrosines were not modified. Moreover, complete reversal of the spectral perturbations upon incubation of the modified proteins with hydroxylamine excluded the possibility of a participation of lysine ε-NH_2 groups and of dicarbethoxylation of the histidines (15). Therefore, the majority of the protein bound N-carbethoxy groups detectable by spectrophotometry could be attributed to monocarbethoxylated histidine residues.

With pig colipase, two carbethoxy groups per mole were incorporated, showing that the two histidines of the molecule were accessible to the reagent. However, their reactivity was quite different and two derivatives with, respectively, one and two carbethoxy groups per mole (1 CE- and 2 CE-colipase) could be isolated. Only the highly reactive histidine was modified in 1 CE-colipase. DEPC treatment of horse colipases A and B led to the fixation at approximately

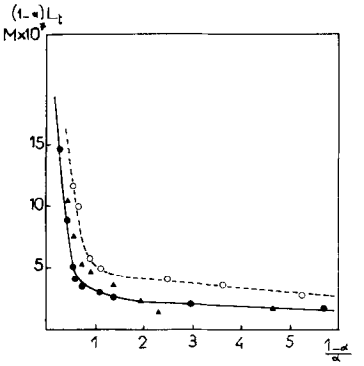

Fig. 1 : Adsorption of native and modified porcine colipases (0,2-10 µM solutions in 0.1 M Tris-HCl pH 7.5 5 mM $CaCl_2$) on 1 g of siliconized glass beads. ●—● , native colipase ; o--o , ▲—▲ , 1 CE- and 2 CE-colipases respectively. Adsorption was followed by determination of colipase activity remaining in supernatant. α , fraction of unadsorbed colipase. L_T, total colipase concentration.

the same rate of one carbethoxy group per mole. This finding is consistent with the presence of a single histidine in both colipases A and B from horse, as previously found by amino acid analysis (9).

Properties of modified colipases.

As shown by fig. 1, the adsorption of porcine 1 CE- and 2 CE-colipases to siliconized glass beads (4) was not essentially different from that of the native protein. The same observation was made in 0.1 mM taurodeoxycholate, showing that intact histidines are not essential for binding of the proteins to hydrophobic interfaces in presence or absence of bile salts. Moreover, the modified colipases reactivated lipase in presence of bile salts, thus proving that their ability to recognize the enzyme at the interface was not lost.

On the other hand, fig. 2A shows that the intrinsic fluorescence of the tyrosines in the porcine cofactor was sharply reduced by carbethoxylation of the histidines (25 % and 50 % reduction, respectively in 1 CE- and 2 CE-colipases compared to the native protein). For horse colipase (fig. 2B), the tryptophan fluorescence (emission maximum at 345 nm), which completely masked that of the tyrosines, was also reduced but to a lesser extent (10 %). These results are consistent with a significant transconformation of the colipase molecule shifting the aromatic residues to a more polar environment.

Moreover, the intrinsic fluorescence intensity of all colipases was affected upon micelle fixation thus confirming the involvement of aromatic residues already suggested by fluorescence quenching (9) and other physical techniques. This effect was different in the native and modified

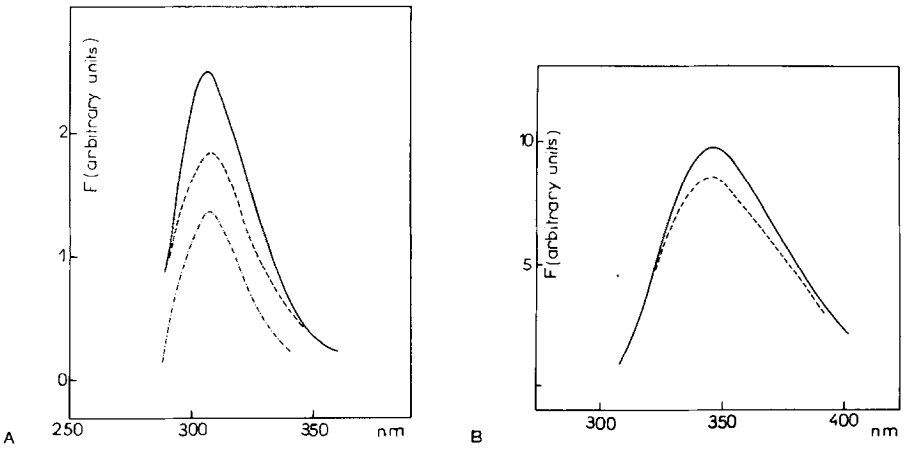

Fig. 2 : Fluorescence spectra of 3 µM colipase solutions in a 20 mM Tris-HCl buffer pH 8.0 NaCl 0.15 M. Fig. 2A : tyrosine spectrum in porcine colipase. Excitation wave length : 274 nm. ——— , native protein ; – – – –, 1 CE-colipase ; –·–·–·–, 2 CE-colipase. Fig. 2B : tryptophan spectrum in horse colipase A. Excitation wave length, 290 nm. ———and – – – – , native and carbethoxylated proteins, respectively.

proteins. For example, the fluorescence of native porcine colipase was enhanced 1.2 fold in 8 mM taurodeoxycholate while that of the 2 CE-derivative was reduced by a factor of 0.9. Under the same conditions, the tryptophan fluorescence was enhanced 1.9 fold in native horse colipase B and only 1.45 fold in the modified molecule. Therefore, carbethoxylation of the colipase histidines is likely to induce a slight transconformation in the aromatic region of the chain without affecting the function.

Acknowledgements : We are grateful to Mrs. J. de Caro for help in peptide purification and to J. Bonicel and Mrs. P. Couchoud for performing the automatic sequence analyses.

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