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The interaction of hydrophobic bile acids with the α_1-proteinase inhibitor

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Abstract

An in vitro complex formation between cholesterol and human α_1-proteinase inhibitor (α_1-antitrypsin, α_1-Pi) has been described. Hydrophobic bile acids were studied for a similar interaction using lithocholic acid (LC) as a prototype of a hydrophobic acid. At a molar ratio of 5:1, LC induced conformational changes of α_1-Pi reflected in an abnormal gel-electrophoretic appearance, loss of anodal immunoreactivity on crossed immunoelectrophoresis, exposition of new antigenic determinant(s) on immunodiffusion, and loss of antiproteinase activity. After 6 h incubation, LC and α_1-Pi form a complex of approximately 200 kDa molecular mass seen following gel-filtration. After prolonged (24 h) interaction a series of large α_1-Pi polymers were seen on SDS-PAGE under reducing conditions followed by Western blotting. Glycolitho-, sulfolitho-, deoxycholic and 3-β-hydroxy-5-cholenoic acids induced similar but less pronounced changes of α_1-Pi, whereas transferrin remained unaffected. Hydrophilic acids lacked effect on α_1-Pi. The results are compatible with a specific, irreversible interaction of α_1-Pi with hydrophobic bile acids affecting its physical and proteinase inhibitory properties. The cholestatic potency of the hydrophobic acids studied and their ability to induce α_1-Pi polymerization may be important in cholestatic conditions.

Key words: α_1-Proteinase inhibitor; Hydrophobic bile acid; Complex; Polymerization

1. Introduction

Members of the serpin (serine proteinase inhibitor) superfamily of highly homologous proteins possess a common tertiary structure. Despite their similarities in substrate specificities, their biological functions differ. α_1-antitrypsin (α_1-AT, α_1-proteinase inhibitor, α_1-Pi) the major plasma proteinase inhibitor, contains a reactive center that specifically reacts with neutrophil elastase. The closely related α_1-antichymotrypsin (ACT) has neutrophil cathepsin G as a target enzyme, whereas the homologous corticosteroid-binding globulin (CBG) lacks an antiproteolytic effect and functions as a transport protein for glucocorticoid hormones [1], though it may also act as an elastase substitute at inflammatory foci [2]. Sequence and gene structure similarities between these proteins suggested to us that α_1-Pi and ACT may have functions similar to those of CBG, a hypothesis deriving support from the isolation of hydrophobic peptides functioning as putative cholesterol carriers from α_1-Pi [3]. In support of this hypothesis we also demonstrated in vitro that at 37°C α_1-Pi and cholesterol at a molar of ratio 1:2 form a 70 kDa complex, increasing in size by polymerization at prolonged incubation times. The interaction resulted in a decline of antiproteinase activity and an abnormal immunoprecipitation pattern of α_1-Pi [4]. Moreover, our results suggested the in vitro complex formation to be dependent on a conformationally constrained reactive site loop. Complex formation was blocked by both direct (heating, proteolytic cleavage) and indirect (disulfide formation) processes affecting this region [5]. The demonstration of an interaction between α_1-Pi and cholesterol suggested that other hydrophobic compounds might behave similarly. Bile acids are the end-products of hepatic cholesterol metabolism and include many compounds exhibiting a wide range of hydrophobicity, depending upon the degree of nuclear hydroxylation and/or side chain amidation [6]. The secondary bile acid, lithocholic acid, particularly in its non-conjugated and non-sulfated form, is formed by bacterial 7 α-dehydroxylation of chenodeoxychrolic acid, and is not only hydrophobic but also, at least in animal models, highly cholestatic when administered intravenously [7]. Here we report on the in vitro interaction of various bile acids, focusing in particular on lithocholic, sulfolithocholic and its unsaturated β-epimer, 3-β-hydroxy-5-cholenoic acid, with the α_1-proteinase inhibitor.

2. Materials and methods

Electrophoretically pure α_1-Pi was isolated from human plasma at our laboratory [4]. Bile acids: cholic, deoxycholic, glycocholic, chenodeoxycholic, taurodeoxycholic, lithocholic, glycolithocholic, sulfolithocholic and 3-β-hydroxy-5-cholenoic acid, were obtained from Sigma, St Louis, MO. [24-^{14}C]Lithocholic acid (specific radioactivity 10 mCi/mmol) was a gift from Prof. Alan Hofmann, San Diego, and was more than 99% pure. Human transferrin, porcine pancreatic elastase type III, *N*-succinyl-(Ala)$_3$-*p*-nitroanilide (SAPNA) were obtained from

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Sigma. Monospecific rabbit antiserum against human α_1-Pi was purchased from DAKO (Denmark) and agarose, Sephadex G-75 superfine, Sephadex G-150, gel filtration calibration kits were from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

2.1. Sample preparation

In the experiments we used stock solutions of α_1-Pi 1.78 mg/ml (3.42×10^{-5} M). Bile acid standard solutions contained 2 mg/ml in 99.5% ethanol. α_1-Pi was mixed in 0.015 M Tris buffer, pH 7.46, containing 0.15 M NaCl with different bile acids at increasing molar ratios (1:1; 1:2; 1:5; 1:10) and incubated for 0.5; 1; 2; 4; 6; 12 or 24 h at 37°C. As the standard experimental protocol, we chose a 1:5 molar ratio of α_1-Pi/bile acid and overnight incubation, because unequivocal and highly reproducible electrophoretic results were obtained under these conditions. Electrophoresis in 1% agarose at pH 8.6, crossed immunoelectrophoresis, and rocket immunoelectrophoresis were performed using standard methods [4]. Double radial immunodiffusion was performed according to Ouchterlony [8]. SDS-PAGE of high molecular weight proteins under reducing and non-reducing conditions was run in a Phast gel gradient of 4–15%. Semi-dry electroblotting of electrophoretically separated proteins was performed in accordance with the manufacturer's instructions (Pharmacia, LKB). Gel filtration on Sephadex G-75 superfine columns was performed as previously described [4]. Gel filtration on a Sephadex G-150 column was used for estimation of the molecular size of α_1-Pi after the addition of unlabeled or labeled [24-^{14}C]lithocholic acid. Samples were prepared under standard experimental conditions prior to gel chromatography. The columns (0.9×60 cm) were equilibrated with the Tris buffer and were run at a flow rate of 3 ml/h at room temperature, 1 ml fractions being collected. The eluted fractions were analyzed spectrophotometrically at 280 nm. Radioactivity was measured with a Packard Tri-Carb 300C liquid screen scincillation system. Electroimmunoassay was performed according to standard procedures [4]. The elastase inhibitory activity of α_1-Pi in native form and after interaction with bile acids was measured as previously described [4].

3. Results

3.1. Screening for bile acid interaction with α_1-Pi by gel electrophoretic techniques

The various bile acid samples were mixed with native α_1-Pi under standard experimental conditions, and reaction mixtures studied by 1% agarose electrophoresis at pH 8.6. As shown in Fig. 1, monohydroxy bile acids; lithocholic, 3-β-hydroxy-5-cholenoic and glycolithocholic acids, had effects on the gel electrophoretic appearance of α_1-Pi. Not only did the native distinct α_1-Pi band become blurred and its mobility towards the anode retarded, but a distinct new band with increased anodal mobility also appeared. The introduction of a sulfate group in 3-α position of lithocholic acid resulted in a similar but less pronounced interaction as was the case with 3-β-hydroxy-5-cholenoic and deoxycholic acids. As can be seen in Fig. 1, neither conjugated dihydroxy- nor unconjugated cheno- or trihydroxy bile acids had any effect on the mobility or shape of the well demarcated α_1-Pi band. When monohydroxy acids were incubated with transferrin, no interaction could be observed (not shown). α_1-Pi-bile acid samples were also analyzed by SDS-PAGE in a 4–15% gel gradient under both reducing and non-reducing conditions followed by Western blotting. As can be seen in Fig. 2A, under non-reducing conditions the interaction of α_1-Pi with lithocholic as well as with sulfolithocholic acids results in the appearance of an immunoreactive extra band with an approximate mass of 200 kDa. After reducing identical samples by adding 4% 2-mercaptoethanol, several strongly immunoreactive bands corresponding to larger molecular masses appeared (Fig. 2B). They persist after prolonged dialysis of the samples. In contrast, no large mass bands were detected after α_1-Pi/cholic acid incubation. The α_1-Pi–bile acid mixtures were also analyzed by crossed immunoelectrophoresis using a monospecific antiserum against human α_1-Pi. As shown in Fig. 3, lithocholic acid affects the α_1-Pi immunoprecipitate pattern in several ways: total immunoreactivity seems to decrease, and there is a tendency toward a double peaked appearance where the cathodal peak is blurred. However, the new anodal band visible in ordinary agarose electrophoretic gels (Fig. 1) is not detected by the antiserum. Glycolithocholic, 3-β-hydroxy-5-cholenoic, and sulfolithocholic acids had less pronounced but similar 'true-to-type' effects as obtained with lithocholic acid. Cholic or other hydrophilic bile acids did not affect the precipitate patterns of α_1-Pi (Fig. 3).

Finally the qualitative changes induced by hydrophobic bile acids were studied by double radial immunodiffusion (Fig. 4). The precipitation patterns clearly show

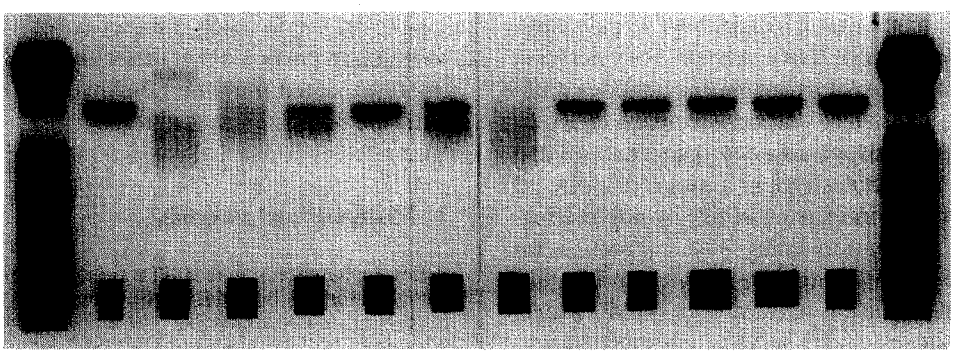

Fig. 1. 1% agarose electrophoresis of native α_1-Pi/bile acid mixtures at pH 8.6. Lanes 1 and 14, normal human plasma pool; lanes 2 and 13, native α_1-Pi; lane 3, α_1-Pi/lithocholic acid; lane 4, α_1-Pi/glycolithocholic acid; lane 5, α_1-Pi/sulfolithocholic; lane 6 α_1-Pi/3β-hydroxy-5-cholenoic acid; lane 7, α_1-Pi/deoxycholic; lane 8, α_1-Pi/cholic acid; lane 9, α_1-Pi/glycocholic; lane 10, α_1-Pi/chenodeoxycholic; lane 11, α_1-Pi/taurocholic acid; lane 12, α_1-Pi in mixture with 2% ethanol (control).

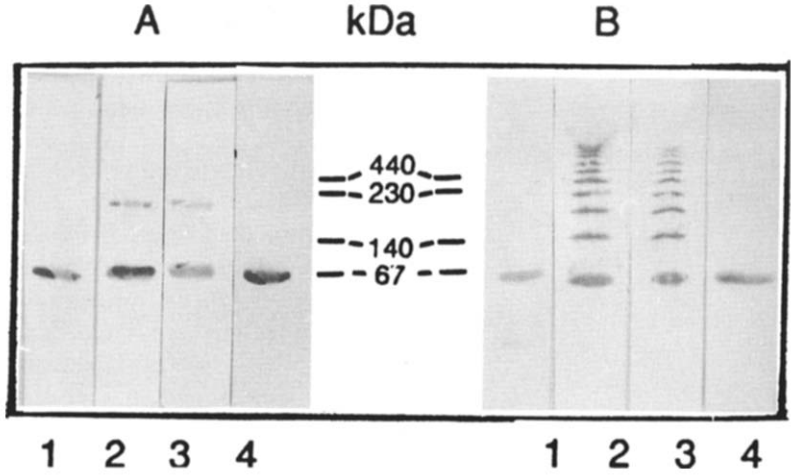

Fig. 2. Interaction between α_1-Pi and bile acids studied by Western blot analysis. Samples were non-reduced (A) before application to SDS-PAGE 4–15% gradient gels and immuno-blotting, or reduced (B) with 4% 2-mercaptoethanol. Lane 1, native α_1-Pi; lane 2, α_1-Pi/lithocholic acid; lane 3, α_1-Pi/sulfolithocholic acid; lane 4, α_1-Pi/cholic acid.

that α_1-Pi interacting with lithocholic acid exposes a new antigenic determinant(s) not visible in native α_1-Pi or after interaction with cholic acid. Sulfolithocholic acid had a similar but less pronounced effect (Fig. 4).

3.2. *Complex formation and polymerization of α_1-Pi*

When native α_1-Pi or α_1-Pi–lithocholic acid mixtures (1:5 molar ratio) were subjected to gel-filtration on Sephadex G-75 or Sephadex G-150 columns, the α_1-Pi–lithocholic acid mixture eluted before native α_1-Pi, indicating complex formation (Fig. 5). After 6 h incubation at 37°C, the complex had a mass of approximately 80 kDa. Prolonged incubation time resulted in larger complexes with molecular masses of about 200 kDa. When gel-filtration fractions containing the largest protein and native α_1-Pi were compared by electroimmunoassay, fractions containing α_1-Pi–lithocholic acid mixture were found to have lost approximately 45% of the native α_1-Pi immunoreactivity (Fig. 5). These results were confirmed and extended by the use of [24-^{14}C]lithocholic acid and gel-filtration on Sephadex G-150 columns. Following 24 h incubation, most of the radioactivity is eluted in the high molecular mass fractions (Fig. 5).

3.3. *Proteinase inhibitory activity of α_1-Pi–bile acid mixtures*

The elastase inhibitory capacity of α_1-Pi–bile acid mixtures as compared to that of native α_1-Pi is shown in Fig. 6. α_1-Pi in mixture with lithocholic acid at a molar ratio of 1:5 lost almost all inhibitory acitivity. Glycolithocholic, 3-β-hydroxy-5-cholenoic acids, produced similar decreases for α_1-Pi activity. Sulfolithocholic as well as deoxycholic acid resulted in a loss of inhibitory activity of approximately 45% and 65%, respectively. At a molar ratio of 1:2 the loss of inhibitory activity was less pronounced but still distinct (Fig. 6). As can also be seen in Fig. 6 the addition of hydrophilic bile acids to α_1-Pi had no effect on α_1-Pi antiproteinase activity.

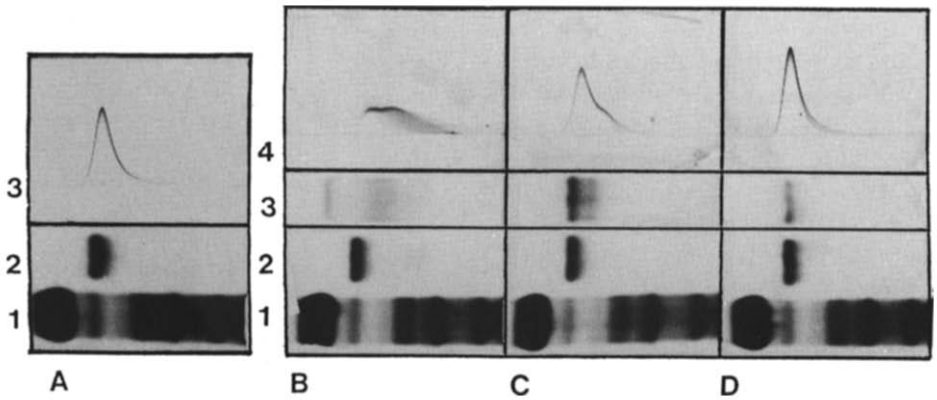

Fig. 3. Crossed immunoelectrophoresis (A) native α_1-Pi and mixtures of α_1-Pi/lithocholic acid (B); α_1-Pi/sulpholithocholic acid (C); α_1-Pi/cholic acid (D). Agarose electrophoresis was carried in the first dimension (anode to the left): A; B; C; D (1) normal plasma pool; (2) native α_1-Pi; B (3) - α_1-Pi/lithocholic acid; C (3) - α_1-Pi/sulfolithocholic acid D (3) α_1-Pi/cholic acid; in the second dimension (anode at top) with agarose gel containing anti-α_1-Pi antiserum: A (3) native α_1-Pi; B (4) α_1-Pi/lithocholic acid; C (4) α_1-Pi/sulfolithocholic acid; D (4) - α_1-Pi/cholic acid.

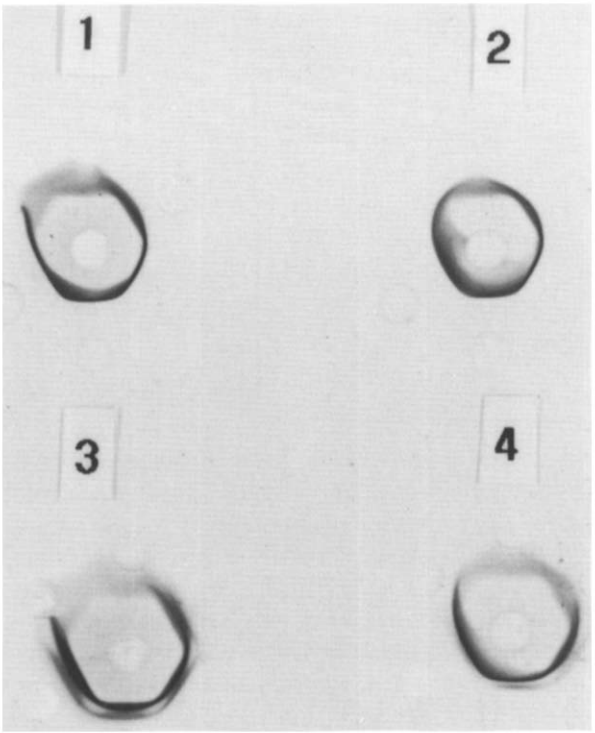

Fig. 4. Double radial immunodiffusion of native α_1-Pi (1); α_1-Pi/cholic acid (2); α_1-Pi/lithocholic acid (3); α_1-Pi/sulfolithocholic acid (4). Center wells contain anti-α_1-Pi antibodies; other wells diluted samples 1:2; 1:4; 1:8; 1:16; 1:32; 1:64.

4. Discussion

Several studies have indicated that hydrophobic bile acids can bind to lipoproteins and albumin [9]. To the best of our knowledge the data presented here constitute the first published evidence of an interaction between monohydroxy bile acids and α_1-Pi. At a molar ratio of 5:1, at 37°C, this interaction distinctly affects the physicochemical characteristics, the immunogenic properties and the biological function of α_1-Pi. The interaction is specific. A non-serpin glycoprotein such as transferrin does not interact under identical experimental conditions. It is evident from the electrophoretic data presented in Fig. 1 that the mixture of α_1-Pi with lithocholic acid, 3-β-hydroxy-5-cholenoic acids, glycolithocholic as well as sulfolithocholic and (to a lesser extent) deoxycholic acids results in the formation of a new anodal band. Analysis of the same samples by crossed immunoelectrophoresis (Fig. 2) clearly shows this 'new anodal band' to lack immunoreactivity. It is also evident from the data in Figs. 1 and 2 that hydrophilic bile acids in mixtures with α_1-Pi lack any effect on the electromobility or immunoprecipitation pattern of α_1-Pi.

Findings in SDS-PAGE followed by Western blotting (Fig. 3) suggest the interaction between α_1-Pi and hydrophobic bile acids to result in the irreversible formation of a series of large molecular mass polymers. Some of these may be trapped in the stacking gel but it is obvious (Fig. 2) that reduction of samples before SDS-PAGE permits penetration into the gel. Under non-reducing conditions α_1-Pi–hydrophobic bile acid samples exhibit only one polymer band with about 200 kDa molecular mass. In contrast, no high-molecular mass polymers have been detected in α_1-Pi cholic acid or other hydrophilic acid mixtures (Fig. 3). The results obtained by gel filtration of identical α_1-Pi bile acids mixtures extend and confirm those obtained with electrophoresis. When mixtures of α_1-Pi with unlabeled or labeled [24-^{14}C]lithocholic acid were analyzed by gel-filtration on Sephadex G-150, α_1-Pi eluted in fractions corresponding to a mass of about 200 kDa (Fig. 5). When labeled lithocholic acid was added, radioactivity was predominately found in the same fractions as α_1-Pi. The limited separation capacity of our gel-filtration systems does not permit separation of the high molecular mass polymers visible after SDS-PAGE electrophoresis (Fig. 3). However, it may be noted that fractions containing α_1-Pi–lithocholic acid mixtures manifested approximately 55% of the immunoreactivity found in native α_1-Pi. One possible explanation of this finding may be that some of the high-molecular mass polymers lack detectable immunoreactivity and may thus correspond to the 'new anodal and non-immune reactive band' seen in ordinary electrophoresis (Figs. 1 and 2).

Again it should be noted that α_1-Pi in mixtures with

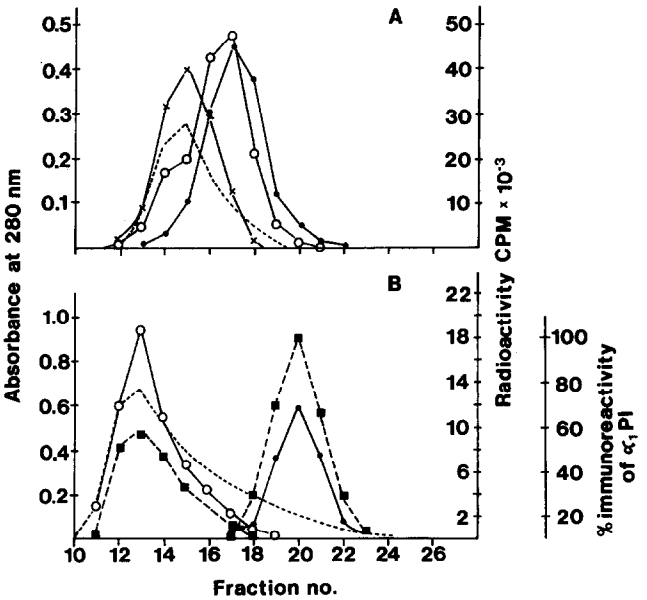

Fig. 5. Gel-filtration of native α_1-Pi and α_1-Pi–lithocholic acid mixtures on Sephadex G-75 superfine (A) and Sephadex G-150 (B) columns. (A) Native α_1-Pi (●—●); α_1-Pi/lithocholic acid mixture at a molar ratio of 1:5 incubated 6h (○—○) and overnight (×–×) at 37°C; α_1-Pi/^{14}C-labeled lithocholic acid at a molar ratio of 1:5, incubated overnight at 37°C (– – –). (B) Native α_1-Pi (●—●); α_1-Pi/lithocholic acid (○—○), α_1-Pi/^{14}C-labeled lithocholic acid (– – –) mixtures at molar ratios of 1:5 incubated overnight at 37°C; percentage of immunoreactivity fractions containing α_1-Pi in mixture with lithocholic acid as compared to native α_1-Pi (■—■)

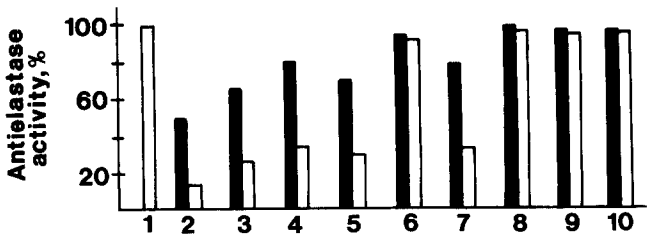

Fig. 6. Antielastase activity of native α_1-Pi and α_1-Pi-bile acids mixtures at molar ratios of 1:2 (black columns) and 1:5 (white columns) 1, native α_1-Pi; 2, α_1-Pi/lithocholic; 3, α_1-Pi/glycolithocholic; 4, α_1-Pi/sulfolithocholic; 5, α_1-Pi/3β-hydroxy-5-cholenoic; 6, α_1-Pi/cholic; 7, α_1-Pi/deoxycholic; 8, α_1-Pi/glycocholic; 9, α_1-Pi/chenodeoxycholic; 10, α_1-Pi/taurocholic acid.

hydrophilic bile acids is characterized by an elution profile identical to that of native α_1-Pi. Our results also demonstrate that the bile acid interaction with α_1-Pi reflects the hydrophobicity of the acids studied and is independent of their detergent and/or micelleforming properties. The physicochemical and immunogenic changes of α_1-Pi induced by hydrophobic bile acids are pronounced and compatible with extensive conformational alterations which are also revealed by the immunodiffusion results. Of particular interest is the finding that interaction between α_1-Pi and lithocholic or sulfolithocholic acid exposes on the α_1-Pi molecule a new epitope(s) recognized by monospecific antiserum against human α_1-Pi (Fig. 5) In contrast no changes of the antigenic structure of α_1-Pi were visible in an α_1-Pi–cholic acid mixture (Fig. 4). Our results are exclusively based on in vitro experiments. Although their possible in vivo importance is at present unknown, it may be appropriate to consider our findings in relation to at least three clinical settings. First, although the primary, causative role of monohydroxy bile acids in human cholestasis has never been unequivocally established [10], it is tempting to speculate that lithocholic acid and its derivates may contribute to cholestasis owing to their ability to form stable, large polymers of α_1-Pi and concomitantly reduce its antielastase activity. We are planning animal experiments, preferentially in rats, including i.v. administration of these acids, with a focus on the polymerization phenomenon we have described. Sulfation of lithocholic acid has been regarded as a potential detoxification mechanism [11,12]. Our findings show that sulfolithocholic acid, despite being more hydrophilic than lithocholic acid and water soluble acts as a polymer-forming compound, though higher concentrations are necessary. Second, several glycoproteins have been proposed to act as so called pronucleating agents in gallstone formation [13]. α_1-Pi has not been studied in this respect but certainly merits attention, again considering its polymerization capability in the presence of hydrophobic bile acids, including lithocholic acid. Third, there is the question of the possible involvement of hydrophobic bile acids interacting with α_1-Pi during fetal development. In α_1-Pi deficiency, PiZ, the abnormally glycosylated protein, owing to an inherent tendency to aggregate, is retained within the endoplasmic reticulum where bile acid biosynthesis also takes place [14]. The molecular basis of this phenomenon, thought to be a loop in sheet polymerization, and which is both concentration and temperature dependent, has recently been elucidated [15]. However, of all newborn children with PiZ-homozygosity, only 10 percent develop a clinically overt neonatal hepatitis syndrome [16]. Some of the affected children are small for gestational age, an anomaly compatible with the presence of a disease process starting already in utero. It is conceivable that hydrophobic bile acids may contribute to a polymerization process at this stage. This might be a random process affecting in particular the 10% of PiZ-homozygous children who happen to have the most immature sulfation and/or the most effective side chain oxidation capacity generating lithocholic and 3β-hydroxy-5-cholenoic acid. This acid represents a considerable portion of the bile acids in meconium and amniotic fluid, is cholestatic and can, as shown here, interact with α_1-Pi and cause polymerization, albeit at higher concentrations than lithocholic acid.

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