Developmental changes in the isoelectric variants of rat hepatic hydroxysteroid sulphotransferase

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Major isoenzymes of androsterone-sulphating sulphotransferase (AD–ST) were isolated from liver cytosols of weanling and young adult female rats and their isoelectric properties were compared. On chromatofocusing the enzyme activity of young adults was eluted over a wider range of pH than was that of weanling rats. The activity at pH 7.8–7.2 (fraction I) is obvious at both ages, whereas the activity eluted over the pH 6.6–5.5 range (fraction II) is much lower in weanlings than in young adults. The AD-ST activities eluted in fractions I and II were separately purified by 3′-phosphoadenosine 5′-phosphate–agarose affinity chromatography at both ages. Two-dimensional gel electrophoresis of the isolated enzyme revealed several subunits with distinct pI values, but with the same molecular mass, namely 30 kDa. The relative levels of the pI 6.7 and pI 7.2 subunits are high and the relative level of the pI 6.1 is low in fraction I. In fraction II, the levels of pI 6.1 and pI 6.7 subunits are high and the level of the pI 7.2 subunit is low. There is no significant difference in the relative levels of the pI variants in each fraction between weanlings and young adults. The N-terminal amino acid sequences of the pI variants are identical within the area determined, irrespective of animal age or pI values. These results demonstrate that the pI variants of AD-ST are derived from the same precursor by post-translational modification or that they are products of closely related, but distinct, genes. The pI 6.1 and 6.7 subunits presumably increased during the development from the weanling stage to adulthood, resulting in the increase in acidic form(s) of AD-ST (fraction II) in adult females.

INTRODUCTION

Hepatic sulphotransferase (ST) transfers sulphate groups from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to various xenobiotics and endogenous compounds. Purification [1–5] and cloning studies [6–9] have provided evidence of multiple hepatic STs in rats and humans. Rat hepatic hydroxysteroid ST activity catalyses sulphation of steroid hormones, bile acids and alcohols with overlapping substrate specificities and have been shown to comprise several isoenzymes [1–3,10]. These ST activities display gender- and age-dependent alterations [11–15] and are known to be under the regulation of gonadal, adrenal [2] and growth hormones [15]. They also exhibit sex-dependent spatial localization in the lobule of the liver [16].

We have previously reported gender- and age-related alteration in the activity of rat hepatic androsterone-sulphating ST (AD-ST), an isoenzyme of hydroxysteroid ST [17]. The activity increased after birth in parallel in both sexes until the weanling stage. Thereafter the activity decreased in males, whereas it declined temporarily in females and increased again to a maximum level in adult females. In previous reports [18,19] we have described isolation of a major isoenzyme of AD-ST from adult female rat liver and demonstrated that it is an oligomer consisting of several isoelectric variants with the same molecular mass, 30 kDa. The most abundant components are subunits of pI 7.2 and 6.7; variants of pI 5.8 and 6.1 are minor constituents. Immunoblot analysis with anti-AD-ST antiserum also revealed that the relative contents of these pI variants in liver cytosols are different in 20-day-old (weanling) and 110-day-old (young adult) rats [19]. In order to address the molecular basis of this developmental change in AD-ST, we isolated the major isoenzyme of AD-ST from the liver cytosols of weanling and young adult females. We compared the elution profiles of the enzyme by chromatography as well as the relative amounts and the N-terminal amino acid sequences of the pI variants.

MATERIALS AND METHODS

Materials

[9,11-^{3}H]Androsterone (AD) (60 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). DEAE-cellulose DE-52 is a product of Whatman Biosystems (Maidstone, Kent, UK). PBE 94, Polybuffer 96 and 74 and agarose–3′-phosphoadenosine 5′-phosphate type 2 (PAP–agarose) were obtained from Pharmacia (Uppsala, Sweden). 5′-AMP and 3′-AMP were from Sigma (St. Louis, MO, U.S.A.). ProBlott, a poly(vinylidene difluoride) (PVDF) membrane was obtained from Applied Biosystems Japan (Tokyo, Japan). 3′-Phosphoadenosine 5′-phosphosulphate (PAPS) was prepared by the method of Singer [20] and its purity was confirmed by h.p.l.c. as described previously [21]. All other reagents were of the highest grade available.

Enzyme assays

The liver homogenates were prepared in buffer A (250 mM sucrose/3 mM 2-mercaptoethanol/0.1 mM EDTA/10 mM Tris/HCl, pH 7.4) from female weanling Wistar rats (20 days of age; 30 animals) and from young adult females (100–110 days of age; seven animals), and cytosolic fractions were obtained as

Abbreviations used: ST, sulphotransferase; AD-ST, androsterone-sulphating sulphotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; PAP, 3′-phosphoadenosine 5′-phosphate; 2DGE, two-dimensional gel electrophoresis; PVDF, poly(vinylidene difluoride); 4-NP, 4-nitrophenol; SMP-2, senescence marker protein 2.

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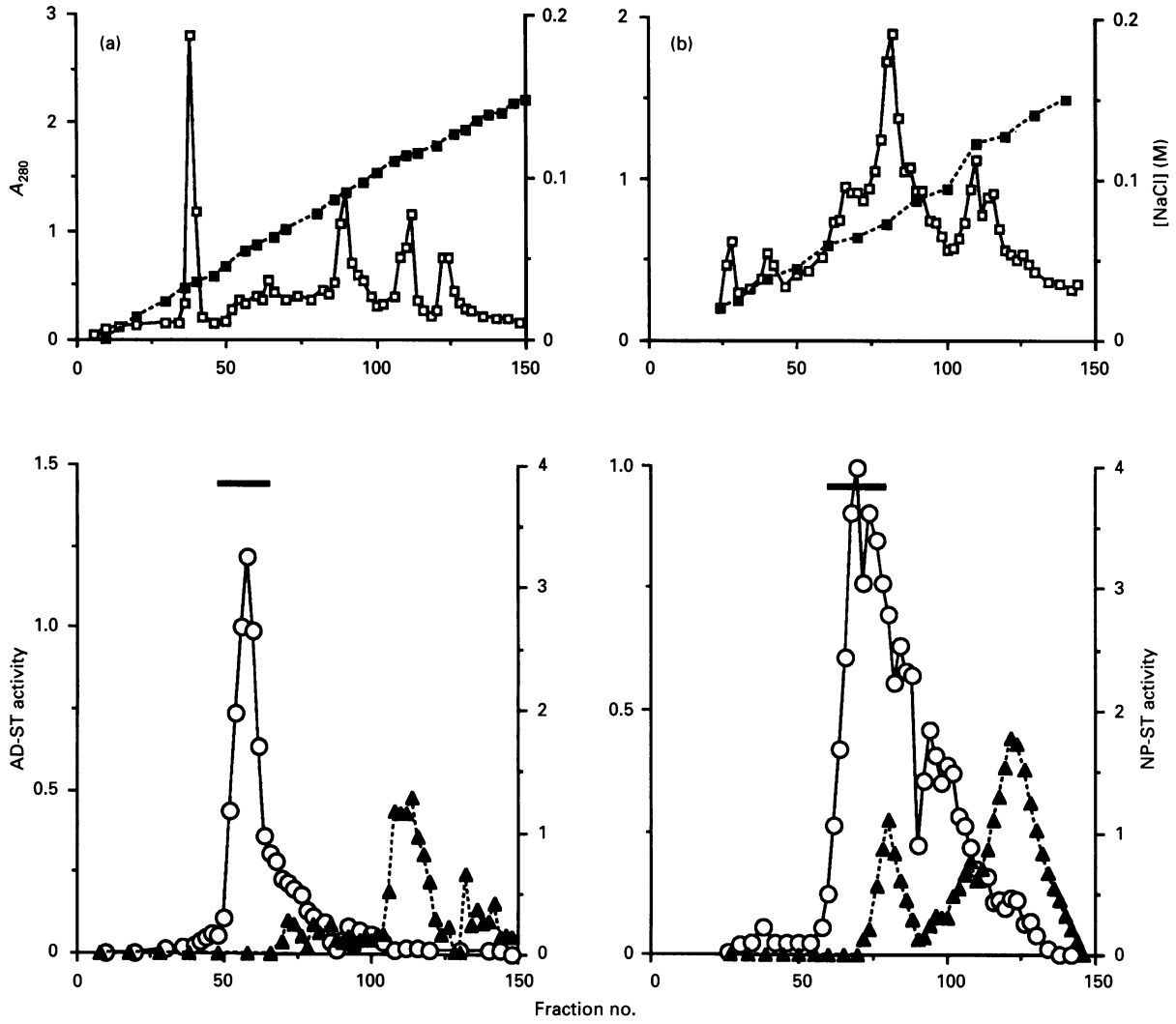

Figure 1 DEAE-cellulose column chromatography of liver cytosolic fractions obtained from weanling (a) and young adult female rats (b)

Details are described in the Materials and methods section. Fractions (18 ml) were assayed for absorbance (□), NaCl concentration (■), AD-ST activity (○) and 4-NP-ST activity (▲) as described in the Materials and methods section. The enzyme activities are expressed in nmol/min per ml. The fractions indicated by bars were pooled for subsequent purification.

previously described [18,22]. The cytosolic fractions contained approx. 16.7 mg of protein/ml (66 ml) and 21.7 mg of protein/ml (67 ml) from the weanlings and young adults respectively. ST activities toward AD and 4-nitrophenol (4-NP) were assayed by the method previously reported [18,22]. Protein concentrations were determined by the method of Bradford [23], with BSA as a standard.

Isolation of AD-ST

DEAE-cellulose chromatography

Liver cytosolic fractions of weanling and young adult female rats were fractionated on a DEAE-cellulose column (2.6 cm × 70 cm) as described previously [18,22]. The column was washed with 2000 ml of buffer A and the activity was eluted with 3000 ml in a linear gradient of 0–0.2 M NaCl in buffer A at a flow rate of 35 ml/h. The recovery of AD-ST activity through this column was about 40% for the samples from animals of both ages. As described in the text, the AD-ST fractions, which contained the highest AD-ST activity and a little 4-NP-ST activity, were separately pooled and concentrated approx. 20-fold by dialysis against 10% poly(ethylene glycol) 20000 in buffer A. The resulting preparations, which included approx. 80 mg of protein for the weanling rats and 100 mg of protein for the young adults were subsequently subjected to chromatofocusing.

Chromatofocusing of AD-ST

The concentrated AD–ST fractions (17–20 ml) were dialysed against a starting buffer (250 mM sucrose/3 mM 2-mercaptoethanol/25 mM Tris/acetic acid, pH 8.4) and applied to a PBE 94 chromatofocusing column (1 cm × 40 cm), which was previously equilibrated with the starting buffer. The AD-ST activity was eluted at a flow rate of 50 ml/h in 350 ml of elution buffer containing 2.7% Polybuffer 96/6.4% Polybuffer 74/250 mM sucrose/3 mM 2-mercaptoethanol (pH 5.0; adjusted with acetic acid), with a recovery of about 45%. As detailed below, the AD-ST activity eluted at pH 7.8 through to pH 7.2

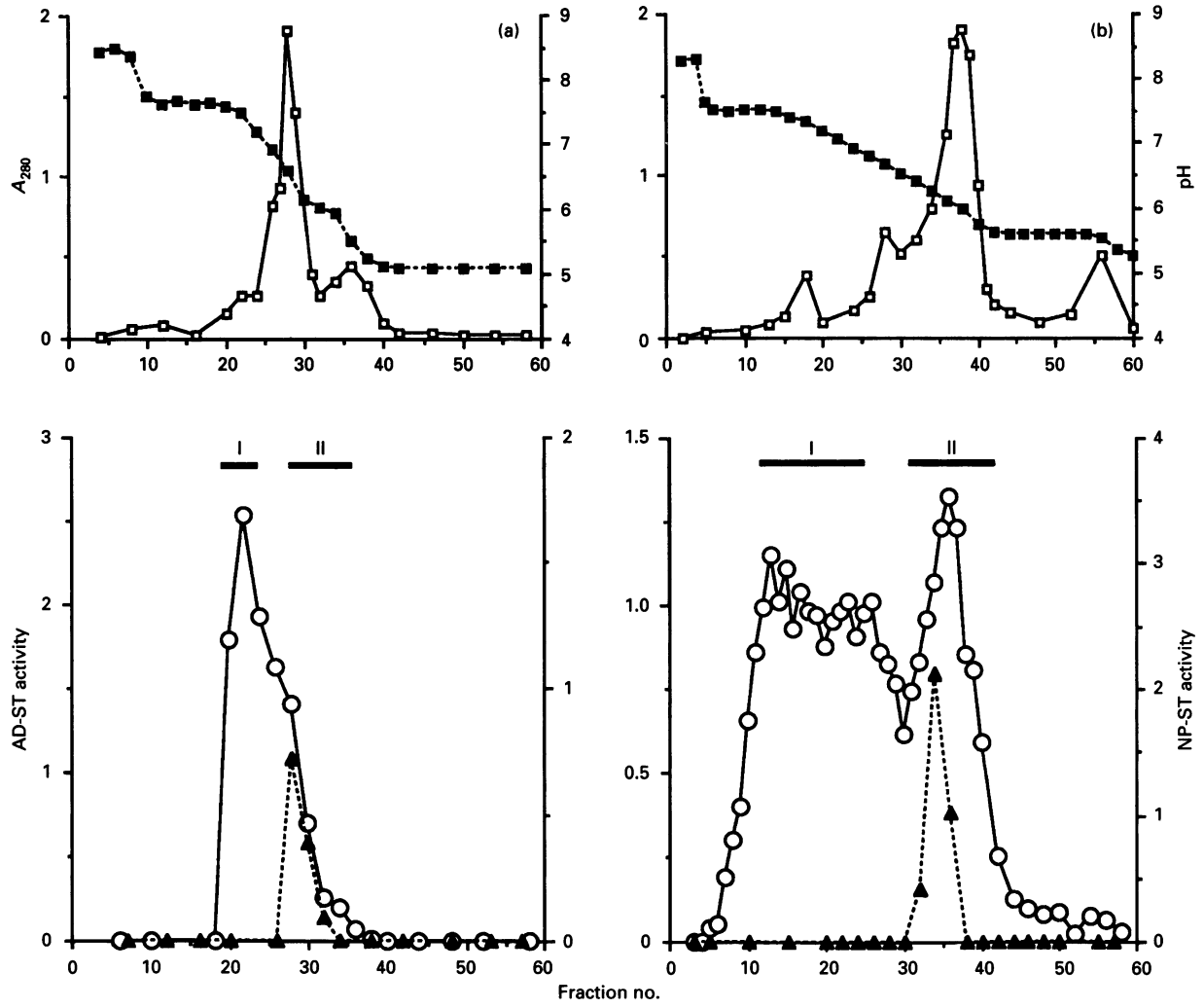

Figure 2 Chromatofocusing of AD-ST fractions obtained by DEAE-cellulose chromatography of weanling (a) and young adult female rats (b) as described in the Materials and methods section

Fractions (6 ml) were assayed for absorbance (□), pH (■), AD-ST activity (○) and 4-NP-ST activity (▲) as described in the Materials and methods section. The enzyme activities are expressed in nmol/min per ml. The fractions indicated by bars (fractions I and II) were pooled for subsequent purification.

and that at pH 6.6 through to pH 5.5 were pooled separately and subjected to PAP–agarose affinity chromatography.

PAP–agarose chromatography

The fractions pooled after chromatofocusing were dialysed against an affinity buffer (250 mM sucrose/3 mM 2-mercaptoethanol/50 mM Tris/HCl, pH 7.4) before application to a PAP–agarose column (1.5 cm × 3 cm). Approx. 30 mg of protein was applied to the column and the AD-ST activity was eluted by a slight modification of the method of Singer [24]. After sequential washings with 125 ml each of 1 mM 5′-AMP and 1 mM 3′-AMP in the affinity buffer, AD-ST was specifically eluted with 125 ml of 4 mM ADP in the buffer.

Electrophoresis

SDS/PAGE was performed as described by Laemmli [25]. Two-dimensional gel electrophoresis (2DGE) was carried out by the method of O'Farrell [26] as described previously [18,19]. In some cases isoelectrofocusing was done under non-denaturing, native conditions in the first dimension, followed by SDS/PAGE in the second dimension. Proteins were stained with Coomassie Brilliant Blue. Densitometry was carried out with Coomassie Blue-stained dried gels using Model CS-9000 scanning densitometer (Shimadzu Corp., Kyoto, Japan). The AD-ST polypeptides were specifically detected by immunoblot analysis with anti-AD-ST antiserum as described previously [19]. The molecular-mass markers used for SDS/PAGE and internal pI markers were the same as those described previously [19].

N-terminal amino acid sequencing

Proteins separated by 2DGE were electroblotted on to a ProBlott PVDF membrane by the method previously reported [27]. After Coomassie Blue staining, protein spots were excised and applied directly to a protein sequencer (Applied Biosystems Japan, model 473A or a Shimadzu Corp. model PSQ-2).

RESULTS

Isolation and comparison of AD-ST from liver cytosols of weanling and young adult female rats

DEAE-cellulose chromatography

The cytosolic fractions of female rat liver were prepared from weanling rats as well as from young adults. The fractions were subjected to DEAE-cellulose column chromatography, and the elution profiles of the AD-ST activity were compared between the two (Figure 1). In weanling rats, virtually only one peak of activity was eluted at about 50 mM NaCl (Figure 1a). On the other hand, the activity in young adults is apparent in two peaks; a major activity peak appeared at about 50 mM NaCl (fractions 60–80), while another minor peak occurred at about 100 mM NaCl (fractions 92–110; Figure 1b). It is noteworthy that between the two ages the elution profile of 4-NP-ST activity is different and that an age-related change in phenol ST isoenzyme composition is apparent. In addition to this, it is of interest that AD-ST activity is eluted in broader peaks in young adults than in weanlings, which may indicate that ST isoenzymes are more heterogeneous in young adults. The fractions which had the highest AD-ST activity and only a little 4-NP-ST activity were eluted at 44–65 mM NaCl in preparations from rats of both ages, so these fractions were pooled (separately for preparations from animals of each age), concentrated and subjected to chromatofocusing for analysis of the isoelectric properties of the enzyme.

Chromatofocusing of AD-ST

The elution patterns of the AD-ST activity from a chromatofocusing column are shown in Figure 2. The activities of weanling and young adult rats were eluted over a wide range of pH, from 7.8 to 5.5, consistently with our previous report [18]. The patterns are, however, markedly different between the two age groups. The activity eluted at pH 6.6–5.5 (fraction II) is obvious in young adults (Figure 2b), whereas that in weanlings is markedly lower (Figure 2a). The activity in the pH gradient from 7.8 to 7.2 (fraction I) is evident at both ages. We previously showed that purified AD-ST comprises several isoelectric variants with similar subunit molecular masses [18,19] and that the relative contents of the variants are different in the liver cytosols of weanling and young adult rats [19]. Fractions I and II from rats of each age were pooled separately and subjected to PAP–agarose affinity chromatography, followed by analysis of the isoelectric variants by 2DGE.

Gel electrophoresis

SDS/PAGE of purified fraction I from rats of both ages resulted in a single protein band with a subunit molecular mass of 30 kDa as reported previously [19], whereas purified fraction II from both ages contained a prominent band of molecular mass of 30 kDa as well as several faint bands (results not shown). 2DGE of fractions I and II of weanling rats exhibited principally three protein spots with pI values of 7.2, 6.7 and 6.1 by Coomassie Blue staining (Figures 3a and 3c). These protein spots are immunoreactive with anti-AD-ST antiserum and proved to be AD-ST isoelectric variants (Figures 4a and 4c). In fraction I, the relative levels of the pI 6.7 and 7.2 spots are both high, and the relative level of the pI 6.1 spot is low (Figure 3a). Densitometric analysis indicated that the relative amounts of the pI 6.7, 7.2 and 6.1 spots were 1.0, 1.2 and 0.08 respectively (Figure 3a). On the other hand, the relative levels of the pI 6.1 and 6.7 variants are

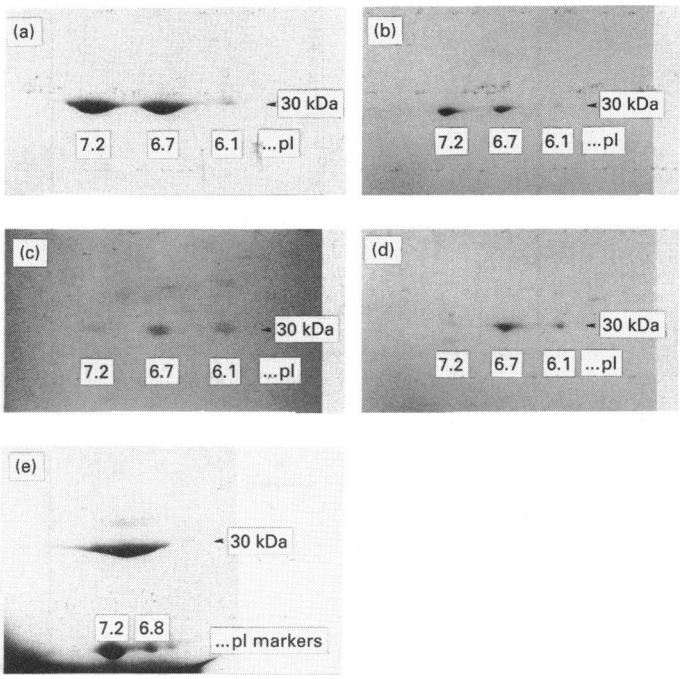

Figure 3 Two-dimensional gel electrophoresis of purified fraction I obtained from weanling (37 μg) (a) and young adult female rats (9 μg) (b) and purified fraction II from weanling rats (17 μg) (c) and young adults (12 μg) (d)

Electrophoresis of purified fraction I from weanlings (59 μg) was also done under non-denaturing conditions in the first dimension, followed by SDS/PAGE in the second dimension (e). Protein spots were stained with Coomassie Blue staining.

high and the spot of pI 7.2 is low in fraction II (Figure 3c). The relative amounts of the pI 6.7, 6.1 and 7.2 spots were 1.0, 0.51 and 0.41 respectively (Figure 3c). These observations are consistent with the chromatofocusing profiles (Figure 2) in which the AD-ST in fraction I was eluted at higher pH than that in fraction II. In fractions I and II of young adult rats similar results were obtained, as shown in Figures 3(b) and 3(d) and Figures 4(b) and 4(d). The relative amounts of pI 6.7, 7.2 and 6.1 spots were 1.0, 1.7 and 0.09 respectively in Figure 3(b), and in Figure 3(d) the relative levels of pI 6.7, 6.1 and 7.2 spots were 1.0, 0.30 and 0.12, respectively. When 2DGE was conducted under non-denaturing conditions in the first dimension, the pI variants were not resolved and migrated as an unresolved elongated protein band (Figure 3e).

These lines of evidence indicate that the relative contents of the pI variants of the major isoenzyme of AD-ST are different in weanling and young adult female rat livers. Thus, in the weanling rats, the AD-ST isoform is primarily composed of the pI 6.7 and 7.2 subunits, whereas in young adults the isoform(s) which consists of the pI 6.1 and 6.7 subunits is also increased. Therefore it was of importance to elucidate the primary structure of these pI variants, so the N-terminal amino acid sequences of the subunits were determined.

N-terminal amino acid sequencing

After separation by 2DGE and transfer on to PVDF membranes, the pI variants were subjected to Edman degradation, as described in the Materials and methods section. As indicated in Table 1, identical N-terminal amino acid sequences were obtained

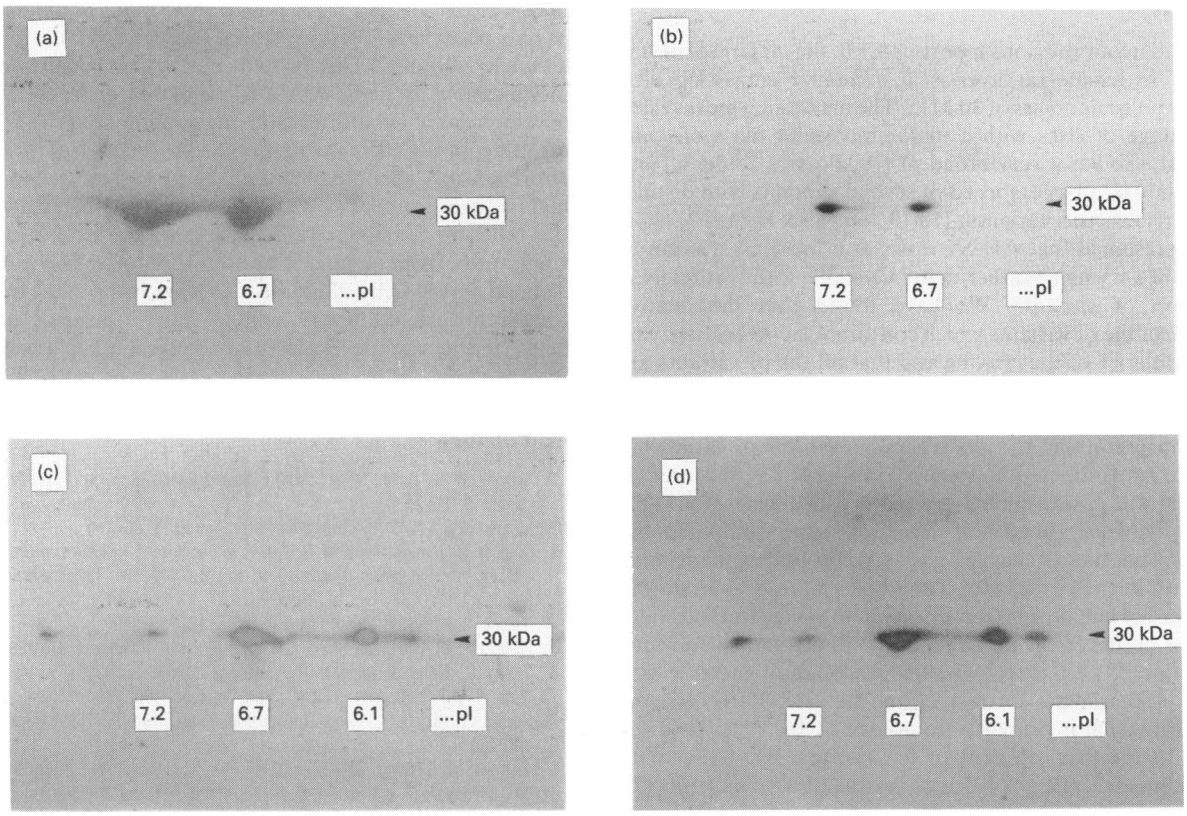

Figure 4 Immunoblot of purified fraction I obtained from weanling (a) and young adult female rats (b) and purified fraction II from weanling rats (c) and young adults (d)

The AD-ST polypeptides were detected as described in the Materials and methods section. Details were the same as in Figure 3. The protein spot with pI value higher than 7.2 is observed as described previously [19]. The identity of this spot is unclear at present, although it is thought to be a variant of AD-ST subunit.

Table 1 N-terminal amino acid sequences of pI variants of AD-ST

The sequences were determined on the 7.2, 6.7 and 6.1 pI variants of fraction I obtained from weanling and young adult rats and the 6.7 and 6.1 pI variants of fraction II from young adult rats. The variants of fraction II in weanlings and the 7.2 pI variant in fraction II of young adults were not determined, because they were not obtainable in sufficient quantity. Asterisks indicate residue ambiguity. Dashes in the amino acid sequences are identical with those of pI 7.2 variant of fraction I obtained from weanling female rats.

		N-terminal amino acid sequence					
pI variant	Position...	1	10	20	30	40	45
Weanlings							
Fraction I							
pI 7.2		PDYTWFEGIP	FPAFGIPKET	LQNVCNKFVV	KEEDLILLTY	PK	
pI 6.7		----------	----------	----------	----------	--SGT	
pI 6.1		----------	----------	----------			
Young adults							
Fraction I							
pI 7.2		----------	----------	----------	------**--	-	
pI 6.7		----------	----------	----------	------		
pI 6.1		----------	-------*-*	---			
Fraction II							
pI 6.7		----------	----------	----------	--*-		
pI 6.1		----------	----------	------			

for all the pI variants determined. The sequences shown in Table 1 are completely identical with the amino acid sequence deduced from the nucleotide sequence of rat liver hydroxysteroid ST (STa) [6], except for the N-terminal methionine residue. They also displayed a high degree of similarity to the sequences of another rat liver isoenzyme, hydroxysteroid ST (ST-20) [28] and senescence marker protein-2 (SMP-2), a putative isoenzyme of rat hepatic hydroxysteroid ST [29].

DISCUSSION

As indicated in our previous reports [18,19], the major isoenzyme of AD-ST in female rat liver is an oligomer consisting of a subunit of molecular mass of 30 kDa. The native enzyme revealed a broad range of sizes with a major molecular mass of some 600 kDa. It also has a very broad pI [18; Figures 2 and 3e] and has been shown to be composed of several subunits with distinct pI values (isoelectric variants) [18,19; see also Figure 3]. It is therefore presumed that AD-ST exists as a loose association of oligomers of varying pI rather than in a stable form with a fixed composition of variants. We have found that the relative proportion of the pI variants which constitute the AD-ST enzyme determines the pI of the enzyme and that all the pI variants are probably responsible for the enzyme activity.

We showed previously [19], by immunoblot analysis with anti-AD-ST antiserum, that the relative amount of the pI variants in the liver cytosols differed between 20- and 110-day-old rats. In the present study, we isolated the major isoenzyme of AD-ST from the livers of animals of these ages and compared the isoelectric properties of the native enzymes as well as the relative composition of the pI variants. The results indicate that, during development of female rats from the weanling stage to adulthood, an increase occurs in the amounts of the pI 6.1 and 6.7 variants of the major AD-ST isoenzyme. A concomitant increase was observed in the acidic form(s) of the native AD-ST enzyme (Figure 2b), which is primarily composed of the pI 6.1 and 6.7 subunits. In the liver cytosols of female rats, AD-ST activity displays a biphasic alteration during postnatal development [17]. The increase in acidic form(s) is presumed to take place through the temporary decline in AD-ST activity at about 40 days of age. The functional significance of this change is unknown at present.

The N-terminal amino acid sequences of the AD-ST subunits are identical, except for the N-terminal methionine, with the sequence deduced from the nucleotide sequence of STa, which is an isoenzyme of rat hepatic hydroxysteroid ST [6]. The N-terminal methionine may be eliminated post-translationally. The sequences also displayed a high degree of similarity to those of ST-20 [28] and SMP-2 [29], both isoforms of rat hepatic hydroxysteroid ST. Therefore, AD-ST could be identical with, or closely related to, these isoenzymes. Recently Otterness et al. [9] reported that human liver dehydroepiandrosterone ST exhibits three protein spots with identical subunit molecular mass, 35 kDa, by 2DGE. They also showed that the partial amino acid sequences of these protein spots are identical. This observation is very similar to our results with AD-ST and represents interspecies conservation of structure and function of hydroxysteroid ST between man and rat. Consistently, the nucleotide sequence of human liver dehydroepiandrosterone ST [9] shows a high degree of sequence similarity to that of rat hydroxysteroid ST [6,28], which is presumed to be identical with AD-ST or a closely related ST isoenzyme.

The N-terminal amino acid sequences of the isoelectric variants of AD-ST are identical within the areas determined, irrespective of animal age or pI value. These results indicate that pI variants may have originated from post-translational modification such as phosphorylation, glycosylation, sulphation or deamination of a single subunit. This would mean that post-translational modification of hepatic AD-ST subunits changes during the development of female rats from the weanling stage to adulthood, resulting in an increase in the acidic form(s) of the enzyme. However, it is also possible that the pI variants are proteins of high sequence similarity with distinct primary structures outside the N-terminal region. In such a case, the acidic pI variants would arise from enhanced transcription of closely related, but distinct, genes or by alternative splicing of primary transcripts. Further investigation will help to clarify the issue of subunit heterogeneity and developmental changes in hepatic AD-ST.

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