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Substrate Specific Sulfatase Activity from Hair Follicles in Recessive X-linked Ichthyosis

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Recessive X-linked ichthyosis (RXLI) has its biochemical basis in a defect of the enzyme steroid sulfatase. Since several studies have reported a simultaneous deficiency of arylsulfatase C and steroid sulfatase it has been hypothesized that both enzymes are identical. In human hair follicles, however, hydrolytic activity for 4-methylumbelliferone sulfate, the substrate for arylsulfatase C, is found, while dehydroepiandrosterone sulfate is not hydrolyzed at all. These findings suggested the possible existence of two different enzymes. In the present paper structure-activity studies and molecular energy calculations are used for the demonstration that the remaining sulfatase activity in hair follicles of RXLI patients can be explained on the basis of the assumption that the enzyme has not lost its total function but has become less efficient. (Received December 9, 1986.)

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Several major forms of ichthyosis can be distinguished on the basis of clinical features and mode of inheritance (1). One of these forms, recessive X-linked ichthyosis (RXLI), is reported to occur in 1 of 6000 males (2). It is now generally accepted that RXLI has its biochemical basis in a defect of the enzyme steroid sulfatase (3, 4). The biochemical basis of the other forms has still to be elucidated. The mechanism by which steroid sulfatase deficiency causes RXLI is related to the accumulation of cholesterol sulfate in the stratum corneum membranes (5, 6). The altered ratio between cholesterol and cholesterol sulfate would change the physical properties of the stratum corneum membranes, resulting in ichthyotic scales.

In recent years various methods have been developed for measuring steroid sulfatase. Several tissues have been used such as cultured fibroblasts (7, 8), leukocytes (9), nails, callus and hair follicles (10). The most commonly employed technique for the measurement of steroid sulfatase activity uses ^{3}H-dehydroepiandrosterone sulfate (DHEAS) as substrate. It is suitable for diagnosis of hemi- and heterozygous individuals with steroid sulfatase deficiency (11), but is regarded rather time-consuming.

Several studies have reported a simultaneous deficiency of arylsulfatase C and steroid sulfatase. It has therefore been hypothesized that both enzymes are identical (12, 13, 14). In human hair follicles, however, hydrolytic activity for 4-methylumbelliferone sulfate, the substrate for arylsulfatase C is found, while dehydroepiandrosterone sulfate is not hydrolyzed at all (15). Therefore, up till now statements with regard to this question remain necessarily speculative. Most studies dealing with the question are not quite comparable due to different experimental conditions.

In the present paper we use structure-activity studies and molecular energy calculations for the demonstration that the remaining sulfatase activity in hair follicles of RXLI patients can be explained on the basis of the assumption that the enzyme has not lost its total function but has become less efficient.

MATERIALS AND METHODS

Patients and controls

Twelve patients were examined in this study. Nine of them were affected members of two families with RXLI and the other three patients had other dermatoses as indicated in Table I. Each case was diagnosed on the basis of clinical appearance, histological aspects and family history. Controls were five healthy male adults.

Enzymatic hydrolysis of dehydroepiandrosterone sulfate and estrone sulfate

The radiometric assay used is a modification of a method described by Faredin et al. (16). Before each experiment ^{3}H-DHEAS (spec. act. 35 Ci/mmol, NEN, Boston) or ^{3}H-estrone sulfate (ES) (spec. act. 40 Ci/mmol, NEN, Boston) was purified by silicagel thin layer chromatography (ethylacetate-ethanol-conc. NH_4OH: 5–5–1). The purified substrate was located with X-ray film, scraped from the silicagel plate and dissolved in 200 µl distilled water. Subsequently 100 µl 2 M NH_4OH and 300 µl saturated $(NH_4)_2SO_4$ were added and DHEAS or ES was extracted three times with ethylacetate. After evaporation of the ethylacetate under a stream of nitrogen, the purified substrate was dissolved in ethanol.

Hairs were plucked at random from the scalp. Only hair follicles with a visible bulb and sheath were used. For each determination 10 hair follicles were immersed in 200 µl incubation medium consisting of 50 mM Tris-HCl buffer (pH 7.4), 0.2 M sucrose and a NADPH generating system (1 mM NADP, 20 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase/ml and 0.5 mM $MgCl_2$) (17). All samples were incubated with 0.4 µCi purified DHEAS or ES for 4 h at 37°C. At the end of the incubation period 100 µl 2M NH_4OH and 300 µl $(NH_4)_2SO_4$ were added. The incubation medium was extracted three times with 500 µl ethylacetate. The organic phases were combined and evaporated to dryness under a stream of nitrogen. The steroids in the residue were dissolved in 100 µl ethanol,

Table I. *Documentation on the patients*

	Age	Age of onset	Family history	Clinical picture
P_1	30♂	From birth on	11 males affected, females unaffected	Extensive brownish scaling, slightly encroaching in the flexures. Face, palms and soles unaffected
P_2	34♂			Slight to moderate scaling, predominantly on the extensor parts of the limbs
P_3	28♂			
P_4	31♂			
P_5	29♂	From birth on	5 males affected, females unaffected	Slight to moderate scaling. Large brownish flakes on the lower part of the legs. Flexures unaffected
P_6	26♂			
P_7	22♂			
P_8	17♂			
P_9	24♂			
P_{10}	30♂	From birth on	2 males and one female affected	Brownish scales all over the body. Flexures involved
P_{11}	28♀	?	–	Atopic dermatitis
P_{12}	51♀	From 12th year	–	Pityriasis rubra pilaris

applied to silicagel thin layer plates and chromatographed in the above-mentioned TLC system. The metabolites were examined autoradiographically by exposure of the plates to X-ray films after spraying with radiographic enhancer. For quantification of the metabolites the areas were cut out and the amount of radioactivity was determined in a LKB Rackbeta scintillation counter, using toluene/PPO/POPOP as scintillation fluid.

Enzymatic hydrolysis of 4-methylumbelliferone sulfate

The determination of the arylsulfatase C activity in hair follicles was essentially the same as described by Meyer et al. (15). For each determination one hair follicle with a visible bulb and sheath was immersed in 50 µl 0.1 M phosphate buffer (pH 8.0), which inhibits the activity of arylsulfatase A and B. The hair follicles were frozen (−20°C) and thawed three times (room temperature) to improve the penetration of the substrate. Subsequently 100 µl 0.4 mM 4-methylumbelliferone sulfate (4-MUS) in 0.1 M phosphate buffer (pH 8.0) was added. After 5 h of incubation at 37°C the reaction was terminated with 1 ml 0.1 M glycine/carbonate buffer (pH 10.3). The fluorescence of 4-methylumbelliferone was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. It must be stressed that the samples had to be diluted ten times in order to obtain a fluorescent signal within the range of our fluorescence spectrophotometer.

Synthesis of equilenin sulfate

The compound was prepared via the method described by Grant & Glen (18), with the notable exception of the work-up procedure, improving the yield significantly (from 69 to 90%). 95 mg d-equilenin was dissolved in 3.5 ml dry pyridine and added dropwise to a stirred solution of chlorosulfonic acid (350 mg, 3 mmol) in a mixture of dry chloroform (3.5 ml) and dry pyridine (1.7 ml) at 0°C. After 6 h at room temperature and 24 h at 40°C no starting material could be observed after thin-layer chromatography (trichloromethane-methanol-acetic acid: 95–20–3) and the mixture was concentrated in vacuo. The residual red oil was dissolved in methanol (13 ml) and treated with decolorizing carbon. After removal of the charcoal the methanolic solution was neutralized to an apparant pH of 7.8 by addition of 1 M sodium hydroxide in methanol. The mixture was evaporated to dryness and the residue was dissolved in n-butanol and washed twice with water. The organic layer was evaporated to dryness. The crude product was reprecipitated by dissolution in methanol (5 ml) and dropwise addition to ether (70 ml). Centrifugation followed by two washings with ether and drying provided the

Histology	Diagnosis
Massive hyperkeratosis, stratum spinosum normal thickness	X-linked ichthyosis
–	X-linked ichthyosis
Hyperkeratosis, acanthosis	Lamellar ichthyosis
Papillomatosis, acanthosis hyperkeratosis, parakeratosis perivascular mononuclear infiltrate	Atopic dermatitis
Follicular hyperkeratosis, acanthosis, hyperkeratosis, perivascular round cell infiltrate	Pityriasis rubra pilaris

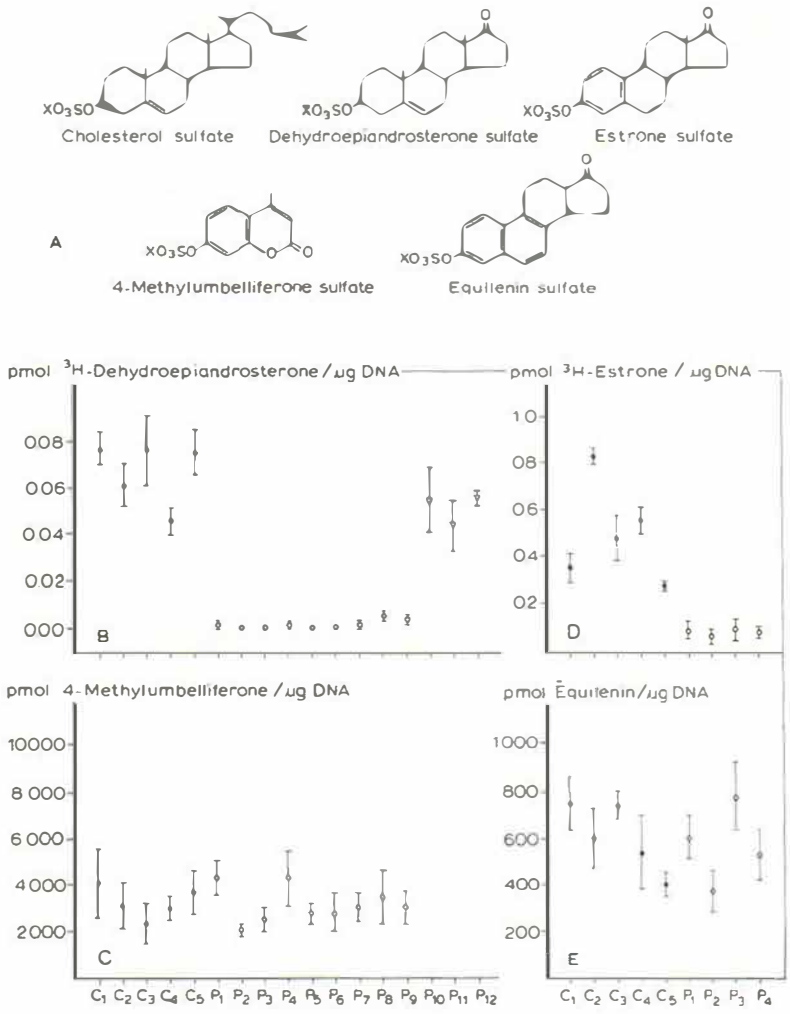

Fig. 1. Determination of sulfatase activity in hair follicles. The data are expressed as the mean value ± standard deviation. C_{1-5} are healthy controls, P_{1-9} are patients with RXLI and P_{10-12} are patients with other dermatoses. The clinical diagnosis is listed in Table I. (A) Structures of the different substrates used for the determination of sulfatase activity. (B) Enzymatic hydrolysis of dehydroepiandrosterone sulfate ($n=5$). (C) Enzymatic hydrolysis of 4-methylumbelliferone sulfate ($n=10$). (D) Enzymatic hydrolysis of estrone sulfate ($n=5$). (E) Enzymatic hydrolysis of equilenin sulfate ($n=5$).

equilenin sulfate as a TLC-homogenous off-white compound; yield 118 mg (90%), $R_f=0.21$ (trichloromethane-methanol-acetic acid: 95–20–3) and $[\alpha]_D^{22}=+69$ ($c=0.25$, H_2O).

Enzymatic hydrolysis of equilenin sulfate

Optimal assay conditions for the sulfatase activity with equilenin sulfate in hair follicles were as follows: 5 hair follicles with visible bulb and sheath were added to 200 µl incubation medium consisting of 50 mM Tris-HCl buffer (pH 7.4), 0.2 M sucrose and a NADPH generating system (1 mM NADP, 20 mM glucose-6-phosphate, 1 unit glucose-6-phophate dehydrogenase/ml and 0.5 mM $MgCl_2$) (17). Subsequently 2 µl of a stock solution of 20 mg/ml equilenin sulfate was added (dissolved in methanol-water: 1–1) and the samples were incubated for 4 h at 37°C. Due to the strong resemblance of the excitation- and emission spectra of equilenin and equilenin sulfate an extraction step was

needed to quantify the amount of equilenin liberated. After the required incubation time the samples were therefore extracted three times with 500 µl n-hexane. The amount of equilenin in the combined extract was determined fluorimetrically at an excitation wavelength of 292 nm and an emission wavelength of 365 nm.

Determination of DNA

Measurement of the DNA content in the hair follicle samples was used to compensate for differences in hair follicle size and quality and was determined as described by Hukkelhoven et al. (19).

Molecular energy force field calculations

Minimal molecular energies of the different desulfatised substrates were obtained with a molecular mechanics computational analysis (20) (MM2-program, Quantum Chemistry Program Exchange, QCPE 13, 395, Indiana University, Bloomington).

RESULTS

In Fig. 1 A the structures of the different substrates used are given together with cholesterol sulfate, a physiologically important substrate for steroid sulfatase in skin, related to stratum corneum cell adhesion (6). Fig. 1 B shows that hair follicles of patients with RXLI (P_{1-9}) are deficient in their steroid sulfatase activity towards DHEAS in comparison to controls (C_{1-5}) or the three patients with other dermatoses (P_{10-12}). The mean value of DHEA liberated is 0.0017±0.004 pmol/µg DNA/hour for the controls and 0.000 pmol/µg DNA/hour for the patients with RXLI. However, with 4-methylumbelliferone sulfate as a substrate, no deficiency of arylsulfatase C could be observed in the same patients and controls (Fig. 1 C). The hair follicles of RXLI patients showed an average activity of 687±275 pmol 4-methylumbelliferone/µg DNA/hour which matches that of the controls: 648±255 pmol/µg DNA/hour.

Steroid structures were selected with the A and B rings resembling the rings of 4-methylumbelliferone sulfate. Estrone sulfate has a comparable A ring while in equilenin sulfate also the B ring shows some resemblance to 4-methylumbelliferone sulfate. Determination of sulfatase activity with estrone sulfate as a substrate gives results comparable to that obtained with DHEAS. Patients and controls can be easily distinguished (Fig. 1 D). Mean sulfatase activity in controls: 0.124±0.047 pmol estrone liberated/µg DNA/hour versus 0.021±0.008 pmol/µg DNA/hour. The use of equilenin sulfate as a substrate however does not allow discrimination between patients and controls (Fig. 1 E). The results of all four assays are summarized in Table II.

Table II. *Sulfatase activity in hair follicles of patients with recessive X-linked ichthyosis and normal controls*

All data are recalculated to pmol product/µg DNA/hour±standard deviation

Substrate	Controls	RXLI
Dehydroepiandrosterone sulfate	0.017±0.004	0.000±0.000
Estrone sulfate,		
normal concentration	0.124±0.047	0.021±0.008
500 times higher concentration	35.6±2.5	9.3±1.8
4-methylumbelliferone sulfate,		
normal concentration	648±255	687±275
40 times lower concentration	13.2±3.3	15.2±1.1
Equilenin sulfate	151±42	150±43

Since the two radiometric assays were performed at substrate concentrations that were an order of magnitude 5 000 lower than those in the two fluorimetric assays, changes in these concentrations were investigated for a possible effect on sulfatase activity in hair follicles of patients with RXLI. The results for estrone sulfate concentrations that were 500 times increased and 4-methylumbelliferone sulfate concentrations that were 40 times decreased are shown in Table II. It is evident that at these concentrations the results are still comparable to the previous situation. Hair follicles of patients with RXLI show activity towards 4-methylumbelliferone sulfate but not towards estrone sulfate as a substrate.

Molecular energy calculations were performed with the aim to investigate whether or not the above results had to be explained by the existence of one or more sulfatases in hair follicles.

According to the Hammond postulate (21) a one step endothermic process will have a transition state (activated complex) with a structure and a geometry very similar to the product to be formed. The relative stability of the endproduct in such a reaction will therefore influence the transition state level and as a consequence the reaction rate. Consequently, the endproduct having the highest stability (lowest molecular energy = MM2 energy) will have the highest reaction rate. In Table III the relative molecular energies of the molecules are depicted.

DISCUSSION

For a dermatologist it is of importance to select therapy as much as possible according to the individual situation of the patient. A molecular approach has often allowed to further subclassify diseases that could not be distinguished on the basis of clinical appearance. In the present paper we use molecular energy calculations in order to explain the presence of arylsulfatase C activity in hair follicles of patients with RXLI.

Assuming that the mechanism of hydrolysis for all sulfates is identical we may compare molecular energies with the aim of predicting reaction velocities, if the molecular structures are not too much different. Structural formulas of estrone sulfate and equilenin sulfate fulfill this criterion. Both molecules differ only in two double bands in the B ring of the steroid. Molecular energies are 10.98 and 6.02 for estrone and equilenin respectively. As such it would be expected that in the normal situation formation of free equilenin would be an order of magnitude higher than that of free estrone formation. A deficient enzyme no longer functioning optimally might just be capable of hydrolyzing equilenin sulfate but

Table III. *Minimal molecular energies of each of the desulfatised substrates, as calculated with the MM2 program (expressed in arbitrary units)*

Force field parameters	DHEA	Estrone	Equilenin	5-Methyl-umbelliferone
Strain	0.63	0.44	0.22	0.16
Stretch-bend	0.14	0.05	−0.03	−0.01
Van der Waals	5.88	5.95	4.85	5.32
Binding	1.41	2.15	0.75	4.14
Torsion	4.24	2.34	0.23	−0.29
Dipole	0.20	0.06	0.00	0.09
MM energy	12.50	10.98	6.02	9.41

would no longer be capable of hydrolyzing estrone sulfate. Comparison of estrone and dehydroepiandrosterone is more difficult. The binding component is higher in estrone than in dehydroepiandrosterone, but the torsion component (see Table III) behaves different and is higher in dehydroepiandrosterone than in estrone. Consequently, the energy difference between both molecules is relatively low. Therefore an important difference in the velocity of hydrolysis is not to be expected on the basis of these theoretical grounds. The molecular structure of 4-methylumbelliferone differs to much from the steroids to allow a comparison on the basis of molecular energy levels.

The above reasoning allows to explain our present observations with regard to different substrates on the basis of one single enzyme system which is aberrant in RXLI patients. It is not yet known whether the same aberration is present in all patients with RXLI. The enzyme steroid sulfatase may well be malfunctioning in different families with RXLI on the basis of different mutations. Moreover, steroid sulfatase may consist of different isoenzymes which are not necessarily all affected by a specific mutation of the enzyme. The controversy described in the literature with regard to the absence of either steroid sulfatase or arylsulfatase C of both enzymes in different tissues should be reviewed in the light of this reasoning.

The clinical appearance of RXLI may differ enormously between patients. It is tempting to speculate that the molecular basis of this phenomenon could be found in the presence of different mutations. Both families in our study appear to have a mutation which allows the hydrolysis of substrates leading to a low energy product at an almost normal rate. Other mutations leading to an even lower rest activity of the enzyme could be theoretically expected to have a more severe clinical appearance. On the basis of our present results the existence of possible different mutations at the same locus (allelism) could be simply checked by measuring in different families the hydrolytic capacity of hair follicle sulfatase towards for example estrone sulfate and equilenin sulfate. This may allow a further subclassification and diagnosis of RXLI.

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