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Purification and Characterization of Corticosteroid Side Chain Isomerase†

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ABSTRACT: Corticosteroid side chain isomerase of rat liver catalyzes the interconversion of the ketol (20-oxo-21-ol) and aldol (20-hydroxy-21-al) forms of the corticosteroid side chain. The enzyme has now been purified to apparent homogeneity from rat liver cytosol by sequential chromatography on anionic, hydroxylapatite, and gel filtration columns. Ketol–aldol isomerization is followed by measuring the exchange of tritium from 21-tritiated steroids with water. The native enzyme is a dimer of MW 44000. The isoelectric point is 4.8 ± 0.1 pH units. The purified enzyme is stimulated by Co^{3+} or Ni^{2+}. The enzyme utilizes 11-deoxycorticosterone, corticosterone, and 17-deoxycortisol as substrate but not cortisol, tetrahydrocortisol, and prednisolone. Tritium–water exchange of (21*S*)-[21-^{3}H]DOC is a pseudo-first-order reaction; 21-^{3}H exchange from the 21*R* isomer proceeds with first-order kinetics only after a lag associated with its epimerization to the 21*S* form.

The structures of the neutral urinary metabolites of cortisol in humans are well established. There are additional metabolites, which make up to 20% of the total excretion products in adults, that have been shown by us to be acids. The major acidic metabolites are 20-hydroxy-21-oic acids, to which we have given the collective name cortoic acids (Bradlow et al., 1973). The 21-oic acids are major metabolites of cortisol and corticosterone in other mammals as well (Monder & Bradlow, 1980). Mice convert corticosterone to acid end products that are structurally similar to the cortoic acids in a sequence of steps that parallel those that occur in man (Han et al., 1983). This pathway of corticosterone metabolism is quantitatively important. About half of the corticosterone secreted by the mouse adrenal is converted to epimeric 20-hydroxy-21-oic acids (Marandici & Monder, 1983; Han et al., 1983). We have found that the main metabolic pathway to the hydroxy acids utilizes a 20-hydroxy-21-aldehyde intermediate formed by rearrangement of the ketol side chain (Martin et al., 1977; Monder et al., 1980a). This is a central intermediate since it sits at a metabolic junction where the choice is made between the conversion of corticosteroids to metabolites with acidic (20-hydroxy-21-oic acid) and those with neutral (20,21-diol) side chains (Monder et al., 1980a,1982; Han et al., 1981; Wermuth & Monder, 1983). This relationship is illustrated in Figure 1. The ketol–aldol interconversion that initiates this process is catalyzed by corticosteroid side chain isomerase (Monder et al., 1980b). In this paper we describe the purification and properties of this enzyme.

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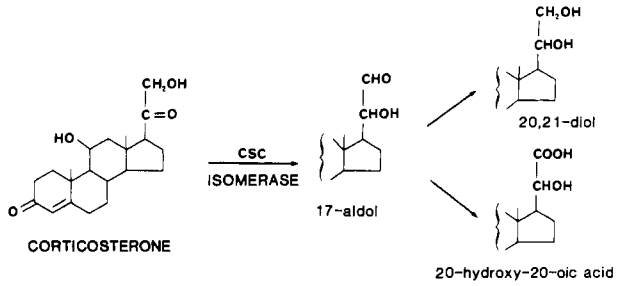

FIGURE 1: Scheme showing the conversion of corticosterone to 21-oic acid metabolites. CSC isomerase, corticosteroid side chain isomerase.

Materials and Methods

Supplies. Steroids were brought from Steraloids (Wilton, NH). (4*S*)-[4-^{3}H]NADH was synthesized as described by Willingham and Monder (1973). NAD and NADH were purchased from Amersham (Arlington Heights, IL).

Bradford protein reagent (Coomassie Brilliant Blue R-250), hydroxylapatite, protein molecular weight standards, and most of the reagents for SDS–PAGE were purchased from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin, βNADH, and alcohol dehydrogenase were bought from Sigma Chemical Co. (St. Louis, MO). Accell QM4 anion exchanger medium was obtained from Waters (Milford, MA). Ampholines and Sephacryl S-30 were purchased from Pharmacia LKB (Piscataway, NJ). Flexible microtiter plates with flat-bottom wells (Falcon 3912) were obtained from VWR Scientific (San Francisco, CA). Optical absorbance of the wells was monitored at designated wavelengths by an Mr 600 Microplate reader (Dynatech Laboratories Inc., Torrance, CA). Standards for protein concentration determinations consisted of aqueous bovine serum albumin diluted serially in the range 1024–0.25 μg/mL.

Animals. Male BALB/c mice, 8 weeks old, were bought from the Charles River Breeding Laboratory (Wilmington, MA). They were maintained for 1–2 weeks at 21–22 °C and fed Purina Rodent Laboratory Chow (Ralston Purina Co., St. Louis, MO) and water ad lib.

Partial Purification of Goat Liver 21-Hydroxysteroid Dehydrogenase (Monder & White, 1963). A 132-g piece of goat liver was homogenized in 4 volumes of 0.1 M sodium phosphate, pH 7.4, and then centrifuged at 1000*g* for 20 min. The supernatant fraction was decanted and fractionated with ammonium sulfate. The precipitate formed between 20% and 50% ammonium sulfate was recovered by centrifugation at 1000*g* for 25 min, resuspended in 25 mL of 0.01 M potassium phosphate, pH 7.0, filtered through Whatman No. 1 filter paper, and applied to a Waters Accell QMA anion exchange column. The material was eluted with a gradient of 0.5 M potassium phosphate buffer, pH 7.0. Fractions of 5 mL were collected. Enzyme activity was detected by measuring the decrease in absorbance at 340 nm of an incubation mixture containing 300 μL of eluant fraction, 30 μL of a 10 mM solution of 21-dehydro-11-deoxycorticosterone in 50% aqueous propylene glycol, 640 μL of 0.1 M potassium phosphate, pH 7.0, and 30 μL of 6 mM NADH. Active fractions emerging between 0.137 and 0.337 M potassium phosphate were recovered.

Synthesis of 21S- and 21R-^{3}H Isomers of 11-Deoxycorticosterone. Corticosteroids with tritium incorporated into position 21 were synthesized according to the stereospecific procedure of Willingham and Monder (1973, 1974) using goat liver 21-hydroxysteroid dehydrogenase as catalyst. The 21*S* isomer was generated by reducing 21-dehydro-11-deoxycorticosterone with [^{3}H]NADH; the 21*R* isomer was made by reducing 21-dehydro-11-deoxy[21-^{3}H]corticosterone with NADH. The stereochemistry of reduction was established as described by Orr et al. (1975). Specific activities of the 21*S* and 21*R* isomers were 97 and 14 μCi/μmol, respectively.

Other radioactive steroids prepared in the same way were 11β,17,21-trihydroxy[21-^{3}H]pregn-4-ene-3,20-dione ([21-^{3}H]cortisol), 11β,21-dihydroxy[21-^{3}H]pregn-4-ene-3,20-dione ([21-^{3}H]corticosterone), 3α,11β,17,21-tetrahydroxy[21-^{3}H]-5β-pregnan-20-one ([21-^{3}H]THF), 17,21-dihydroxy[21-^{3}H]pregn-4-ene-3,20-dione ([21-^{3}H]S), and 11β,17,21-trihydroxy[21-^{3}H]pregna-1,4-diene-3,20-dione ([21-^{3}H]prednisolone). Purification of the steroids was done on silica gel coated thin-layer plates with graded mixtures of chloroform and methanol as resolving solvents. Homogeneity of the steroids was confirmed by chromatographic analysis using the Bioscan System 200 imaging scanner (Bioscan, Washington, DC).

Specific activities of the steroids were calculated on the basis of optical absorption maxima with solvent molar extinction coefficients as indicated by Engel (1963) for the respective steroids. Values obtained were in the range of 12–26 mCi/mmol.

Protein Assay. Direct assay of chromatographic effluents was performed by continuous spectrophotometric monitoring at 280 nm. Discontinuous assay was performed by the Bradford method (Bradford, 1976). When the concentration of protein was less than 100 μg/mL, we utilized a modification of the method of Brogdon (1984) with improved sensitivity.

Bradford reagent was diluted 1:4 with distilled water and filtered immediately before use. Diluted reagent was added to the protein solutions in the wells of the microtiter plate with a Gilson Pipetman dispenser Model R-200 (Rainin Instruments, Woburn, MA). Reagent was kept in the barrel of a 20-cm^3 syringe attached directly to the dispenser. The ejector of the dispenser was extended with a 20-μL plastic pipet tip with a small opening. Thus, when the piston of the pipet was depressed with sufficient vigor (avoiding splashing and bubbles), the reagent and sample were mixed by the energy of the expelled reagent. Further stirring was not necessary. The plates were read by the plate reader set in the automatic mode and in the dual-wavelength optical system (λ_T/λ_R) of 5:3. The test filter T (#5) and reference filter R (#3) pass wavelengths of 630 and 490 nm, respectively.

Data were analyzed by computer programs developed in this laboratory for the IBM PC/XT computer using Enzfitter software (Biosoft, Milltown, NJ) or on the VAX computer at Rockefeller University using a program developed by Dr. Glen Gunsalus of The Population Council. By use of standard curves prepared with bovine serum albumin, a lower limit of detection of 40 ng of protein was attained.

Isomerase Assay. For analysis of corticosteroid side chain isomerase, we monitored the exchange of tritium at C-21 with water. Each incubation vessel contained 1 nmol (90 000 dpm) of [21-^{3}H]DOC in 5 μL of methanol, 0.05 mL of enzyme, and 0.445 mL of 0.025 M Tris, pH 8.0, in a total volume of 0.5 mL incubated at 37 °C for 2 h. Tritium in water was determined after lyophilization by scintillation analysis of the lyophilizate. Modifications are indicated in the text. Velocity is expressed as nmol min^{-1} (mg of protein)$^{-1}$.

Antibody Production. Antibody to homogeneous corticosteroid side chain isomerase was produced in female New Zealand white rabbits. After blood was collected for the preparation of preimmune serum, each animal was injected subcutaneously at multiple sites with a total of 50 μg of antigen in Freund's complete adjuvant. Blood samples were collected

after 6 weeks. A booster dose of 50 μg of antigen per animal was administered in incomplete Freund's adjuvant. Two weeks later, all animals showed the presence of antibodies. A second booster dose of 50 μg of isomerase in Freund's incomplete adjuvant was injected, and the animals were killed with an air bolus 2 weeks later. The serum was recovered after the blood had been allowed to clot overnight at 4 °C. Sera were stored as 1-mL aliquots at −20 °C. Two-dimensional double immunodiffusion was performed with the Ouchterlony (1968) technique.

Purification of Corticosteroid Side Chain Isomerase. Mice were killed by cervical dislocation. Livers were quickly dissected out, care being taken to remove the gall bladder. Tissues were prepared and utilized immediately. Mouse livers were rinsed with saline, blotted dry, and homogenized in 4 volumes of 0.25 M sucrose in 0.01 M sodium phosphate, pH 7.0, in a Teflon glass homogenizer. The homogenate was centrifuged at 105000*g* for 90 min. The clear supernatant was carefully removed with a fine-tipped Pasteur pipet, avoiding the upper lipid layer. The recovered cytosol was stored at −20 °C if not used immediately. All purification steps were performed at 0–4 °C.

Supernatants were held at ca. 2 °C for 2–3 days, allowing an inactive sediment to form with no loss in total activity, and then centrifuged at 105000*g* for 90 min. The pellet was discarded. The supernatant was then filtered through a 0.22-μm filter (Millex-GV, Millipore). The filtered protein was passed through a Waters Accell QMA column, 1.5 cm × 15 cm, equilibrated with 0.01 M potassium phosphate, pH 7.0. Elution of protein was achieved with a convex gradient of 0.6 M potassium phosphate, pH 7.0. The profile is shown in Figure 2a. The active fractions were pooled, diluted in 3 volumes of 0.01 M phosphate, pH 7.0, and passed through a hydroxylapatite column, 1.5 cm × 10 cm (HTP, Bio-Rad). Enzyme was eluted with a convex gradient of 0.01–0.5 M potassium phosphate, pH 7.0 (Figure 2b).

The active peak emerging from the hydroxylapatite column was diluted with 4 volumes of 0.01 M potassium phosphate, pH 7.0, and passed through a second QMA column, with a gradient of potassium phosphate, pH 7.0, in the range of 0.01–0.5 M buffer (Figure 2c). Fractions corresponding to active enzyme were concentrated to a volume of 1 mL (Centricon 30 microconcentrator, Amicon Corp.).

The concentrated enzyme solution was applied to a 1.5 × 75 cm column of Sephacryl S-300 (Pharmacia). Elution was performed with 0.05 M sodium phosphate, pH 7.0, containing 0.15 M sodium chloride (Figure 2d). Fractions were monitored by SDS–PAGE, and those containing a single band (fractions 22–33) were pooled. The inset to Figure 2d shows that the protein in the pooled fractions migrated as a homogeneous band in sodium dodecyl sulfate–polyacrylamide gel electrophoretograms (SDS–PAGE).

Isoelectric Focusing. Isoelectric focusing was performed on 5% blended Ampholine (LKB, Uppsala, Sweden) in the pH range of 4.0–6.5. The anode was 1 M sodium hydroxide, and the cathod was 1 M phosphoric acid. Surface pH values were monitored at 0.5-cm intervals with an Ingold flat-top electrode. Samples of 40 μL of enzyme were applied on 0.5 × 1 cm paper wicks near the cathode. Electrophoresis was performed at 200 V for 17 h. pH was measured at the end of the run. To detect protein, the gel was stained with Coomassie Blue R-250.

Amino Acid Analysis. The amino acid composition of one preparation of CSC isomerase was determined at the Protein Sequencing Facility at Rockefeller University. Two other

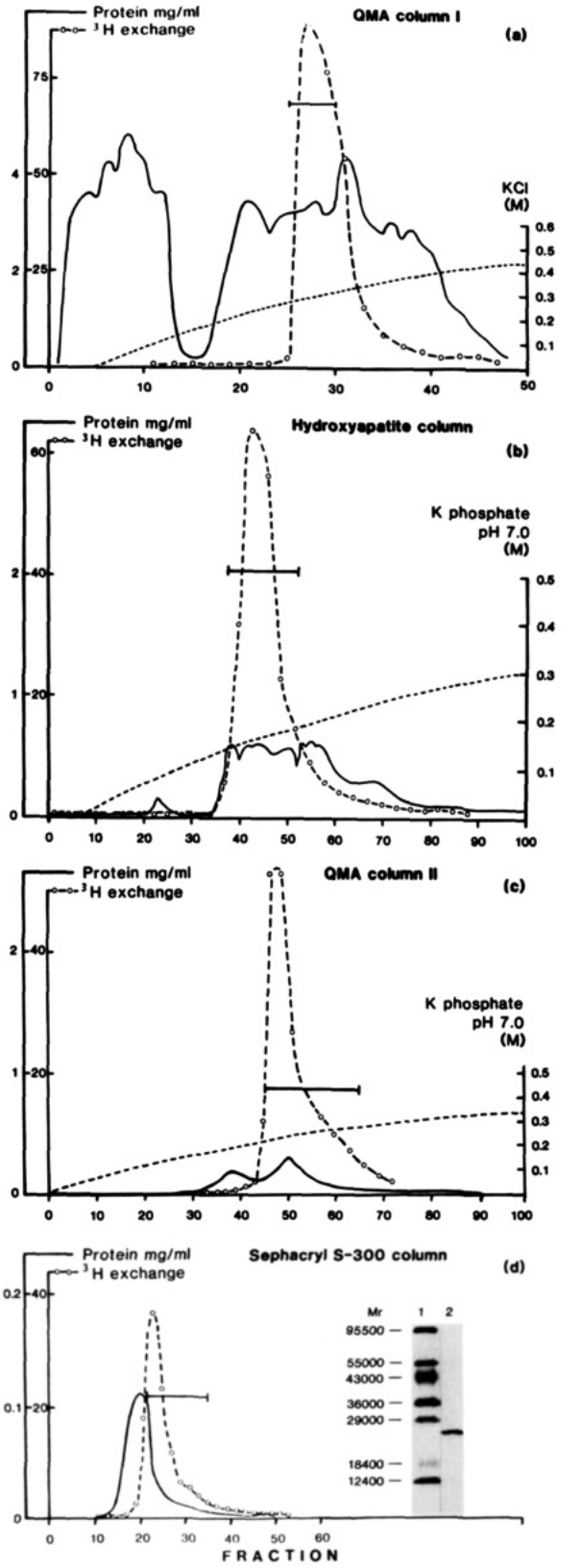

FIGURE 2: Fractionation patterns of corticosteroid side chain isomerase from elution columns: (—) protein (mg/mL); (O) enzyme activity expressed as tritium–protium exchange rate; (– –) salt gradient. The figures correspond to the purification sequence outlined in Table I and described in the text. (a) QMA column I; (b) hydroxylapatite column; (c) QMA column II; (d) Sephacryl S-300 column. Horizontal bars in (a)–(c) show the portions of the column effluents collected and used in the following step. Bar in (d) shows the part of the effluents collected that contained homogeneous enzyme. Lane 1 of the inset to (d) shows the molecular weight markers stained with Coomassie Blue; lane 2 shows the protein pattern of the final purification step visualized with silver stain.

Table I: Purification of Corticosteroid Side Chain Isomerase

step	vol (mL)	total protein (mg)	total act. (pmol/min)	specific act. [pmol min^{-1} (mg of pol)$^{-1}$]	yield (%)	purification (x-fold)
(1) supernatant	24	370	2465	6.7	100	1
(2) QMA Accell	15	49.0	1542	31.5	62.6	4.7
(3) hydroxylapatite	10	7.39	1058	143.2	42.9	21.5
(4) QMA Accell	12	4.40	1004	228.2	40.7	34.2
(5) Sephacryl S-300	17	0.61	492	803.7	20.0	120.6

isomerase preparations were analyzed in the laboratory of Prof. James M. Manning. Hydrolysis of 1 μg of sample was performed in 6 N hydrochloric acid containing phenol at 110 °C for 22 h. Quantitation of the amino acids was performed on phenylthiocarbamyl derivatives by reversed-phase high-performance liquid chromatography and detection in the ultraviolet range as described by Atherton (1989). The quantity of protein used in the hydrolysis (1 μg) yielded a composition that under the conditions described was ±10% of expected values on the basis of experience with other proteins (Bidlingmeyer et al., 1984; Atherton, 1989). Routine controls were included for instrument performance, random contaminants, and hydrolytic destruction.

Tryptophan was determined according to Simpson (1976). Hydrolysis was performed at 110 °C for 20 h in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole as catalyst. Sample was loaded into the chromatograph after the hydrolysate had been adjusted to pH 2 with 10 N sodium hydroxide. Precolumn derivatization was performed with ninhydrin. Cysteine was determined as cysteic acid after oxidation of sulfhydryl groups with performic acid according to the method of Moore (1963).

Results

The purification of corticosteroid side chain isomerase is summarized in Table I. Recoveries averaged 20% of the starting activity, with a 120-fold increase in specific activity. The elution profiles showing the patterns of emergence of enzyme from the columns used in purification steps 2–5 are shown in Figure 2. An SDS–PAGE profile of the purified enzyme obtained in the final step is shown in the inset of Figure 2d. The electrophoretogram, visualized with silver stain, showed a single homogeneous band.

Native gel electrophoresis of the homogeneous protein resulted in the migration of a band, visualized with Coomassie Blue, which coincided with the localization of active enzyme eluted from parallel gels electrophoresed concurrently under the same conditions. These profiles are shown in Figure 3. In the preparation shown, a faint Coomassie Blue positive contaminant was seen in the gel. This material had no enzyme activity.

Enzyme Stability. The purified enzyme lost less than 10% of its activity when held at 0–4 °C for 6 months in 0.01 M sodium phosphate, pH 7.0. Activity was not affected by sodium azide added as a preservative. Isomerase was stable at room temperature, as well. Figure 4 shows that inactivation was slow at temperatures up to 65 °C; above 65 °C, activity was lost rapidly. Enzyme was inactivated within 60 s at 100 °C.

Molecular Weight. The apparent molecular weight of native homogeneous isomerase was determined after passage through a Superose 6 HPLC gel filtration column. Values were determined by interpolation of retention times onto a regression line corresponding to the molecular weights of five standard proteins. The molecular mass of isomerase was 44 400 ± 1450 (SEM) Da. Gel filtration of the isomerase on a Sephacryl S-300 column under similar conditions gave a value of 44 000 Da.

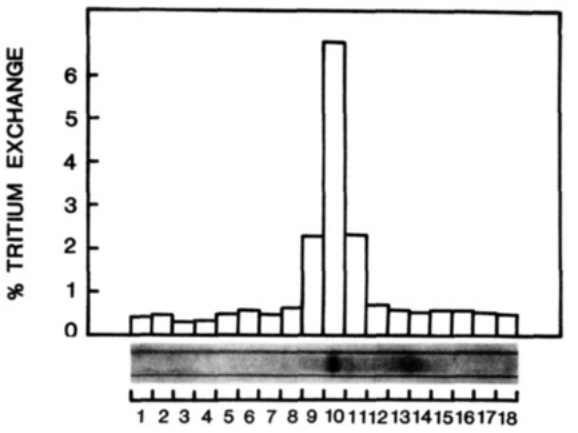

FIGURE 3: Comparison of electrophoretic mobility of isomerase activity and protein. Two cylindrical gel rods were loaded with 8 μg of isomerase (right side of figure). One, stained with Coomassie Blue, is reproduced in the lower part of the figure. The other was cut into 18 0.5-cm segments. Each was homogenized in 0.9 mL of 0.025 M Tris, pH 8.0, and incubated with 11.5 nmol of tritium-labeled DOC for 2 h. The incubation mixtures were lyophilized, and percent tritium loss was determined after measurement of the tritium retained in the condensate. The results are shown in the upper part of the figure.

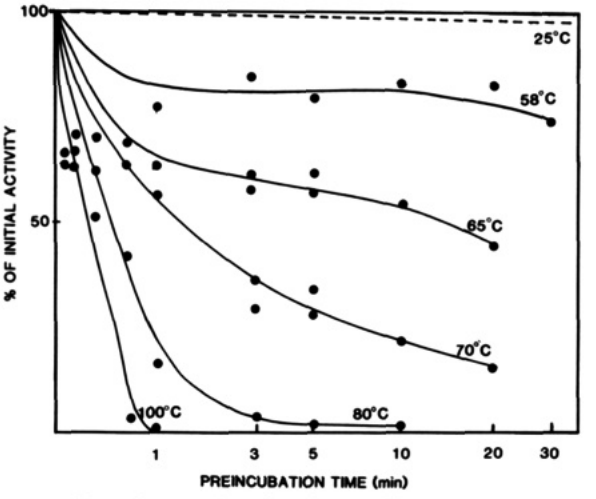

FIGURE 4: Heat denaturation of corticosteroid side chain isomerase. Duplicate aliquots containing 5 μg of purified enzyme in 550 μL of 10 mM potassium phosphate, pH 7.0, were preincubated at the indicated temperatures for the times shown. The solutions were then incubated with 445 μL of 25 mM Tris, pH 8.0, and 0.75 nmol of 21[3]-H-labeled 11-deoxycorticosterone for 2 h at 37 °C. The results are expressed as percent of initial activity.

The purified enzyme migrated on SDS–PAGE as a monomer, 23 500 ± 100 Da, on the basis of a fitting curve established by running simultaneously seven standards of known molecular mass. Calculations were performed with the fitting program of Plikaytis et al. (1986). The mass ratio of native

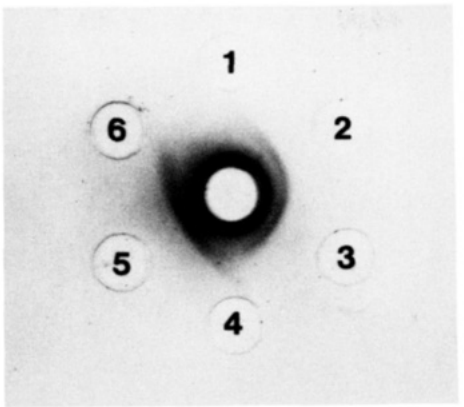

FIGURE 5: Double-radial immunodiffusion of corticosteroid side chain isomerase. Each well contained a final volume of 5 μL. Peripheral wells contained (1) 9 μg of isomerase; (2) 21 μg of isomerase; (3) 36 μg of isomerase; (4) 0.01 M potassium phosphate, pH 7.0; (5) mouse blood serum diluted 1:20 with phosphate, pH 7.0; (6) heart cytosol diluted 1:20 with phosphate, pH 7.0. The center well contained 5 μL of antibody to mouse liver isomerase. Diffusion proceeded for 4 days. Visualization of precipitin bands was enhanced with Cowle's reagent (Cowle & Cline, 1977).

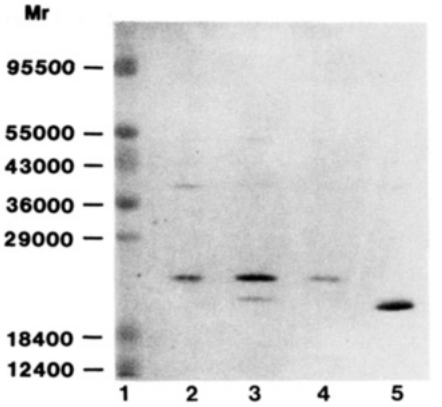

FIGURE 6: Western blots of isomerase after electrophoresis on SDS-PAGE. After blocking with 2% bovine serum albumin and incubating in 1% antibody, antigen antibody complexes on the nitrocellulose absorbant were visualized with protein A-horseradish peroxidase reagent. (Lane 1) molecular weight standards; (lane 2) liver cytosol; (lane 3) partially purified isomerase (to step 4); (lane 4) purified isomerase; (lane 5) purified isomerase maintained at −20 °C for 2 years.

to denatured isomerase was 1.9, close to the value required for a dimeric native form.

Isoelectric Point. The isoelectric point of isomerase was determined to be 4.8 ± 0.1 by flat-bed gel isoelectric focusing in the pH range of 4.0–6.5.

Carbohydrate Content. From the procedure of Hawkes (1982), no carbohydrate was detected in the enzyme.

Double Immunodiffusion and Western Blots. Figure 5 shows that in double immunodiffusion determinations the antiserum raised against purified side chain isomerase gave a single precipitin line to its own antigen (wells 1–3). Blood serum generated a single fusion line (well 5) and a spur indicating a cross-reacting component. Mouse blood serum had active isomerase (C. Monder and A. Marandici, unpublished experiments). No precipitin line was detected with heart cytosol (well 6).

Western blot analyses were performed of isomerase separated under denaturing conditions by SDS–PAGE (Burnette, 1981). Antibody was detected with horseradish peroxidase–antiimmunoglobulin. Figure 6 shows an immunoprecipitin band at 23 000 daltons and two very faint bands at 36 000 and 46 000 Da in liver cytosol (lane 2). Lane 3 shows a pattern for a partially purified preparation of liver isomerase (Table I, purification step 4) held for 1 year at −20 °C. The faint higher molecular mass bands observed in unfractionated liver cytosol were present, and an additional band at lower molecular mass (ca. 20 000 Da) was observed. Purified enzyme showed a single band at 23 000 Da (lane 4). Purified enzyme preparations that had been stored at −20 °C for more than 6 months retained full activity but showed a marked shift in electrophoretic mobility to an apparent lower molecular mass. The preparation displayed in lane 5 was 2 years old and fully active. Its mobility originally was identical with that of enzyme in lane 4.

Dependence of Rate on Protein Concentration. Velocity of tritium exchange was proportional to protein concentration up to 5 μg/mL homogeneous enzyme in incubations containing 1.8 nmol of [21-^{3}H]DOC.

Steroid Specificity. Six steroids with tritium incorporated at position 21 were incubated with homogeneous isomerase, and the rates of exchange of tritium with water were determined. Isomerase catalyzed transfer of tritium to water with 11-deoxy[21-^{3}H]corticosterone ([21-^{3}H]DOC), [21-^{3}H]-corticosterone, and 11-deoxy[21-^{3}H]cortisol as substrates. [21-^{3}H]Cortisol, [21-^{3}H]tetrahydrocortisone, and [21-^{3}H]-prednisolone were not substrates. The most active substrate was [21-^{3}H]DOC; [21-^{3}H]corticosterone and 11-deoxy[21-^{3}H]cortisol were detritiated at 20% of the rate of [21-^{3}H]DOC.

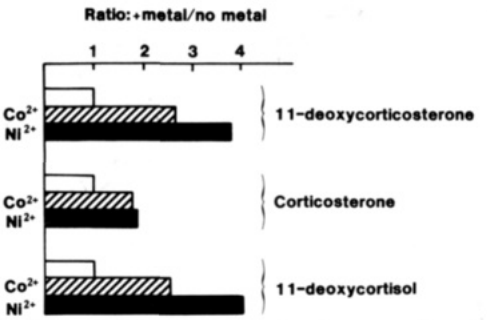

FIGURE 7: Effect of cobaltous and nickelous ions on activity of purified side chain isomerase. The assay system contained 5 μg of enzyme, 22 mM Tris, and 0.01 mM cationic solution (2.9 μg of cobaltous or nickelous cation). Enzyme activities were simultaneously determined in parallel systems either containing or free of added cation. The values indicate *x*-fold increase in activity relative to the incubation performed without added cation.

pH Dependence. The effects of pH on the activities of the isomerase with 11-deoxycorticosterone, corticosterone, and 11-deoxycortisol as substrates were generally consistent and similar to those reported earlier with partially purified enzyme from mouse, hamster, and human livers (Purkaystha et al., 1982; Martin et al., 1977; Martin & Monder, 1976). Activity in acetate/phosphate buffers increased from pH 4 to pH 7; a plateau of activity occurred between pH 7 and pH 9.5. Above pH 9.5, spontaneous enolization of the side chain, with attendant loss of tritium, became prominent, making measurement of enzyme activity uncertain at these higher pH values.

No enzyme activity was detected in citrate buffer up to pH 5.8. Activity at pH 8 was greater in Tris than in phosphate buffer. Glycine–sodium hydroxide was inhibitory.

Effects of Co^{2+} and Ni^{2+}. We have shown previously that cobaltous and nickelous ions stimulated isomerase activity of cytosol (Iohan & Monder, 1984; Purkaystha & Monder, 1981). In Figure 7, it is seen that these cations stimulated the purified enzyme, as well. The percent increased activity was of comparable magnitude to those of 11-deoxycortico-

Table II: Amino Acid Composition of Corticosteroid Side Chain Isomerase

	residues/100 residues[c]		residues/100 residues[c]
Asx[a]	13.17 ± 0.07	Tyr	2.95 ± 0.27
Glx[b]	9.61 ± 0.07	Val	3.32 ± 0.10
Ser	5.87 ± 0.27	Met	1.71 ± 0.06
Gly	10.23 ± 0.31	Cys	2.07 ± 0.12
His	0.80 ± 0.27	Ile	4.33 ± 0.06
Arg	2.49 ± 0.05	Leu	8.25 ± 0.06
Thr	6.48 ± 0.07	Phe	5.62 ± 0.09
Ala	5.86 ± 0.17	Lys	8.66 ± 0.28
Pro	4.26 ± 0.08		

[a] Asp and Asn are not distinguished. [b] Glu and Gln are not distinguished. [c] Means and standard errors for three determinations. Two preparations were hydrolyzed with hydrochloric acid, and one was hydrolyzed with methanesulfonic acid as described in the text. Since the amino acid concentrations determined by both methods were similar, all data were combined. Tryptophan is represented as a single determination. Cysteine is the average of two determinations.

sterone, corticosterone, or 17-deoxycortisol as substrate.

Amino Acid Composition. The amino acid composition of corticosteroid side chain isomerase is summarized in Table II. Tryptophan was present in low concentration under hydrolysis conditions devised to minimize its destruction. Under other hydrolysis conditions, it was destroyed and could not be detected. The values for most amino acids were the same after hydrochloric acid or methanesulfonic acid hydrolysis. Therefore, the values were pooled to provide a mean value. The polarity index (Capaldi & Vanderkooi, 1972) is 47.1, equivalent to the mean value for a soluble protein (47 ± 6). Basic amino acids comprised 12% of the total.

Attempts at protein sequencing were unsuccessful, since the N-terminal amino acid could not be derivatized. Consequently, automated Edman degradation (Hunkapiller & Hood, 1978) could not be initiated.

Time Dependence of Isomerase-Catalyzed Tritium–Protium Exchange from (21R)-[21-^{3}H]DOC and (21S)-[21-^{3}H]DOC. We have reported that (21*R*)-[21-^{3}H]DOC was converted to (21*S*)-[21-^{3}H]DOC by an epimerase mechanism that appeared to be an obligatory accompaniment to the isomerase (Monder et al., 1980b). Figure 8 compares the time dependence of 21-^{3}H–H_2O exchange for the 21*R* and 21*S* forms of [21-^{3}H]DOC. As we had observed in our earlier experiments with partially purified enzyme, detritiation of (21*R*)-[21-^{3}H]DOC proceeded after an initial lag, while tritium–water exchange as substrate with (21*S*)-[21-^{3}H]DOC was initiated with no detectable lag. After the initial lag period, both *S* and *R* forms of the steroid underwent tritium exchange at the same rate. The data, plotted in the form of the first-order equation $kt = \log [1/(1 - x)]$, where x is the fractional extent of reaction at time t, yielded a straight line for the 21*S* form, consistent with a first-order reaction rate. The rate of reaction of the 21*R* form, following the initial lag, was also first order and indistinguishable from that of the 21*S* form.

Kinetics. The effect of substrate concentration on the rate of tritium–water exchange followed classical Michaelis–Menten kinetics when (21*S*)-[21-^{3}H]DOC was the substrate.

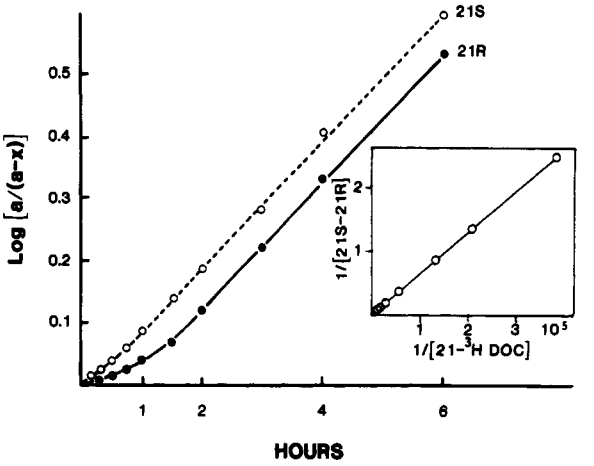

FIGURE 8: Comparison of the rate of tritium exchange with water by the 21*R* and 21*S* isomers of 21-^{3}H-labeled 11-deoxycorticosterone. Inset: Kinetics of the epimerase reaction. The difference in net conversion of 21*S* and 21*R* forms of [21-^{3}H]DOC after 1 h of incubation represents the lag due to epimerization. Variation of the increment, 21*S* minus 21*R*, with substrate concentration is displayed as a double-reciprocal plot.

Subsequent to the initial lag in tritium exchange with (21*R*)-[21-^{3}H]DOC as substrate, the rates for *R* and *S* epimers were the same, and their K_m and V_{max} values were identical. The displacement of the substrate dependence curves was due to the lag interval, which we have interpreted to be a consequence of epimerase activity. A plot of the substrate dependence of the lag interval was used to calculate epimerase kinetics (inset of Figure 8). A summary of velocity constants K_m and V_{max} is presented in Table III. For isomerase, values are presented for 11-deoxycorticosterone, corticosterone, and 17α-hydroxy-11-deoxycortiosterone. For epimerase, the values shown are for 11-deoxycorticosterone as substrate.

Discussion

Corticosteroid side chain isomerase, an obligatory participant in the metabolism of corticosterone in the mouse, catalyzes the rearrangement of the ketol side chain to the aldol form. The overall process is driven forward by aldehyde dehydrogenase, which oxidizes the 21-aldehyde to a carboxylic acid, yielding the 20-hydroxy-21-oic acid (Monder et al., 1982; Martin & Monder, 1978), or by aldehyde reductase, which reduces the aldehyde to an alcohol and generates the 20,21-diol side chain (Wermuth & Monder, 1983; Lippman & Monder, 1978). The expected oxidized and reduced products are formed from 3-oxo-11β,20-dihydroxypregn-4-en-21-al in vivo (Monder et al., 1980a). Because of the importance of isomerase in the metabolism of corticosterone in the mouse, we considered it important to purify it and to determine if the properties of the purified and crude enzymes correspond. The properties of the purified mouse liver enzyme did not differ from those of the crude cytosolic enzyme in any major way. Response to cobaltous or nickelous ions, pH optimum, substrate specificity, and temperature stability were similar. The estimated molecular weight of the crude enzyme was about

Table III: Kinetic Constants for Isomerase and Epimerase Activities

substrate	mode	v_{max} [nmol min^{-1} (mg of protein)$^{-1}$][b]	K_m (M × 10^4)[b]
11-deoxy[21-^{3}H]corticosterone	isomerase	2.82 ± 0.11	2.03 ± 0.17
[21-^{3}H]corticosterone	isomerase	2.85 ± 0.07	15.2 ± 0.52
17α-hydroxy-11-deoxy[21-^{3}H]corticosterone	isomerase	16.0 ± 0.50	3.52 ± 0.01
11-deoxy[21-^{3}H]corticosterone	epimerase[a]	1.09 ± 0.04	1.18 ± 0.41

[a] Epimerase activity was proportional to the time interval required for the rate of tritium–protium exchange of the 21*R*-^{3}H epimer to equal that of the 21*S*-^{3}H epimer, as illustrated in Figure 8. [b] Values are the means ± SD for three determinations.

twice that of the purified preparation, suggesting that it may form aggregates. Western blot analysis of liver cytosol revealed the presence of a faint antigenic component corresponding to the dimeric form of the enzyme, possibly the consequence of incomplete dissociation in SDS. Alternatively, the 46K band may represent an unrelated cross-reacting cytosolic component. Purification revealed other properties that were described by us in crude preparations, but that we could not be sure were intrinsic to the enzyme, and others that could be only studied adequately with the pure enzyme. For example, we concluded earlier that stimulation of isomerase by cobaltous ions was in part due to removal of an inhibitor from the incubation system (Iohan & Monder, 1984). That cobalt also stimulated the enzyme directly has now been shown with purified isomerase. The possibility that the acceleration of C-21 tritium exchange with water is due to an interaction of cobaltous ions with the steroid side chain, leading to a stable enediol (Eger et al., 1972a,b), was excluded by us in earlier experiments (Purkaystha & Monder, 1981).

The preferred substrate in vitro is 11-deoxycorticosterone. The significance of this preference is unclear, since DOC is not a major adrenal secretory product nor is it generated in quantitatively significant amounts by the peripheral 21-hydroxylation of progesterone. The effectiveness of corticosterone as substrate for the pure enzyme is consistent with our observation that the mouse converts 50% of its corticosterone to 20-hydroxy-21-oic acid metabolites, in vivo (Han et al., 1983).

We showed that the conversion of the ketol side chain to the aldol form proceeded through an enediol intermediate and that the exchange of tritium in [21-^{3}H]cortisol with water was a valid measure of the ketol–aldol interconversion (Monder et al., 1980b; Purkaystha et al., 1982). We also documented the stereospecificity of this exchange and showed that the 21*S*-^{3}H label was specifically exchanged. We presented evidence that the 21*R* form was exchanged after epimerization. In the current experiments, the exchange of 21-tritium with water by purified enzyme followed the pattern described by us previously. Neither double immunodiffusion nor Western blot analyses revealed more than one antigenic component corresponding to isomerase–epimerase. We have thus again failed to separate the isomerase and epimerase.

Although our results do not exclude the copurification of separate isomerase and epimerase activities, they remain consistent with a mechanism that we have described (Monder et al., 1980b) in which a single active site was responsible for both isomerization and epimerization. The immunological homogeneity of the enzyme is in accord with our proposed one-site mechanism. Corticosteroid side chain isomerase illustrates an uncommon example, in which both isomerase and epimerase activities appear to be the properties of a single enzyme (Noltman, 1972; Adams, 1976; Glaser, 1972). The mediation of the epimerase may explain how the 20(*S*)-hydroxy epimer of (20*S*)-11β,17,20-trihydroxy-3-oxopregn-4-en-21-al (aldol corresponding to cortisol) is converted to both 20α- and 20β-hydroxy epimers of the 20,21-diol end products in vivo (Monder et al., 1975). Inversion of configuration at C-20 is an important step in the metabolism of the corticosteroid side chain. It would be of interest to determine the extent to which 20(*R*)- and 20(*S*)-hydroxy end products are due to the action of epimerases as opposed to the mediation of specific 20-reductase. The physiological processes that direct isomerase and epimerase activities of the enzyme and that control the stereochemistry of corticosteroid metabolites at C-20 remain to be established.

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Registry No. Corticosteroid side-chain isomerase, 75139-73-0; 11-deoxycorticosterone, 64-85-7; corticosterone, 50-22-6; 17α-hydroxy-11-deoxycorticosterone, 152-58-9.

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Isolation and Characterization of a Complementary DNA Clone Coding for the $E_1\beta$ Subunit of the Bovine Branched-Chain α-Ketoacid Dehydrogenase Complex: Complete Amino Acid Sequence of the Precursor Protein and Its Proteolytic Processing†

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ABSTRACT: A 1.7-kb cDNA clone encoding the entire precursor of the $E_1\beta$ subunit of the branched-chain α-ketoacid dehydrogenase (BCKDH) complex was isolated from a bovine liver cDNA library by screening with a mixture of synthetic oligonucleotide probes corresponding to the C-terminal five-residue sequence of the mature $E_1\beta$ subunit. A partial amino acid sequence was determined by Edman degradation of the intact subunit and the peptides generated by cleavage at the lysyl bonds. Nucleotide sequence analysis revealed that the isolated cDNA clone contained the 5′-untranslated sequence of 186 nucleotides, the translated sequence of 1176 nucleotides, and the 3′-untranslated sequence of 306 nucleotides with a poly(A) tail. A type AATAAA polyadenylation signal was located 17 nucleotides upstream of the start of a poly(A) tail. Comparison of the amino acid sequence predicted from the nucleotide sequence of the cDNA insert of the clone with the partial amino acid sequence of the mature BCKDH $E_1\beta$ subunit showed that the cDNA insert encodes for a 342 amino acid subunit with M_r 37 745 and that the subunit is synthesized as the precursor with a leader sequence of 50 amino acids and processed at the N-terminus. Northern blot analysis using the cDNA insert as a probe showed the presence of a 1.8-1.9-kb mRNA in bovine liver, suggesting that the insert covers nearly a full length of mRNA. Alignment of the deduced amino acid sequence of bovine BCKDH $E_1\beta$ with that of the human pyruvate dehydrogenase (PDH) complex $E_1\beta$ subunit revealed a high degree of sequence homology throughout the two enzymes. The structure and function of mammalian α-ketoacid dehydrogenase complexes are apparently highly conserved.

Mammalian branched-chain α-ketoacid dehydrogenase (BCKDH)[1] (EC 1.2.4.4) is a mitochondrial multienzyme complex that catalyzes the oxidative decarboxylation of branched-chain α-keto acids derived by transamination of branched-chain amino acids such as valine, leucine, and isoleucine:

$$\text{RCOCOOH} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{TPP, Mg}^{2+}} \text{RCOCoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The BCKDH complex consists of three catalytic components: branched-chain α-ketoacid decarboxylase (E_1), dihydrolipoyl transacylase (E_2), and dihydrolipoamide dehydrogensae (E_3) (Pettit et al., 1978; Danner et. al., 1979; Heffelfinger et al., 1983). The enzyme complex also contains two specific regulatory enzymes, a kinase (Lau et al., 1982; Odessey, 1982; Paxton & Harris, 1982) and a phosphatase (Fatania et al., 1983; Damuni et al., 1984), that regulate the catalytic activity. E_1 is composed of two subunits, α and β, with M_r of 46 500 and 37 500, respectively (Heffelfinger et

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[1] Abbreviations: BCKDH, branched-chain α-ketoacid dehydrogenase; PDH, pyruvate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; bp, base pair(s); kb, kilobase(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MSUD, maple syrup urine disease; SSC, 0.15 M NaCl containing 0.015 M trisodium citrate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin derivative; *S*-CAM, *S*-carbamoylmethyl; TFA, trifluoroacetic acid; TPP, thiamin pyrophosphate.

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