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Evidence for Distinct Dehydrogenase and Isomerase Sites within a Single 3β-Hydroxysteroid Dehydrogenase/5-Ene–4-Ene Isomerase Protein

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ABSTRACT: Complementary DNA encoding human 3β-hydroxysteroid dehydrogenase/5-ene–4-ene isomerase (3β-HSD) has been expressed in transfected GH_4C_1 with use of the cytomegalovirus promoter. The activity of the expressed protein clearly shows that both dehydrogenase and isomerase enzymatic activities are present within a single protein. However, such findings do not indicate whether the two activities reside within one or two closely related catalytic sites. With use of [^{3}H]-5-androstenedione, the intermediate compound in dehydroepiandrosterone (DHEA) transformation into 4-androstenedione by 3β-HSD, the present study shows that 4MA (*N*,*N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide) and its analogues inhibit DHEA oxidation competitively while they exert a noncompetitive inhibition of the isomerization of 5-androstenedione to 4-androstenedione with an approximately 1000-fold higher K_i value. The present results thus strongly suggest that dehydrogenase and isomerase activities are present at separate sites on the 3β-HSD protein. In addition, using 5α-dihydrotestosterone (DHT) and 5α-androstane-3β,17β-diol as substrates for dehydrogenase activity only, we have found that dehydrogenase activity is reversibly and competitively inhibited by 4MA. Such data suggest that the irreversible step in the transformation of DHEA to 4-androstenedione is due to a separate site possessing isomerase activity that converts the 5-ene-3-keto to a much more stable 4-ene-3-keto configuration.

The activity of 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/steroid 5-ene–4-ene isomerase (EC 5.3.3.1), hereafter called 3β-HSD, catalyzes the transformation of 5-ene-3β-hydroxysteroids to the corresponding 4-ene-3-keto configuration (Samuels et al., 1951) and is therefore an essential step in the biosynthesis of all classes of hormonal steroids, namely, progesterone, glucocorticoids, mineralocorticoids, androgens, and estrogens. While different proteins responsible for these two reactions have been isolated separately from bacterial sources (Talalay & Wang, 1955), the two activities appear to reside within a single protein in mammalian tissues as observed for the enzyme purified from bovine ovaries (Cheatum & Warren, 1955) as well as from human placenta (Luu-The et al., 1988, 1989, 1990; Lachance et al., 1990), ovine adrenals (Ford & Engel, 1974), rat adrenals (Ishii-Ohba et al., 1987) and testes (Ishii-Ohba et al., 1986), and bovine adrenals (Eastman & Neville, 1987; Inano et al., 1990).

In addition to being required for the synthesis of all classes of steroids, 3β-HSD activity could well play a role in the preferential transformation of steroid precursors into mineralocorticoids, glucocorticoids, progesterone, or sex steroids. In fact, there is still a debate about the presence of one or more 3β-HSD(s), and clinical data suggest the existence of more than one 3β-HSD in the human (del Carmen Cravioto et al., 1986). The existence of three different substrate-specific 5-ene–4-ene isomerases has been suggested in bovine adrenals (Edwald et al., 1964).

In order to obtain more information about the mechanism of action of 3β-HSD, we have expressed human placental 3β-HSD cDNA (Luu-The et al., 1989) in mammalian cells and we have synthesized [^{3}H]-5-androstenedione, the intermediate product in DHEA transformation into 4-androstenedione. In addition, with the use of 3β-HSD purified from human placenta (Luu-The et al., 1990), the present data show that 4MA inhibits competitively dehydrogenase activity while it is noncompetitive for the isomerase site. On the other hand, trilostane, another potent inhibitor of 3β-HSD, inhibits competitively both dehydrogenase and isomerase activities. The present results thus suggest that the two enzymatic sites are distinct within a single 3β-HSD protein.

Materials and Methods

Materials. NAD^+ and $NADP^+$ were purchased from Sigma Chemical Co. [4,7-^{3}H]Pregnenolone, [1,2-^{3}H]-dehydroepiandrosterone, 5α-[1,2,6,7-^{3}H]dihydrotestosterone, 5α[1,2-^{3}H]androstane-3β,17β-diol, [^{14}C]dehydroepiandrosterone, and [^{14}C]pregnenolone were obtained from New England Nuclear. TLC silica gel plates were from Merck,

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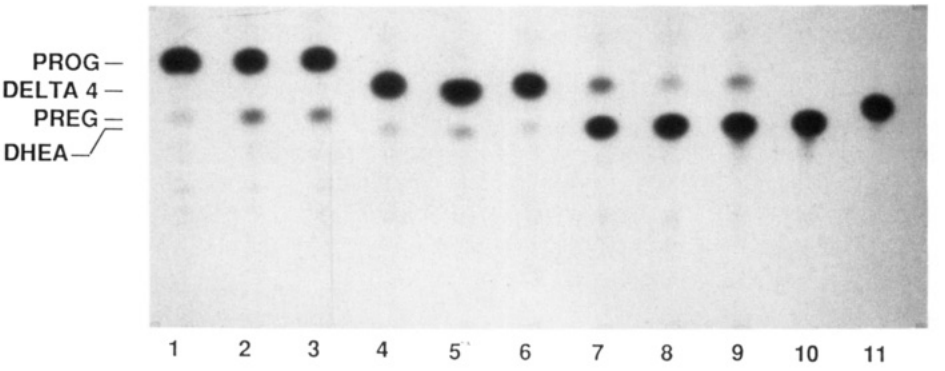

FIGURE 1: Autoradiograph showing dual activity of transfected human 3β-HSD. Transfected GH_4C_1 cells were incubated as described under Materials and Methods in the presence of 1 μM [^{14}C]pregnenolone (lanes 1, 2, and 3), 1 μM [^{14}C]DHEA (lanes 4, 5, and 6), or 1 μM [^{14}C]DHEA in the presence of 1 μM 4MA (lanes 7, 8, and 9). Lanes 10 and 11 represent control cells incubated with 1 μM [^{14}C]DHEA and [^{14}C]pregnenolone, respectively. Cells were transfected with 30 μg (lanes 1, 4, and 7), 15 μg (lanes 2, 5, and 8), and 5 μg (lanes 3, 6, and 9), respectively.

Darmstadt, FRG. All nonradioactive steroids were purchased from Steraloids Ltd. Trilostane (2α-cyano-4α,5α-epoxy-17β-hydroxyandrostan-3-one) was kindly provided by Sterling-Winthrop Research Institute, Renslaer, New York. 4MA, 4MAPC (4-aza-4-methyl-3-oxo-5α-pregnane-20(*S*)-carboxylate), 4A1E (*N*,*N*-diethyl-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide), and DIPA (*N*,*N*-bis(1-methylene-ethyl)-3-oxo-4-aza-5α-androstane-17β-carboxamide) were generous gifts from Merck Sharp & Dohme Research Laboratories, Rahway, NJ. All restriction enzymes as well as T4 DNA ligase were obtained from Pharmacia.

Preparation of Expression Vector. An *Eco*RI–*Eco*RI fragment (1.5 kbp) of human placenta 3β-HSD cDNA (Luu-The et al., 1989) containing the entire 3β-HSD coding region as well as the 3′-untranslated region was inserted downstream to the cytomegalovirus (CMV) promoter in the pCMV vector and amplified by use of *E. coli* DH5α competent cells. Recombinant plasmids (pCMV–3β–HSD) were prepared by the alkaline lysis procedure (Maniatis et al., 1982) and purified by two cesium chloride–ethidium bromide density gradient centrifugations.

Transfection in Mammalian Cells. GH_4C_1 cells, a rate pituitary tumor cell line that secretes prolactin and growth hormone (Tashjian, 1979), were grown in Dulbecco's-modified Eagle's Medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 international units of penicillin/mL, 100 μg/mL streptomycin, and 10% (v/v) fetal bovine serum (Hyclone, Logan, UT) under a humidified atmosphere of air–CO_2 (95%/5%) at 37 °C. Cells were plated at 10^4 cells/cm^2 in triplicate in 60-mm dishes and transfected with the indicated amounts of the pCMV-3β-HSD expression vector using the calcium–phosphate procedure (Kingston et al., 1987). Control cells were transfected with 5 μg of pCMV without the 3β-HSD cDNA insert. After 24 h, media were removed and cells were washed twice with PBS and fresh medium was added. After 24 h of incubation, the cells were harvested.

Enzymatic Assay. Transfected cells (10^6 cells) or purified enzyme (1 μg) was incubated at 37 °C in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 1 mM NAD^+ for 1 h in the presence of the indicated substrate or inhibitor. Steroids were dissolved in ethanol and added to the medium at the constant final concentration of 1%. Immediately after incubation, steroids were extracted twice from the incubation mixture with 2 mL of methylene dichloride. The extract was then dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen. The steroid products were separated by TLC on silica gel in the benzene–acetone (4/1 v/v) developing system and were identified by comparison with authentic steroids. Radioactivity was measured with a liquid scintillation spectrometer (Beckman LS 3801).

Synthesis of [1,2-^{3}H]Androst-5-ene-3,17-dione. The reaction was carried out in a closed system kept under slightly positive Ar atmosphere. Five milligrams of DHEA in 500 μL of ice-cold stabilized acetone was mixed with 1 mCi of [1,2-^{3}H]DHEA (50 Ci/mmol), and 5 mL of Jones reagent was added. After 15 min, the reaction was stopped by adding 3 mL of ice-cold water. After remaining in the cold room (4 °C) overnight, the precipitate was filtered, washed, dried in vacuo, and purified through LH-20 columns. The purified compound showed no absorption in 240 nm, thus indicating the absence of contamination from 4-androstenedione. Authenticity of [1,2-^{3}H]androst-5-ene-3,17-dione was determined by HPLC, melting point, and recrystallization to constant specific activity. The final yield was 3.5 mg (70%) with a specific activity of 50 μCi/mmol.

RESULTS

As shown in Figure 1 (lanes 10 and 11), control GH_4C_1 cells do not contain detectable 3β-HSD activity, thus offering an appropriate model for studying the activity of various types of transfected 3β-HSD. After transfection with the pCMV vector containing the 3β-HSD cDNA fragment that encodes the human 3β-HSD protein of 42 126 Da (Luu-The et al., 1989), the resulting cells efficiently transfer DHEA and pregnenolone to 4-androstenedione and progesterone, respectively (Figure 1). Such results confirm that the unique protein of 42 kDa contains both 3β-hydroxysteroid dehydrogenase and 5-ene–4-ene isomerase activities and that it can use C21 as well as C19 steroids as substrates. Addition of 4MA, a compound recently shown as an inhibitor of purified human 3β-HSD (Takahashi et al., 1990a,b) is also a potent inhibitor of the activity of the expressed 3β-HSD (Figure 1).

With use of 5-androstene-3,17-dione, a substrate specific for 5-ene–4-ene isomerase, the data in Figure 2 show that 4MA specifically inhibits dehydrogenase activity while it has a minimal effect on isomerase activity. It can be seen in Figure 3 that the three 4MA analogues, 4MAPC, 4A1E, and DIPA, which contain the aza group, show a pattern of inhibitory effect similar to that of 4MA limited to dehydrogenase activity. From the use of DHEA as a substrate, analysis by Dixon plot shows that 4MA, 4MAPC, 4A1E, and DIPA are competitive inhibitors with K_i values of 56 nM, 3.6 μM, 4.4 μM, and 120 μM, respectively (Table I). On the other hand, when 5-androstenedione is used as substrate, the same compounds exert a noncompetitive inhibitory effect at much higher concentrations (Table I) with K_i values of 55 μM, 240 μM, 550 μM, and 930 μM, respectively.

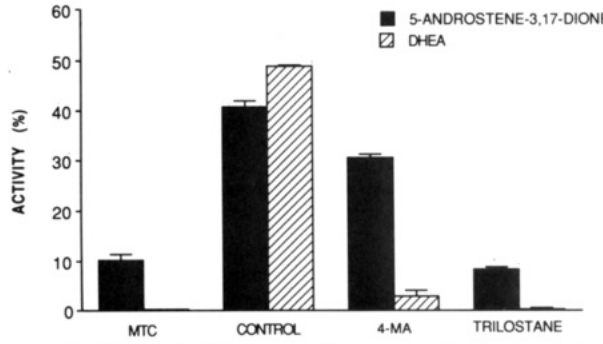

FIGURE 2: Effect of 4MA and trilostane on the dual activity of transfected human 3β-HSD. Transfected cells were incubated as described under Materials and Methods in the presence of 1 μM [^{3}H]-5-androstene-3,17-dione (solid bar) or [^{3}H]DHEA (shaded bar), respectively. MTC stands for mock transfected cells. 4MA and trilostane dissolved in ethanol were added at the concentration of 10 μM, with the final concentration of ethanol being 1%.

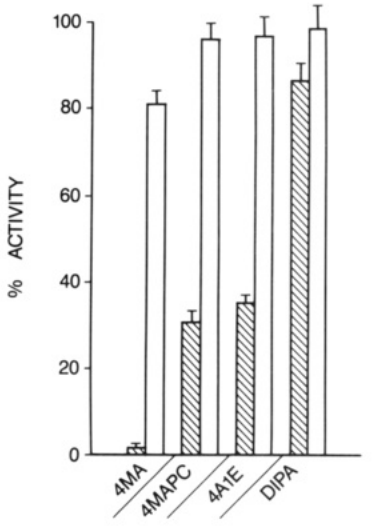

FIGURE 3: Effect of 4MA and its analogues on the activity of purified human placental microsomal 3β-HSD. Incubation was performed as described under Materials and Methods in the presence of 20 μM DHEA (shaded bar) or 20 μM 5-androstene-3,17-dione (open bar) and the indicated inhibitors at the concentration of 10 μM. The 100% activity corresponds to incubation in the presence of substrate alone. The velocity in this case is 2.4 nmol/(min·mg of protein). Each value was obtained from duplicate experiments.

Table I: Inhibition Constants for Trilostane and 4MA and Its Analogues[a]

inhibitor	K_i (μM) substrate	
	DHEA	5-androstene-3,17-dione
trilostane	0.036[b]	0.16[b]
4MA	0.056[b]	55[c]
4MAPC	3.6[b]	240[c]
4AIE	4.4[b]	550[c]
DIPA	120[b]	930[c]

[a] Inhibition experiments were performed, in duplicate, at two fixed substrate concentrations of 20 and 100 μM for [^{3}H]DHEA and 5 and 10 μM for [^{3}H]-5-androstene-3,17-dione, respectively. K_i values were calculated by Dixon plot analysis. V_{max} values were calculated at 2.4 and 25 nmol/(mg·mg of protein) for DHEA and 5-androstene-3,17-dione, respectively. [b] Competitive inhibition. [c] Noncompetitive inhibition.

In order to examine with precision whether trilostane, a well-known competitive inhibitor of 3β-HSD, has the same effect as 4MA on both DHEA and 5-androstene-3,17-dione transformation, its effect on DHEA and 5-androstene-3,17-dione transformation has been studied by Dixon plot analysis.

Table II: Inhibition and Affinity Constants of 4MA Inhibition for Saturated Substrates and Cofactor of 3β-HSD[a]

constants	substrates DHT	5α-androstane-3β,17β-diol
K_m[b] (μM)	5.1	ND
K_i[c] (nM)	16	3.7
V_{max}[b]	17	ND

[a] V_{max} values are expressed as nanomoles per minute per milligram of protein. [b] K_m and V_{max} values were determined by Lineweaver–Burk plot analysis. [c] K_i values for DHT and 5α-androstane-3β,17β-diol were determined by Dixon plot analysis, while K_i values for NADH were determined with the use of Lineweaver–Burk plot analysis. ND, not determined.

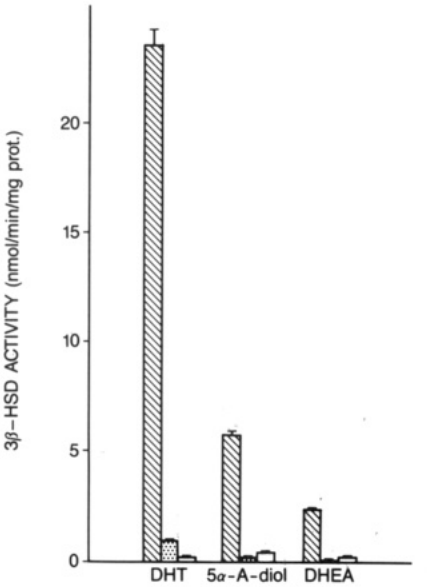

FIGURE 4: Effect of trilostane and 4MA on the transformation of DHT, 5α-androstane-3β,17β-diol, and DHEA by purified human placental microsomal 3β-HSD. The incubation was performed as described under Materials and Methods in the presence of 20 μM of substrate alone (shaded bar), substrate and 10 μM trilostane (dotted bar), or substrate and 10 μM 4MA (open bar). Each value was obtained from duplicate experiments.

Contrary to 4MA, trilostane inhibits both reactions competitively at K_i values of 36 nM and 160 nM, respectively (Table I).

In order to obtain further evidence for the distinct dehydrogenase and isomerase sites, we have studied the kinetics of 3β-HSD activity with and androstane substrates dihydrotestosterone and 5α-androstane-3β,17β-diol, which lack the ethylene group and are thus substrates exclusively for dehydrogenase activity. As illustrated in Table II, purified 3β-HSD efficiently interconverts dihydrotestosterone and 5α-androstane-3β,17β-diol with a maximal turnover higher than that obtained for the transformation of DHEA itself. It can be seen that this interconversion is strongly inhibited by trilostane and 4MA (Figure 4). Data obtained from Lineweaver–Burk plot analysis (Table II) indicate that 4MA inhibits competitively DHT as well as 5α-androstane-3β,17β-diol transformation with K_i values of 16 nM and 37 nM, respectively. In fact, Dixon plot analysis shows that 4MA inhibits both DHT and 5α-androstane-3β,17β-diol transformation in a competitive manner with K_i values of 16 nM and 3.7 nM, respectively.

Although the rate of transformation using DHT as a substrate is higher than that of DHEA, DHEA has higher affinity for the active site as demonstrated by mutual competitive inhibition (Table III), with the K_i values for DHEA and DHT

Table III: Mutual Competition of DHEA and DHT on 3β-HSD[a]

	K_i (μM)	
	substrate	
	DHEA	DHT
DHEA		3.3
DHT	30.6	

[a] Competition experiments were performed, in duplicate, at two fixed substrate concentrations of 5 μM and 20 μM [^{3}H]DHEA or [^{3}H]-5-androstene-3,17-dione. K_i values were calculated by Dixon plot analysis.

being 3.3 μM and 30.6 μM, respectively. The higher turnover but lower affinity of DHT for the site responsible for the transformation of DHEA agrees with the finding that this compound binds only to the dehydrogenase site, whereas DHEA binds to both the dehydrogenase and isomerase sites.

Discussion

Although data obtained with 3β-HSD purified from mammalian sources (Cheatum & Warren, 1955; Ford & Engel, 1974; Ishii-Ohba et al., 1986, 1987; Eastman & Neville, 1987; Inano et al., 1990; Luu-The et al., 1988, 1989, 1990; del Carmen Cravioto et al., 1986) strongly suggest that 3β-hydroxysteroid dehydrogenase and 5-ene–4-ene isomerase activities are included in the same enzyme, the finding of two activities catalyzed by separate proteins from bacterial sources has brought some uncertainty about the presence of one or two proteins responsible for 3β-HSD activity.

Data obtained with the human placental 3β-HSD cDNA expressed in mammalian cells (Figure 1) clearly demonstrate that 3β-hydroxysteroid dehydrogenase and 5-ene–4-ene isomerase activities reside within a single 42-kDa protein, thus supporting the observations of Lachance et al. (1990) and Zhao et al. (1990, 1991). A major tool in defining the presence of one or two sites is the availability of 5-androstene-3,17-dione, the product of dehydrogenase and a substrate for isomerase activity. This compound is rather difficult to obtain since it can be easily transformed to the much more stable 4-androstene-3,17-dione configuration. Taking special care in carrying out the oxidation of [^{3}H]DHEA by the Jone's reagent under a strictly inert atmosphere, we were successful in obtaining a high yield of [^{3}H]-5-androstene-3,17-dione.

The availability of tritiated 5-androstene-3,17-dione gave us the opportunity of differentiate the inhibitory effect of 4MA and its analogues on the dehydrogenase and isomerase activities. In fact, while these compounds are potent and competitive inhibitors of the transformation of DHEA (Table I), which is a substrate for dehydrogenase, they exert a noncompetitive inhibition on the transformation of 5-androstene-3,17-dione with approximately 1000-fold higher K_i values. On the contrary, trilostane inhibits competitively both the dehydrogenase and isomerase activities. Since both 4MA and its analogues and trilostane inhibit competitively the transformation of DHEA by 3β-HSD, it seems reasonable to expect that the same compounds would also inhibit 5-androstene-3,17-dione transformation competitively. However, our results indicate that while trilostane inhibits 5-androstene-3,17-dione competitively, 4MA is noncompetitive, thus suggesting that 4MA and its analogues bind to the dehydrogenase site, which is distinct from the isomerase site. Moreover, study of the mutual competition between DHT and DHEA (Table II) indicates that DHEA is a better competitor than DHT for the active site. In fact, K_i values of 3.3 μM and 30.6 μM were found when DHEA and DHT were used as competitors, respectively. Such a difference in K_i values might be explained by the fact that DHEA binds to both dehydrogenase and isomerase sites whereas DHT binds to the dehydrogenase site only. The present data also show that the rates of transformation of 5α-dihydrotestosterone and 5α-androstane-3β,17β-diol, which do not contain the ethylene group and are only substrates for the dehydrogenase activity, are higher than the transformation of DHEA and pregnenolone, which bind to both the dehydrogenase and isomerase sites.

Since DHT and 5α-androstane-3β,17β-diol are interconverted by 3β-hydroxysteroid dehydrogenase, the present data also indicate that the dehydrogenase step is reversible while the isomerase activity is irreversible. Comparison of the structures of DHEA, 5α-androstane-3β,17β-diol, and DHT suggests that binding to the isomerase site requires the A and B rings to be more planar and rigid, due to the existence of the 5-ene bond. Trilostane, which contains an epoxy group at 4α,5α, is likely to possess the rigidity and planar conformation required to bind to the isomerase site whereas 4MA, which contains the androstane structure, is less likely to bind to the isomerase site.

Acknowledgments

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Registry No. 4MA, 73671-86-0; 4MAPC, 92472-61-2; 4AIE, 92472-70-3; DIPA, 135226-28-7; DHEA, 53-43-0; [1,2-^{3}H]DHEA, 110906-04-2; DHT, 521-18-6; 5α-androstane-3β,17β-diol, 571-20-0; trilosane, 13647-35-3; [1,2-^{3}H]androst-5-ene-3,17-dione, 135226-27-6.

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Structural Organization of Aldehyde Dehydrogenases Probed by Limited Proteolysis†

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ABSTRACT: Incubation of cytosolic and mitochondrial aldehyde dehydrogenases with trypsin or Glu-C protease under native conditions causes a time-dependent loss of dehydrogenase activity and the production of protein fragments. For evaluation of the results, termination of the reactions with a specific protease inhibitor is especially important in the case of the Glu-C protease. Cleavage site determination by SDS/polyacrylamide gel electrophoresis and sequence analysis identified protease-sensitive amino acid residues at two internal regions spanning positions 248–268 (region 1) and 397–399 (region 2) and at positions in the N-terminal segment (region 3). Region 1 encompasses several cleavages and is sensitive to both proteases in both aldehyde dehydrogenases. Further, it is in a conserved segment and correlates with reactive residues and regions ascribed functional roles. It also correlates with exon borders in the corresponding genes. Combined, the results define region 1 as an important and highly accessible segment of the protein. Region 2 is also adjacent to a conserved segment but lacks further correlation with special properties and appears just to represent an accessible region. The internally cleaved subunits retain a tetrameric configuration as calculated from exclusion chromatography and polyacrylamide gel electrophoresis under native conditions, suggesting that the quaternary structure is not dependent on covalently linked domains within the subunits. Furthermore, the fragments can bind to AMP–Sepharose, suggesting that some functional properties are retained within the cleaved tetramers. However, cleavage at position 35 appears to cause a large fragment (36–263) to be released from the tetramer, suggesting a role of an N-terminal segment or arm (at or before region 3) in subunit interactions.

Aldehyde dehydrogenase is an oxidoreductase with a wide variety of aldehyde substrates (Blackwell et al., 1989). The NAD^+-dependent cytosolic and mitochondrial mammalian enzymes are homotetramers with known primary structures, analyzed at the protein level for the two forms from both human (Hempel et al., 1984, 1985) and horse (von Bahr-Lindström et al., 1984; Johansson et al., 1988) liver. Corresponding cDNA and genomic structures have been analyzed for these and other mammalian forms of the two types of enzyme (Hsu et al., 1985, 1988, 1989; Braun et al., 1987; Dunn et al., 1989; Farrês et al., 1989), as well as for prokaryotic aldehyde dehydrogenases from three species (Pickett et al., 1987; O'Connell & Kelly, 1989; Kok et al., 1989). Furthermore, another type of aldehyde dehydrogenase, dimeric and active also with $NADP^+$, has been structurally characterized from rat hepatocarcinoma and from liver after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Jones et al., 1988; Hempel et al., 1989). It appears identical with or highly similar to forms from normal bladder, stomach, and other organs (Lindahl, 1986; Algar & Holmes, 1989; Eckey et al., 1990; Yin et al., 1991). While the two tetrameric mammalian enzyme types exhibit 68% residue identity, the dimeric type is clearly different, representing another class with a residue identity at only about the 30% level. Consequently, at least three mammalian forms of aldehyde dehydrogenase exist with largely known interrelationships.

Structural comparisons of the tetrameric enzymes gave an initial estimate of the organization of the molecules, localizing likely segments for coenzyme binding, and active-site residues (Hempel et al., 1985), as later also concluded from knowledge of the dimeric enzyme (Hempel et al., 1989). In spite of this structural knowledge, there is little information available on the tertiary structure and only limited insight into the functional organization of the enzyme molecule. Important roles have been ascribed to Lys-487 from natural variants of the mitochondrial enzyme (Yoshida et al., 1984), and to positions

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