Enzymatic Characteristics and Subcellular Distribution of a Short-Chain Dehydrogenase/Reductase Family Protein, P26h, in Hamster Testis and Epididymis

Syuhei Ishikura,[‡] Noriyuki Usami,[‡] Kouei Kitahara,[‡] Tomoya Isaji,[‡] Koji Oda,[§] Junichi Nakagawa,[§] and Akira Hara*,[‡]

Biochemistry Laboratory, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-8585, Japan, and Taisho Pharmaceutical Company Ltd., Yoshino-machi, Omiya, Saitama 330-8530, Japan

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ABSTRACT: A hamster sperm 26 kDa protein (P26h) is strikingly homologous with mouse lung carbonyl reductase (MLCR) and is highly expressed in the testis, but its physiological functions in the testis are unknown. We show that recombinant P26h resembles NADP(H)-dependent MLCR in the tetrameric structure, broad substrate specificity, inhibitor sensitivity, and activation by arachidonic acid, but differs in a preference for NAD(H) and high efficiency for the oxidoreduction between 5α-androstane-3α,17β-diol ($k_{cat}/K_M = 243\ s^{-1}\ mM^{-1}$) and 5α-dihydrotestosterone ($k_{cat}/K_M = 377\ s^{-1}\ mM^{-1}$). The replacement of Ser38-Leu39-Ile40 in P26h with the corresponding sequence (Thr38-Arg39-Thr40) of MLCR led to a switch in favor of NADP(H) specificity, suggesting the key role of the residues in the coenzyme specificity. While the P26h mRNA was detected only in the testis of the mature hamster tissues, its enzyme activity was found mainly in the mitochondrial fraction of the testis and in the nuclear fraction of the epididymis on subcellular fractionation, in which a mitochondrial enzyme, isocitrate dehydrogenase, exhibited a similar distribution pattern. The enzyme activity of P26h in the two tissue subcellular fractions was effectively solubilized by mixing with 1% Triton X-100 and 0.2 M KCl, and enhanced more than 10-fold. The enzymes purified from the two tissue fractions exhibited almost the same structural and catalytic properties as those of the recombinant P26h. These results suggest that P26h mainly exists as a tetrameric dehydrogenase in mitochondria of testicular cells and plays a role in controlling the intracellular concentration of a potent androgen, 5α-dihydrotestosterone, during spermatogenesis, in which it may be incorporated in mitochondrial sheaths of spermatozoa.

A 26 kDa sperm protein (P26h)[1] was first identified as a membrane protein in hamster epididymis (*1*). The protein has been reported to be abundant in the luminal fluid of the proximal region of the epididymis and to accumulate on spermatozoa during epididymal maturation (*2*, *3*). Since antibodies against P26h inhibit sperm−zona pellucida binding (*4*), the protein has been thought to play a role in sperm−egg interactions. Recently, Gaudreault et al. (*5*) have cloned the cDNA for P26h from a hamster testicular cDNA library and shown that this protein is a member of the short-chain dehydrogenase/reductase (SDR) superfamily (*6*). This superfamily is a rapidly growing group of enzymes containing more than 1000 members with different substrate specificity and intracellular localization (*6*, *7*). Although the degree of residue identity between different SDR family members ranges from 15 to 30%, P26h is 87% identical with mouse lung carbonyl reductase (MLCR, EC 1.1.1.184), which is a tetramer catalyzing the reduction of various carbonyl compounds and the oxidation of secondary alcohols in the presence of NADP(H) (*8*, *9*). The enzymatic and structural properties of P26h have not been reported to date, although it possesses the functional residues in the catalysis, coenzyme binding, and subunit association shown by the crystal structure of MLCR (*10*).

The mRNA for P26h has been predominantly detected in hamster testis and at a very low level in the epididymis (*5*), which suggests a dual origin of this sperm protein. While the localization of P26h in spermatozoa and epididymis of hamster has been investigated as described above, distribution of the protein in the testis and its relationship with the sperm protein are unknown. In this study, we have compared the enzymatic characteristics and substrate specificity of the recombinant P26h with those of NADP(H)-preferring MLCR, and the data show that this protein utilizes NAD(H) as the preferable coenzyme and efficiently catalyzes the oxidoreduction between 5α-androstane-3α,17β-diol and 5α-dihydrotestosterone, despite the similarity of other properties of the two proteins. The structural determinants for the NAD(H) specificity of P26h were also examined by preparing

* To whom correspondence should be addressed: Biochemistry Laboratory, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-8585, Japan. Phone or fax: +81 58 237 8586. E-mail: hara@gifu-pu.ac.jp.

[‡] Gifu Pharmaceutical University.

[§] Taisho Pharmaceutical Co. Ltd.

[1] Abbreviations: CHX, cyclohex-2-en-1-ol; 5α-dihydrotestosterone, 5α-androstan-17β-ol-3-one; L39R, P26h where Leu39 is replaced with Arg; MLCR, mouse lung carbonyl reductase; PCR, polymerase chain reaction; P26h, hamster sperm 26 kDa protein; RT, reverse transcription; SDR, short-chain dehydrogenase/reductase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TRT, P26h where the Ser38-Leu39-Ile40 sequence is replaced with Thr-Arg-Thr; PLCR, pig lung carbonyl reductase; GLCR, guinea pig lung carbonyl reductase; SD, specificity determinant.

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the L39R and S38T/L39R/I40T mutant enzymes (TRT), of which the latter mutant resulted in a switch of the coenzyme specificity. Furthermore, we report the subcellular distribution of P26h in hamster testis and epididymis, and the properties of the proteins purified from the subcellular fractions of the two tissues.

EXPERIMENTAL PROCEDURES

Materials. Pyridine nucleotide coenzymes and pI markers were obtained from Oriental Yeast (Tokyo, Japan). Steroids, retinoids, and arachidonic acid sodium salt were from Sigma Chemicals. Prostaglandins were from Cayman Chemicals. Sepiapterin was from Schricks Laboratories (Jona, Switzerland). Restriction and DNA-modifying enzymes were purchased from Stratagene and Takara (Kusatsu, Japan). *Escherichia coli* cells were from Toyobo (Tokyo, Japan). Concanavalin A, plasmids, M_r markers, and resins for column chromatography were from Amersham Pharmacia Biotech. All other chemicals were of the highest grade that could be obtained commercially.

cDNA Isolation and Site-Directed Mutagenesis. P26h cDNA was prepared by RT-PCR. Isolation of total RNA from hamster testis (100 mg) and RT-PCR were carried out as described previously (*9*). PCR was performed for 30 cycles using *Pfu* polymerase and the following primers. P26h-N (5′-CCGAATTCATGAAGCTGATGAATTTCA-3′) corresponds to nucleotides 1−16 of the coding regions of P26h cDNA (*5*) and includes an underlined *Eco*RI site. P26h-C (5′-GG*G*AATTCTTAGGAGGCCAGGTAACCA-3′) is complementary to nucleotides 717−735 of the cDNA and includes an underlined *Eco*RI site. The PCR products were digested with the endonuclease and subcloned into pKK223-3 expression plasmids (pKKP26h) at the restriction site.

Mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene) and the pKKP26h expression plasmid as the template according to protocols described by the manufacturer. The sense primers for preparing L39R and TRT were 5′-GGTGGCCGTGTCA*CG-C*ATCAACGAAGACCTGG-3′ and 5′-GGTGGCCGTG*A-CACGCACC*AACGAAGACCTGG-3′, respectively, where the italics denotes the mutated codons. The complete coding regions of the cDNAs in the expression plasmids were sequenced as described previously (*9*) to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

Expression and Purification of Recombinant Proteins. Expression of the recombinant enzymes in *E. coli* JM109 cells and preparation of the cell extract were carried out as described previously (*9*). The following procedures for the purification of the recombinant P26h and the mutant enzymes were performed at 4 °C. The precipitate of the crude cell extract was collected between 45 and 95% $(NH_4)_2SO_4$ saturation by centrifugation at 12000*g* for 15 min, dissolved in buffer A [10 mM Tris-HCl (pH 8.0) containing 0.1 M KCl, 2 mM EDTA, and 1 mM 2-mercaptoethanol], and dialyzed against the same buffer. The enzyme was applied to a Sephadex G-100 column (3 cm × 70 cm) equilibrated with buffer A. The enzyme fractions were concentrated by ultrafiltration using an Amicon YM-10 membrane, dialyzed against buffer A without KCl, and applied to a Q-Sepharose (2 cm × 15 cm) equilibrated with the buffer. The enzyme was eluted with a linear gradient of 0 to 0.1 M NaCl in the buffer. The enzyme fractions were applied to a Blue-Sepharose column (1.5 cm × 5 cm) equilibrated with buffer A without KCl. The column was washed with the buffer containing 0.1 M NaCl, and the enzyme was eluted with the buffer containing 0.1 M NaCl and 1.0 mM NAD^+. Recombinant MLCR was purified as previously described (*9*).

Subcellular Fractionation. Brain, lung, heart, liver, kidney, spleen, testis, and epididymis of 8−10-week-old golden hamsters were excised. The tissues were homogenized in 4 volumes of 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5), using a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 600*g* for 10 min. The precipitate (nuclear fraction) was washed once with the sucrose solution. The supernatants were combined and centrifuged at 9000*g* for 10 min, and the precipitate (mitochondrial fraction) was washed once with the sucrose solution. The 9000*g* supernatants were combined and centrifuged at 105000*g* for 1 h to obtain microsomal and cytosolic fractions. All procedures, including the homogenization and centrifugation, were performed at 0−4 °C. All particulate fractions were suspended in the sucrose solution and analyzed for protein and enzyme activity. The protein concentration was determined by Bradford's method (*11*) using bovine serum albumin as a standard, and enzyme activity in the particulate fractions was assayed after the fractions were treated with 1% Triton X-100 for 1 h.

Solubilization and Purification of P26h from Subcellular Fractions. The mitochondrial fraction of the testis and the nuclear fraction of the epididymis were diluted 4-fold with 0.25 M sucrose, and proteins in the fractions were solubilized by following procedures. For sonication, the fraction was sonicated in an ice bath for 2 min at 6 kHz using a Microson sonicator (Heat Systems). For freezing and thawing, the fraction was frozen at −35 °C and then thawed at 25 °C. This was repeated three times. For the detergent and salt treatment, aliquots of 10% (w/v) Triton X-100 and/or 2 M KCl were added to the fraction and the mixtures were incubated in an ice bath for 1 h. The enzyme activity in the treated mixture was first assayed as described below, and then the solubilized proteins (solubilized supernatant) were separated from the unsolubilized portion by centrifugation at 12000*g* for 15 min at 4 °C.

P26h in the mitochondrial fraction of the testis and the nuclear fraction of the epididymis were solubilized with 1% Triton X-100 and 0.2 M KCl as described above. The solubilized supernatant was diluted with 2 volumes of 0.25 M sucrose and passed through a Blue-Sepharose column that had been equilibrated with 0.25 M sucrose containing 0.1 M KCl. After the column was washed with 10 mM Tris-HCl (pH 8.0) containing 0.1 M KCl, 1 mM EDTA, and 2 mM 2-mercaptoethanol (buffer A), the adsorbed P26h was eluted with the buffer supplemented with 1 mM NAD^+. The protein fractions were concentrated by ultrafiltration and then purified by consecutive Sephadex G-100 and Q-Sepharose chromatographies as described for the recombinant P26h.

Enzyme Assay. Dehydrogenase and reductase activities of P26h were assayed by measuring the rate of change in NADPH absorbance at 340 nm. The standard reaction mixture for the dehydrogenase activity consisted of 0.1 M potassium phosphate buffer (pH 7.0), 1.0 mM NAD^+, and

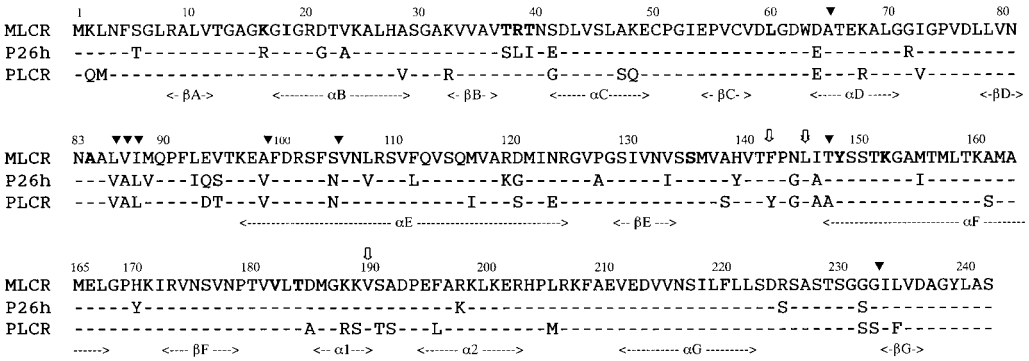

FIGURE 1: Alignment of amino acid sequences of MLCR, P26h, and pig lung carbonyl reductase (PLCR). The secondary structure identification is based on the crystal structure of MLCR (complex with NADPH and 2-propanol), in which the residues involved in the binding NADPH and the catalytic triad (Ser136, Tyr149, and Lys153) are highlighted in bold and those near 2-propanol are indicated by arrows. Identical amino acids between MLCR and other proteins are denoted with hyphens. Black arrowheads indicate the residues which are conserved in P26h and PLCR but not in MLCR.

10 mM cyclohex-2-en-1-ol (CHX), and enzyme, in a total volume of 2.0 mL. The activity in the *E. coli* extract and during the purification was assayed with 0.1 M glycine-NaOH buffer (pH 10.0) instead of the phosphate buffer. The reductase activity was determined with 0.1 mM NADH and 0.2 mM 4-nitroacetophenone as the coenzyme and substrate, respectively, unless otherwise noted. The steroids, which were tested as substrates, were first dissolved in methanol, diluted with 0.01% Triton X-100, and added into the reaction mixture, in which the final concentration of Triton X-100 was 0.005% and that of methanol was <0.2%. The concentrations of Triton X-100 and methanol did not affect the activity of P26h.

The activities of glucose-6-phosphate dehydrogenase (a cytosolic marker; *12*), NAD^+-dependent isocitrate dehydrogenase (a mitochondrial marker; *13*), and NADPH−cytochrome *c* reductase (a microsomal marker; *14*) were assayed spectrophotometrically. One unit of these oxidoreductases was defined as the amount that catalyzes reduction or formation of 1 μmol of NAD(P)H per minute at 25 °C, except that 1 unit of NADPH−cytochrome *c* reductase was expressed as 1 μmol of reduced cytochrome *c* formed per minute at 25 °C. The activities of retinol dehydrogenase, retinal reductase (*15*), and sepiapterin reductase (*16*) were assayed as described previously.

The apparent K_M and V_{max} values for coenzymes and substrates in the presence of a fixed concentration of either coenzyme or substrate were directly determined by fitting to the Michaelis−Menten equation. The kinetic mechanism and constants of the 5α-dihydrotestosterone reduction were analyzed according to the method of Cleland (*17*). The initial velocities were fitted to the equation

$$v = VAB/(AB + K_A B + K_B A + K_{IA}K_B)$$

where v is the initial velocity, V is the maximum velocity at saturating substrate concentrations, A and B are the two substrate concentrations, K_A and K_B are their corresponding Michaelis constants, and K_{IA} is the dissociation constant of substrate A.

Computer Modeling. The model structure of P26h was constructed on the basis of the crystal structure of MLCR (*10*) and sequence alignment shown in Figure 1, using the protein modeling package Insight-II/Homology (Molecular Simulations Inc., San Diego, CA).

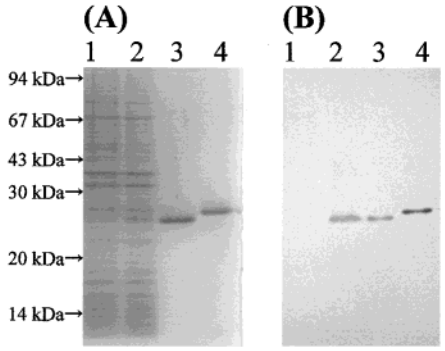

FIGURE 2: SDS−PAGE and Western blot analysis of the *E. coli* extract, purified recombinant P26h, and recombinant MLCR. Samples are the extracts (15 μg of protein each) of the cells transformed with pKK223-3 (lane 1) and pKKp26h (lane 2) and the purified preparations (1.0 μg each) of P26h (lane 3) and MLCR (lane 4). The proteins in the SDS−PAGE gel (A) were stained with Coomassie brilliant blue R-250 and detected by immunoblot using the anti-MLCR antibody (B). Positions of molecular mass standards are indicated.

Other Analytical Methods. SDS−PAGE on 12.5% slab gels (*18*), isoelectric focusing on 7.5% polyacrylamide disk gels (*19*), and analytical gel filtration on a Superdex 200 HR column (*8*) were carried out as described previously. Western blot analysis using the anti-MLCR IgG was performed by the method of Towbin et al. (*20*) with a minor modification (*9*). For analysis of expression of mRNA for P26h in hamster tissues, the total RNA samples were prepared from the tissues and RT-PCR was carried out as described above, except that PCR was performed using *Taq* polymerase. The identification of the reaction products of the 5α-dihydrotestosterone reduction was carried out by thin-layer chromatography using several solvent systems described by Gibb and Jeffery (*21*).

RESULTS

Properties of Recombinant P26h. The extract of the *E. coli* cells transfected with the pKKP26h construct contained a 26 kDa protein that cross-reacted with the anti-MLCR IgG and migrated slightly faster than MLCR (Figure 2). This protein was not detected in the extract of the cells transfected with the vector (pKK223-3). The extract of the cells transfected with the pKKP26h vector also exhibited NAD^+-linked dehydrogenase activity (0.33 unit/mg) for CHX, one of the representative substrates for MLCR (*8*, *9*), ∼9-fold

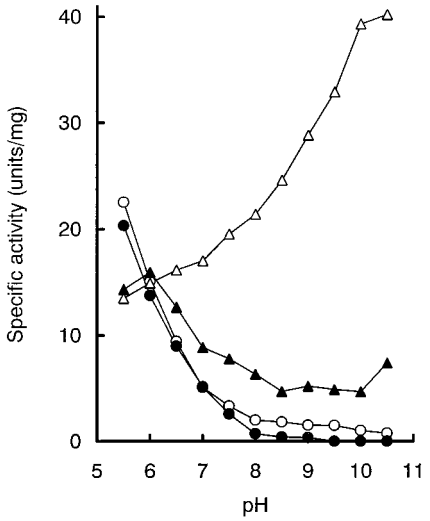

FIGURE 3: pH dependency of 4-nitroacetophenone reduction and CHX oxidation by recombinant P26h. The reductase activity was measured with 0.2 mM 4-nitroacetophenone and 0.1 mM NADH (○) or NADPH (●), and the dehydrogenase activity was measured with 10 mM CHX and 1 mM NAD^+ (△) or 2 mM $NADP^+$ (▲).

higher than the $NADP^+$-linked activity. The CHX dehydrogenase was purified to homogeneity by ammonium sulfate fractionation and consecutive column chromatographies on Sephadex G-100, Q-Sepharose, and Blue-Sepharose. From 1 L of cultured cells, 1.5 mg of enzyme was obtained, in a 26% yield, with a specific activity of 29.5 units/mg at 25 °C. SDS−PAGE revealed a single 26 kDa protein band that cross-reacted with the anti-MLCR IgG as detected in the crude extract, indicating the identity of the purified CHX dehydrogenase with P26h. On gel isoelectric focusing, the enzyme was focused at pH ∼10. The pI value is similar to that of P26h purified in hamster spermatozoa (*22*). In addition, gel exclusion chromatography of the enzyme on a Superdex 200 HR column resulted in a single peak with an apparent M_r of ∼100000, demonstrating a tetrameric structure for P26h.

The NAD^+-linked CHX dehydrogenase activity of the recombinant P26h was elevated by increasing the pH from 5.5 to 10.5, whereas the $NADP^+$-linked activity was low and exhibited a broad pH optimum at ∼6.0 (Figure 3). The K_M values for NAD^+ determined at pH 6.0 and 7.0 were 55 and 68 μM, respectively, and the respective values for $NADP^+$ were 1.8 and 1.4 mM. Although the reason for the difference in the pH dependency of the NAD^+- and $NADP^+$-linked activities remains unknown, the K_M values for the coenzymes indicated a pronounced preference of P26h for NAD^+. P26h reduced 4-nitroacetophenone at pH <8.0 in the reverse reaction with either NADH or NADPH as the coenzyme, and also exhibited lower K_M values for NADH (3.0 μM) at pH 7.0 than for NADPH (79 μM). Since the pH dependencies of NAD(P)(H)-linked activities were different, kinetic constants for substrates were measured with NAD(H), the preferable coenzymes, at a physiological pH of 7.0 (Table 1).

P26h was found to reduce various aldehydes and ketones, including oxosteroids. Since most of the carbonyl compounds are the substrates for MLCR, the kinetic constants were compared between P26h and MLCR. The K_M, V_{max}, and V_{max}/K_M values for the nonsteroidal compounds, except 4-nitroacetophenone, were similar for the two enzymes, whereas P26h reduced the oxosteroids more efficiently than did MLCR. In the reverse reaction, P26h exhibited higher V_{max}/K_M values for secondary alcohols, except chloral hydrate, than MLCR. Especially, it oxidized both 5α- and 5β-forms of androstan-3α-ol-17-one and androstane-3α,17β-diol that were very poor substrates for MLCR. Of the alcohols, 5α-androstane-3α,-17β-diol was the best substrate, exhibiting the lowest K_M and highest V_{max}/K_M values. P26h did not oxidize steroids with a 17α- or 17β-OH group (17α-epitestosterone, 17α-estradiol, 17β-estradiol, estriol, and testosterone), those with a 3β-OH group (5α-androstan-3β-ol-17-one, 5β-pregnan-3β-ol-20-one, 5-pregnen-3β-ol-20-one, and dihydroepiandrosterone), those with a 11β-OH group (corticosterone, cortisol, and deoxycorticosterone), and those with a 20α- or 20β-OH group (4-pregnen-20β-ol-3-one, 4-pregnen-17α,20β-diol-3-one, and 4-pregnen-17α,20α-diol-3-one). In the SDR family, several enzymes in the metabolism of prostaglandins (*23*), retinoids (*24*), and sepiapterin (*16*) have been reported to exhibit carbonyl reductase or 3α-hydroxysteroid dehydrogenase activity. However, both P26h and MLCR did not reduce or oxidize 50−100 μM prostaglandins (E_2, $F_{2\beta}$, and D_2), 100 μM retinoids (*all-trans*-retinol and *all-trans*-retinal) and 100 μM sepiapterin, in addition to 0.1−50 mM primary alcohols (1-propanol, 1-butanol, farnesol, and geraniol) and 10−50 mM sugars (D-glucuronic acid, D-xylose, L-xylulose D-glucose, D-sorbitol, D-threitol, L-arabitol, erythritol, inositol, xylitol, galactitol, and mannitol).

The reduced product of 5α-dihydrotestosterone after exposure to P26h was identified with 5α-androstane-3α,17β-diol by thin-layer chromatography. This, together with the above substrate specificity, indicated that P26h efficiently catalyzes the reversible conversion between 3α-OH and 3-oxo groups on the androstanes, in addition to having MLCR-like carbonyl reductase activity. To determine the precise kinetic parameters for the coenzymes and substrates, initial velocity measurements for the forward (NAD^+-linked 5α-androstane-3α,17β-diol oxidation) and reverse (NADH-linked 5α-dihydrotestosterone reduction) directions were performed. The double-reciprocal plots of initial velocity versus NAD^+ concentration at five fixed levels of 5α-androstane-3α,17β-diol yielded a series of intersecting lines. Similar patterns of initial velocity were observed in the reverse reaction (data not shown). The results are consistent with a reaction mechanism that proceeds in a sequential manner. The kinetic constants, calculated from the secondary plots of the initial rate data, were as follows. The Michaelis constants for NAD^+, 5α-androstane-3α,17β-diol, NADH, and 5α-dihydrotestosterone were 30, 3.4, 3.4, and 3.5 μM, respectively; dissociation constants for NAD^+ and NADH were 28 and 18 μM, respectively, and V_{max} values for the oxidation and reduction were 1.96 and 2.96 units/mg, respectively. The k_{cat} values for the 5α-androstane-3α,17β-diol oxidation and 5α-dihydrotestosterone reduction were calculated to be 1.28 and 0.85 s^{-1}, respectively, on the basis of the P26h subunit molecular mass of 26 kDa, and the respective k_{cat}/K_M values were 377 and 243 s^{-1} mM^{-1}. When these values were inserted into the Haldane relationship (*25*), the equilibrium constant for the reaction was calculated to be 0.27.

The effects of known inhibitors for MLCR and daidzein on the NADH-linked 4-nitroacetophenone reductase activity

Table 1: Comparison of Substrate Specificities of P26h and MLCR[a]

substrate	P26h K_M (mM)	P26h V_{max} (units/mg)	P26h V_{max}/K_M (units mg^{-1} mM^{-1})	MLCR K_M (mM)	MLCR V_{max} (units/mg)	MLCR V_{max}/K_M (units mg^{-1} mM^{-1})
reduction						
acetone	0.065	3.74	57	0.064	2.73	42.7
2-butanone	0.011	2.96	269	0.007	1.60	216
cyclohexaone	0.032	5.2	162	0.009	2.76	303
cyclohex-2-en-1-one	0.19	2.98	15.2	0.33	1.73	5.2
indan-1-one	0.18	1.36	11	0.18	1.04	5.8
pyridine-3-aldehyde	0.033	5.0	152	0.017	2.77	162
pyridine-4-aldehyde	0.071	4.09	58	0.13	2.38	18
4-nitroacetophenone	0.005	6.30	1260	0.015	2.50	166
menadione	0.050	6.95	139	0.064	6.91	108
5α-androstane-3,17-dione	0.010	2.25	225	–	(0.10)	–
5β-androstane-3,17-dione	0.004	2.81	702	0.024	5.24	218
5α-dihydrotestosterone	0.004	2.06	515	0.036	0.81	22
5β-androstan-17β-ol-3-one	0.004	3.98	995	0.071	1.62	23
oxidation						
2-propanol	0.78	2.95	3.8	7.1	0.36	0.05
(*S*)-2-butanol	0.072	7.77	108	0.11	0.74	0.74
chloral hydrate	1.9	3.87	2.0	4.4	19.3	4.4
cyclohexanol	0.19	2.82	14.8	0.49	0.30	0.61
CHX	0.078	26.0	335	0.28	6.3	23
(*S*)-indan-1-ol	0.23	40.5	176	0.49	10.7	22
(*R*)-indan-1-ol	0.55	50.6	92	2.94	27.9	9.9
5α-androstan-3α-ol-17-one	0.020	0.81	41	–	(0.01)	–
5β-androstan-3α-ol-17-one	0.029	1.90	66	–	(0.02)	–
5α-androstane-3α,17β-diol	0.005	1.72	340	–	(0.01)	–
5β-androstane-3α,17β-diol	0.045	1.62	36	–	(0.03)	–

[a] The kinetic constants of P26h for substrates were determined with 0.1 mM NADH or 1.0 mM NAD^+ as the coenzyme, and the coenzymes used for the analysis for MLCR were 0.1 mM NADPH and 0.25 mM $NADP^+$. The apparent K_M values of P26h for $NAD(P)^+$ and NAD(P)H were determined with 10 mM CHX and 0.2 mM 4-nitroacetophenone, respectively, as the substrate. The values in parentheses are specific activities with 25 μM 5α- and 5β-androstane-3α,17β-diols, and 50 μM other steroids.

Table 2: Effects of Inhibitors for MLCR on the Reductase Activity of P26h[a]

inhibitor	concentration (mM)	inhibition of P26h (%)	inhibition of MLCR (%)
cibacron blue	0.01	71	56
daidzein	0.01	72	56
quercitrin	0.05	45	41
pyrazole	0.1	71	35
4-methylpyrazole	0.1	69	19
2-mercaptoethanol	0.1	36	27
benzoic acid	1.0	10	13

[a] The activity was assayed in 0.1 M potassium phosphate (pH 7.0) containing 0.2 mM 4-nitroacetophenone and 0.1 mM NADH for P26h or NADPH for MLCR.

of P26h were examined to compare the inhibitor sensitivity between the two enzymes. All the inhibitors depressed the activity, and the inhibition percentages were almost the same as those of the NADPH-linked activity of MLCR, except that pyrazole and 4-methylpyrazole showed more potent inhibition for P26h than for MLCR (Table 2). The activity of P26h was also enhanced by the addition of arachidonic acid and 1,10-phenanthroline, which are the efficient activators for MLCR (*9*), but the NADPH-linked activity of P26h was not (Figure 4). This is in contrast to the activation of MLCR, where the activators stimulated the NADPH-linked activity but not the NADH-linked activity.

Kinetic Alteration by Mutagenesis. One of the significant differences in properties between P26h and MLCR was the coenzyme specificity as described above. The subunits of the two enzymes are composed of 244 amino acids, and show differences of 32 amino acid residues, of which Thr38, Arg39, and Thr40 of the MLCR sequence, which interact with the 2′-phosphate of NADPH (*10*), are replaced with Ser, Leu, and Ile, respectively, in the P26h sequence (Figure 1). To elucidate structural determinants for the NAD(H) specificity of P26h, we prepared two P26h mutants, L39R and TRT (replacing the Ser38-Leu39-Ile40 sequence with the corresponding residues, Thr-Arg-Thr, of MLCR), and compared the kinetic constants in both the forward (with CHX as the substrate) and reverse (4-nitroacetophenone as the substrate) reactions between the wild-type and mutant enzymes (Table 3). The most striking alteration by the mutations was to increase the K_M values for NAD(H) and to decrease the K_M values for NADP(H). Especially, the triple mutation of TRT resulted in a significant decrease in the K_M values for NADP(H) which are comparable to those reported with MLCR that prefers NADP(H) as the coenzyme (*9*). In addition, the catalytic efficiency (V_{max}/K_M) of TRT with NADP(H) was more than 100-fold higher than that of the wild type with NAD(H).

The mutations also significantly influenced the activation by arachidonic acid and 1,10-phenanthroline (Figure 4). The stimulatory effect of the NADH-linked activity observed with wild-type P26h was largely decreased or disappeared with the L39R and TRT mutations, whereas the NADPH-linked activity of L39R was slightly activated by the activators and that of TRT greatly stimulated. The activation of wild-type P26h and TRT by 16 μM arachidonic acid resulted in an ∼2-fold increase in the K_M values for the coenzymes. A similar increase in the K_M value for NADPH by the activation has been observed with MLCR (*26*). The results, together with the K_M values of the mutant enzymes for the coenzymes

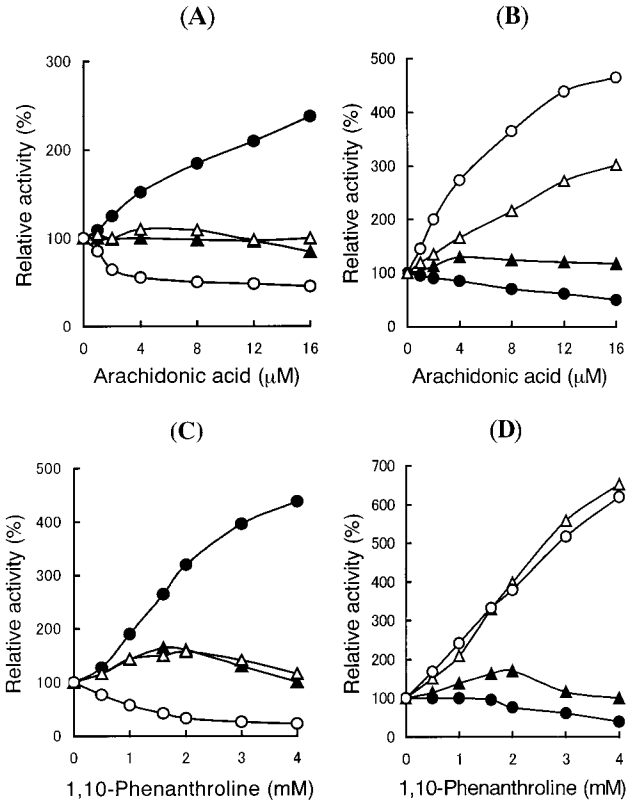

FIGURE 4: Effects of arachidonic acid and 1,10-phenanthroline on NADH- and NADPH-linked 4-nitroacetophenone reductase activities of wild-type and mutant P26h proteins and MLCR. The activities of wild-type P26h (●), L39R (▲), TRT (△), and MLCR (○) were assayed with 0.1 mM NADH in panels A and C or 0.1 mM NADPH in panels B and D. The activity without the activator was taken to be 100%.

in Table 3, indicated that the stimulation by the activators occurs in the reactions mediated by only the high-affinity coenzymes, and suggested that the binding of the activators leads to rapid release of the oxidized coenzyme products.

Distribution of P26h in Subcellular Fractions of Hamster Tissues. The distribution of P26h in the subcellular fractions of hamster tissues was examined by assaying the NAD^+-linked CHX dehydrogenase activity and by Western blot analysis. The high enzyme activity was detected in the mitochondrial fraction of the testis and in the nuclear fraction of the epididymis, which corresponded to 76 and 82%, respectively, of the sums of total activities recovered in all the subcellular fractions of the two tissues (Figure 5). The total amount of enzyme activity in the testis was ~9-fold higher than that in the epididymis because of the difference in the tissue weights. Western blot analysis also revealed that P26h is enriched in the two fractions of the tissues, and the single 26 kDa band indicated that MLCR-like protein, which is expected to migrate slower than P26h (Figure 2), is not present in the two tissues. No NAD^+-linked CHX dehydrogenase activity was detected in the nuclear and mitochondrial fractions of the other tissues; however, the moderate activity of 26 munits/mg was observed in the cytosolic fraction of liver, and low activities of 0.3−2.0 munits/mg were detected in the cytosolic fractions of heart, lung, kidney, spleen, and brain, and in the microsomal fractions of heart and liver. The moderate or low enzyme activities of these tissue fractions may be due to other dehydrogenase(s), because no immunoreactive protein with the anti-MLCR antibody was observed on Western blot analysis of these fractions. The results suggest that P26h is present specifically in the testis and epididymis. However, the origin of P26h in the nuclear fraction of the epididymis remains unknown because of the testis-specific expression of mRNA for P26h (*5*) that was also confirmed by the RT-PCR with gene-specific primers (data not shown).

When the activities of several marker enzymes of the organelles were assayed, NAD^+-dependent isocitrate dehydrogenase, a mitochondrial marker enzyme, was enriched in the nuclear fraction of the epididymis, and in both nuclear and mitochondrial fractions of the testis (Figure 5). It should be noted that in the subcellular fractionation of other tissues, such as liver and kidney, more than 80% of the NAD^+-dependent isocitrate dehydrogenase activity was recovered in the mitochondrial fractions. The effects of various treatments on solubilization of the NAD^+-linked CHX dehydrogenase activity were further compared between the testis mitochondrial and epididymis nuclear fractions to determine whether P26h exists as different forms. Both the two subcellular fractions prepared with 0.25 M sucrose exhibited low enzyme activity before the various treatments (Table 4). Sonication and repeated freezing and thawing enhanced the total activity of the testis mitochondrial fraction by 9- and 4-fold, respectively, and more than 50% of the activity was solubilized. Treatments with 0.5% Nonidet P-40 and 1% Triton X-100 resulted in ~13-fold enhancement of the total activity, but the amounts of released enzyme activity were lower than with sonication. Since P26h has been solubilized from hamster sperm by the detergents containing 0.15 M NaCl (*1*, *22*), the mitochondrial fraction was treated with the detergents in the presence of 0.2 M KCl. While extraction with 0.2 M KCl alone had almost no effect on the enzyme activity of the fraction, almost complete solubilization of the enzyme activity was achieved by treatments with the detergents and 0.2 M KCl. The effects of the treatments on the mitochondrial enzyme activity of testis were similar to those on the enzyme activity in the nuclear fraction of the epididymis, except the treatments with detergents alone released low amounts of the activity from the fraction.

To compare the properties of P26h in the fractions of the testicular mitochondria and epididymal nuclei, this protein was purified from the solubilized supernatants of the two particulate fractions by 1% Triton X-100 and 0.2 M KCl. The purification procedure used for the recombinant protein was modified to remove the detergent. The protein in the solubilized sample was first adsorbed on a Blue-Sepharose column, which was then extensively washed with the buffer without the detergent before the enzyme was eluted with the buffer containing NAD^+. The enzyme fraction was further purified by consecutive column chromatographies on Sephadex G-100 and Q-Sepharose. The final preparations purified from the mitochondrial fractions of testis (4 g) and the nuclear fractions of epididymis (1 g) exhibited specific activities of 13.0 and 9.0 units/mg, respectively, and the respective yields were 11 and 2%. The two preparations were not homogeneous on SDS−PAGE analysis, but the major protein migrated at the same mobility (26 kDa) as the recombinant P26h, and cross-reacted with the anti-MLCR IgG. In addition, the recombinant, testicular, and epididymal enzymes or the mixture of the three preparations exhibited

Table 3: Alteration of Kinetic Constants for Coenzymes and Substrates in the Forward and Reverse Reactions by Mutants of L39R and TRT[a]

coenzyme	parameter	WT	L39R	ratio (L39R/WT)	TRT	ratio (TRT/WT)
NAD^+	K_M for NAD^+ (mM)	0.068	1.4	21	0.62	9.2
	V_{max} (units/mg)	17	19	1.1	49	2.8
	V_{max}/K_M (units mg^{-1} mM^{-1})	256	14	0.05	78	0.3
	K_M for CHX (mM)	0.078	0.057	0.7	0.066	0.9
$NADP^+$	K_M for $NADP^+$ (mM)	1.4	0.37	0.3	0.003	0.002
	V_{max} (units/mg)	15	22	1.4	14	0.9
	V_{max}/K_M (units mg^{-1} mM^{-1})	11	59	5.5	4666	424
	K_M for CHX (mM)	0.054	0.054	1.0	0.078	1.4
NADH	K_M for NADH (mM)	0.003	0.013	4.1	0.018	5.8
	V_{max} (units/mg)	5.1	5.1	1.0	17	3.4
	V_{max}/K_M (units mg^{-1} mM^{-1})	1700	388	0.2	927	0.5
	K_M for 4NAP (mM)	0.005	0.018	3.6	0.016	3.2
NADPH	K_M for NADPH (mM)	0.079	0.011	0.1	0.0008	0.01
	V_{max} (units/mg)	9.3	9.9	1.1	9.4	1.0
	V_{max}/K_M (units mg^{-1} mM^{-1})	118	877	7.5	11750	96
	K_M for 4NAP (mM)	0.013	0.039	3.0	0.008	0.6

[a] The K_M values for coenzymes and V_{max} values were determined in the presence of 10 mM CHX or 0.2 mM 4-nitroacetophenone (4NAP), and the K_M values for the substrates were in the presence of 1.0 mM $NAD(P)^+$ or 0.1 mM NAD(P)H.

single bands at an identical pI value of 10.0, when NAD^+-linked CHX dehydrogenase activity was stained on isoelectric focusing analysis (data not shown).

Both the testicular and epididymal enzymes exhibited pH dependency of the CHX dehydrogenase activity similar to that for recombinant P26h. The K_M values for NAD^+ and $NADP^+$ were 66 μM and 1.4 mM, respectively, for the testicular enzyme, and the respective values of the epididymal enzyme were 67 μM and 1.8 mM. The two enzymes also oxidized 5α-androstane-3α,17β-diol at the same low K_M value of 4 μM in the presence of 1.0 mM NAD^+. The NADH-linked 4-nitroacetophenone reductase activities of the two enzymes were inhibited to ~20−30% of the respective control values by 10 μM daidzein and 0.1 mM pyrazole, and activated ~3-fold by 16 μM arachidonic acid. The enzyme activities of the recombinant, testicular, and epididymal P26hs were not affected by the addition of 5 μg of concanavalin A or the preincubation of the enzymes (1 μg) with 5 μg of concanavalin A in 20 mM Tris-HCl (pH 7.5) for 30 min at 4 °C. In addition, these enzyme preparations did not adsorb on a ConA−Sepharose column that had been equilibrated with the Tris-HCl buffer containing 0.1 M NaCl.

DISCUSSION

The enzymatic properties of recombinant P26h characterized in this present study indicate that the protein exhibits high reductase activity for various carbonyl compounds, like those of MLCR (*8*, *9*) and lung carbonyl reductases of guinea pigs (GLCR; *27*) and pigs (PLCR; *28*). In addition, P26h resembles the lung carbonyl reductases in terms of intracellular localization (with respect to testicular cells), tetrameric structure, inhibitor sensitivity, and activation by arachidonic acid and 1,10-phenanthroline. However, there are some differences between P26h and MLCR. (1) The most striking difference is the coenzyme specificity: P26h exhibited a preference for NAD(H) to NADP(H), in contrast to the NADP(H)-preferring MLCR. (2) P26h efficiently reduced the 3-oxo group of 5α- and 5β-androstanes. This steroid specificity differs from that of MLCR for 3-oxo-5β-steroids (*8*) and the low activity of GLCR toward the oxosteroids (*27*). Although the specificity of P26h is similar to that of PLCR (*28*), the V_{max}/K_M values for the steroids of P26h at pH 7.0 are much higher than those of PLCR determined at the pH optimum of 6.0. (3) P26h exhibited high dehydrogenase activity not only for the xenobiotic alcohol substrates of the lung carbonyl reductases but also for 3α-hydroxysteroids that are poor substrates for MLCR or have been described not to be oxidized by GLCR and PLCR (*8*, *27*, *28*). Thus, P26h is an NAD^+-dependent secondary alcohol dehydrogenase with 3α-hydroxysteroid dehydrogenase activity, rather than carbonyl reductase.

The amino acid sequence of P26h is 87% identical with that of MLCR, and thus should have a three-dimensional structure very similar to that of MLCR (*10*). P26h should also have a quaternary structure similar to that of MLCR, because it possesses Arg203 that has been shown to play an important role in subunit association of MLCR. The prediction is further supported by the fact that the expressed recombinant P26h was tetrameric and maintained enzymatic activity similar to that of MLCR as described above.

As we have shown, P26h takes NAD(H) as the coenzyme. In many NAD(H)-dependent enzymes, including the SDR family proteins, the critical determinant for the coenzyme specificity is the negatively charged residue, usually Asp, at the C-terminus of the second β-strand of the $\beta\alpha\beta$ (Rossmann) fold (*10*, *29*−*32*). However, there is no such acidic residue in this region of P26h (Figure 1). The unique way of recognizing NAD(H) of P26h was, therefore, further examined through the three-dimensional homology model of P26h built on the basis of the X-ray structure of MLCR which binds NADP(H). In MLCR, the NADP(H) specificity is due to the electrostatic interactions of the two basic residues, Lys17 and Arg39, and hydrogen bond interactions of Thr38 and Thr40 with the 2′-phosphate of the coenzyme (*10*, *26*, *33*). In the P26h sequence, however, Lys17, Thr38, Arg39, and Thr40 of MLCR are replaced with Arg, Ser, Leu, and Ile, respectively, although all the other residues of MLCR interacting with the coenzyme molecule are conserved. Of the four replacements, the change at position 17 may not contribute to the NAD(H) specificity of P26h, because the mutation of Lys17 to Arg in MLCR has no effect on the kinetic constants for the coenzymes (*26*). On the other hand, the changes at positions 39 and 40 will have drastic effects.

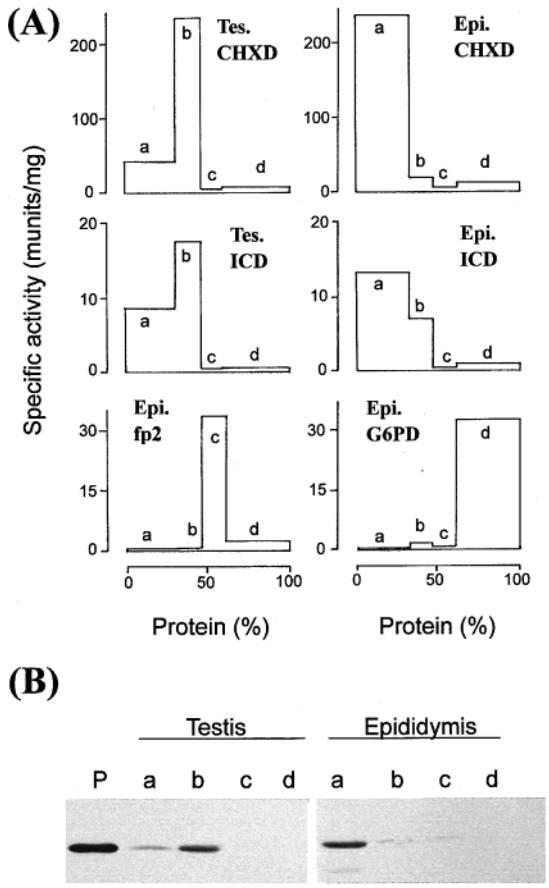

FIGURE 5: Distribution of P26h in the subcellular fractions prepared from hamster testis and epididymis. (A) Distribution pattern of the activities of NAD^+-linked CHX dehydrogenase and marker enzymes. The fractions are represented according to the order in which they are separated, i.e., from left to right, (a) nuclear fraction, (b) mitochondrial fraction, (c) microsomal fraction, and (d) cytosolic fraction. For testis (Tes), the patterns of the CHX dehydrogenase (CHXD) and isocitrate dehydrogenase (ICD) are shown, because NADPH−cytochrome *c* reductase (fp2) and glucose-6-phosphate dehydrogenase (G6PD) were mainly distributed in the microsomal and cytosolic fractions, respectively, like the patterns in epididymis (Epi). The values represent the means of two experiments. (B) Western blot analysis of the subcellular fractions of the testis (left) and epididymis (right) using the anti-MLCR antibody: lane P, recombinant P26h; lane a, nuclear fraction; lane b, mitochondrial fraction; lane c, microsomal fraction; and lane d, cytosolic fraction.

MLCR has electrostatic and hydrogen bond interactions with the 2′-phosphate of NADP(H) through Arg39 and Thr40. On the contrary, P26h generates steric hindrance and/or hydrophobic environments through the neutral and bulky side chains of Leu39 and Ile40, which would prevent the approach of the 2′-phosphate group (Figure 6). The importance of these residues is supported by our experimental result that the complete inversion of the coenzyme specificity of P26h can be achieved by the triple mutation of Ser38, Leu39, and Ile40 into the corresponding residues of MLCR (Thr, Arg, Thr, respectively), but not by the single mutation of Leu39 to Arg. Thus, the manner in which P26h recognizes NAD(H) is quite different from that of the other NAD(H)-dependent enzymes reported so far, and this study is the first report that reveals the key role of the residues other than well-known Asp in the NAD(H) specificity.

The reactivity toward steroids varies among P26h and three lung carbonyl reductases, those of mouse, guinea pig, and

Table 4: Solubilization of NAD^+-Linked CHX Dehydrogenase Activity from the Mitochondrial Fraction of Testis and Nuclear Fraction of Epididymis

treatment	step[a]	testicular mitochondrial fraction (%)[b]	epididymal nuclear fraction (%)[b]
none	mixture	100	100
	supernatant	4	5
sonication	mixture	938	413
	supernatant	710	290
freezing and thawing	mixture	390	333
	supernatant	211	215
0.2 M KCl	mixture	100	27
	supernatant	65	37
0.5% Nonidet P-40	mixture	1310	1258
	supernatant	504	77
0.5% Nonidet P-40 and 0.2 M KCl	mixture	1324	1433
	supernatant	1211	1408
1% Triton X-100	mixture	1255	1308
	supernatant	514	79
1% Triton X-100 and 0.2 M KCl	mixture	1279	1417
	supernatant	1200	1375

[a] Mixture represents the mitochondrial or nuclear fractions treated, and supernatant is the 12000*g* supernatant of the mixture. [b] The value represents the relative activity in the mixture or supernate, in which the activity in the nontreated mixture was taken as 100%.

pig. With respect to the reduction of oxosteroids, P26h, as well as PLCR, accepts 5α- and 5β-androstanes as the substrates, while MLCR accepts only 5β-steroids, despite the amino acid sequences of P26h, PLCR (*34*), and MLCR (*9*) being more than 85% identical to each other. Therefore, the residues, which are conserved in both P26h and PLCR but not in MLCR (Figure 1, marked with arrowheads), can be candidates for the specificity determinant (SD) residues which are responsible for the difference in the steroid specificity. In the search for the SD residues, it is reasonable to focus on the substrate-binding site. The crystal structures of MLCR (*10*) and the other SDR family proteins (*35*−*38*) suggest that the substrate-binding site is composed of the residues located in the three helices (αF, αG1, and αG2), the βD−αE, βE−αF, and βF−αGl loops, and the C-terminal segment. Among them, candidates for the SD residues can be found in the βD−αE loop and in the βE−αF loop. Such residues in the P26h sequence are Val86, Ala87, and Leu88 (in the βD−αE loop) and Gly145 and Ala147 (in the βE−αF loop). Of these residues, Ala87 is the nearest to 2-propanol in the three-dimensional model of P26h (Figure 6), and may play a role in the binding of both 5α- and 5β-androstanes. In comparison to the Val side chain of MLCR at the corresponding position, the Ala side chain common to P26h and PLCR is smaller and generates additional room in the putative substrate-binding pocket. This room in the substrate-binding pocket of P26h or PLCR would make it possible to accept 5α-androstanes as well as 5β-androstanes, while the bulky Val side chain of MLCR could be a steric hindrance for 5α-androstanes entering into the binding pocket. Another candidate of the amino acid residues responsible for the substrate binding is Gly145 in the βE−αF loop, although this site is a little distant from 2-propanol. The introduction of Gly would increase the flexibility of the βE−αF loop, which may allow the approach and adaptation of both 5α- and 5β-androstanes to the substrate-binding

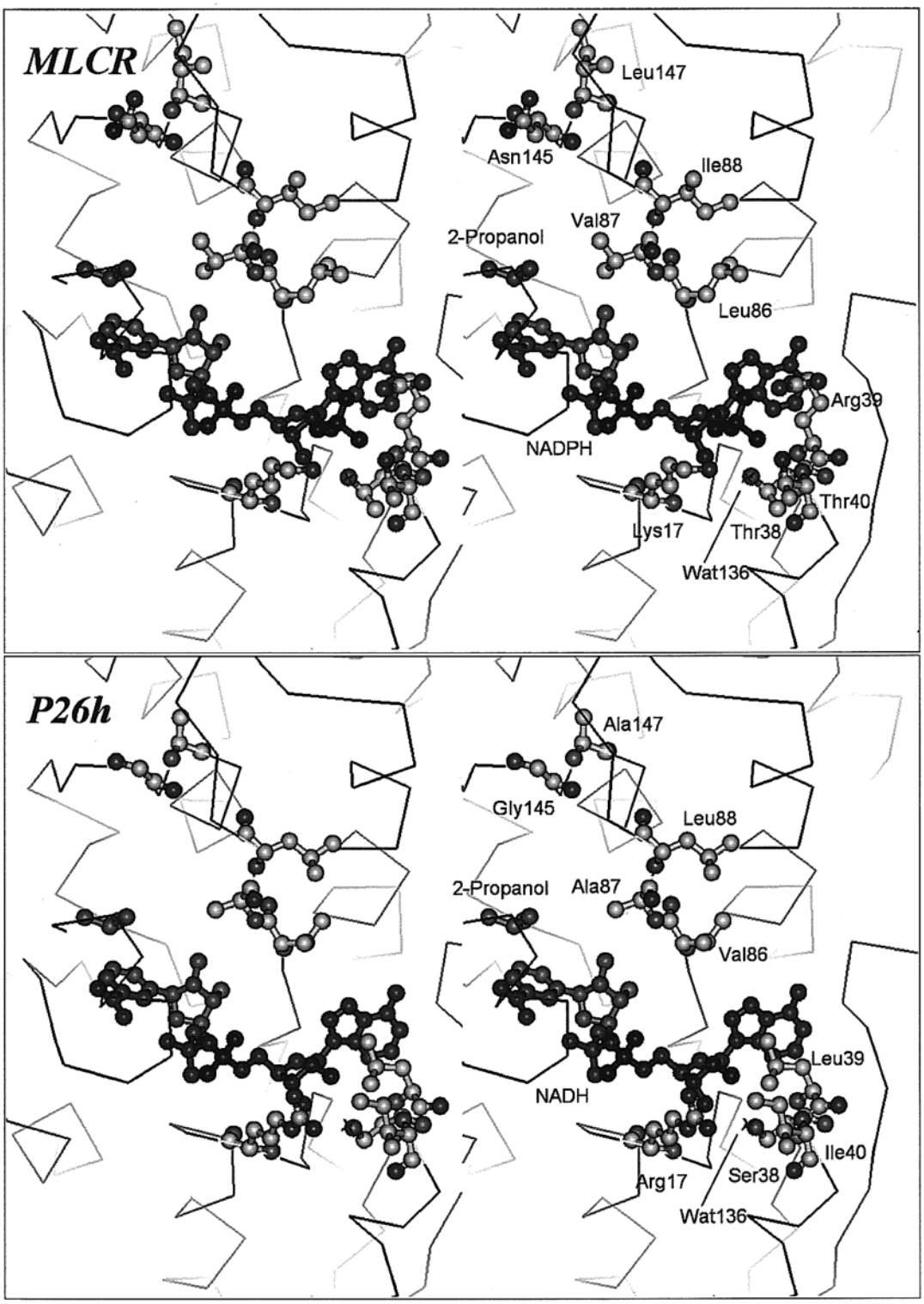

FIGURE 6: Comparison of the residues involved in the binding of the 2′-phosphate of NADPH between the ternary complexes of MLCR (with NADPH and 2-propanol) and the P26h model (with NADH and 2-propanol). The NAD(P)H and 2-propanol molecules are depicted in dark gray and black, respectively, and the water molecule (Wat136) that bridges the 2′-phoshate group and the side chain of Thr38 in MLCR is shown as a cross. The residues which are possibly responsible for the difference in substrate specificity of the two enzymes are also shown around 2-propanol.

pocket of P26h and PLCR. In this sense, Ala147 in P26h and PLCR, which is Ile in MLCR, can also be a candidate. This residue exists at the terminus of the βE−αF loop and is packed densely with the hydrophobic side chains of the neighboring monomer in the case of MLCR, but would not be so densely packed in the case of P26h and PLCR. The room could increase the flexibility of Ala147 and thus the βE−αF loop, too. Thus, Ala87, Gly145, and Ala147 are the candidates for the SD residues for the reduction of 5α- and 5β-oxosteroids. In addition to the three residues, other residues that are present only in P26h but not in MLCR and PLCR might be coordinately responsible for the high

dehydrogenase activity, which is one of the unique properties of this protein.

P26h was described to be bound on the surface covering the acrosomal cap of spermatozoon (*4*), but P26h mRNA has been shown to be predominantly expressed in spermatogenic cells of hamster testis and at a low level in the corpus epididymis by an in situ hybridization study (*5*). From these findings, P26h has been thought to be secreted by the testis and/or corpus epididymis and to accumulate on the membrane of spermatozoa during epididymal maturation. However, we propose that P26h is a tetrameric protein in the mitochondrial matrix of the testicular cells and epididymal cells (probably spermatozoa) by the following biochemical characterization of this protein and histological difference of the testis and epididymis. (1) The results of the subcellular distribution of P26h indicated that this protein exists as a mitochondrial protein in testicular cells. Although the enzyme activity was recovered mostly in the nuclear fraction in the case of the epididymis, most of the activity of the mitochondrial marker enzyme, isocitrate dehydrogenase, was also detected in this subcellular fraction. (2) The enzyme activity of P26h in the testicular, mitochondrial, and epididymal nuclear fractions exhibited high latency, and P26hs purified from the two subcellular fractions exhibited almost the same molecular weights, isoelectric points, and enzymatic properties as those of the recombinant P26h. This implies that the P26h molecule is not modified after being translated from the P26h mRNA and exists inside the membranes. (3) MLCR is a mitochondrial protein (*39*), and its N-terminal sequence (residues 1−21) acts as a noncleavable signal to transport the protein into the mitochondrial matrix (*9*). The N-terminal sequence of P26h is almost the same as that of MLCR, except that there are two replacements (Ser6 with Thr and Lys17 with Arg, Figure 1) that do not affect the role of the sequence as the noncleavable signal (*40*). (4) In the last phase of spermatogenesis, the Golgi region and mitochondria of the round spermatid in the testis are utilized to form the head cap and middle piece (mitochondrial sheath), respectively, of spermatozoon, which is stored in the epididymis to acquire the capacity for fertilization (*41*). This, together with the findings described above, suggests that P26h is mostly localized in the mitochondria of the testicular spermatids and the mitochondrial sheaths of the epididymal mature spermatozoa. The high yield of P26h in the nuclear fraction of the epididymis may be the result of its localization in the mature spermatozoa, which are resistant to the ordinary homogenization employed in this study.

P26h has been believed to be a glycoprotein because of evidence of its binding to concanavalin A (*1*), and recently reported to be phosphatidylinositol anchored to the sperm membrane (*42*). Phosphatidylinositol-anchored protein is generally synthesized on the endoplasmic reticulum, and processed in the lumen of the organelle as follows: the removal of its N-terminal leader peptide, the transfer of the glycosyl−phoshatidylinsitol moiety on an amino acid of its C-terminal region, and hydrolysis of the C-terminal hydrophobic region below the phosphatidylinositol-anchored amino acid (*43*). However, there is no consensus sequence for N-glycosylation in the P26h sequence, and the protein previously purified from hamster spermatozoa retains the N-terminal sequence deduced from its cDNA (*5*), which supports the mitochondrial localization of this protein as described above. If the C-terminal region of P26h would be cleaved after the transfer of the glycosyl−phosphatidylinositol moiety, the modified P26h may not form correct quaternary structure and lose the enzymatic properties, because the C-terminal part of the protein is probably important for the subunit association as described in the MLCR structure (*10*). Furthermore, the proteins expressed in *E. coli* and purified from the testis and epididymis did not adsorb to the ConA−Sepharose column, and their enzyme activities were not affected by concanavalin A. Therefore, most of P26h molecules may not exist in phosphatidylinositol-anchored proteins at least in the testicular cells, although some of them can be attached with the glycosyl−phoshatidylinositol moiety in epididymal cells before being transported into mitochondria.

Our insight into the localization of P26h also differs from those proposed by immunohistochemical studies with polyclonal antibodies against P26h (*4*, *42*). The polyclonal antibodies have been reported to cross-react with a human epididymal protein, P34H, whose amino acid sequence is 65% identical with that of P26h (*44*, *45*). We molecularly cloned a protein whose amino acid sequence was 89% identical against P34H from hamster liver and found that the P34H-like protein was distributed in many tissues, including the testis and epididymis (unpublished experiments). It should be noted that the antibodies against MLCR used in this study cross-reacted with P26h, but not with the hamster P34H-like protein. Thus, it might be possible that the immunoreactive P34H-like protein is detected in the studies on the localization of P26h (*4*, *42*). In these previous experiments, the spermatozoa were incubated with the antisera against P26h, which might react with proteins on the surface of the membranes but not with those inside the membranes. To resolve the discrepancy in the localization of P26h in spermatozoon between this study and previous studies, its ultrastructural localization using electron microscopy and monoclonal or monospecific antibody against this protein is, however, required.

The study present here shows that in the testis and epididymis P26h exists in mitochondria as a tetrameric NAD^+-dependent dehydrogenase. Especially, P26h efficiently catalyzed the oxidoreduction between 5α-androstane-3α,17β-diol and 5α-dihydrotestosterone. The k_{cat}/K_M or V_{max}/K_M values for the steroids are higher than or comparable to those reported with mammalian cytosolic 3α-hydroxysteroid dehydrogenases (*46*−*49*), and are much greater than those of human L-3-hydroxyacyl-CoA dehydrogenase which has been also reported to act as a mitochondrial 3α-hydroxysteroid dehydrogenase (*50*). Testosterone is able to initiate, maintain, and reinitiate the formation of spermatozoa, and the testicular effects of testosterone are mediated by its metabolite, 5α-dihydrotestosterone, as well as by estradiol (*51*). We propose that P26h plays a role in controlling the concentration of the potent androgen, 5α-dihydrotestosterone, in the spermatogenic cells during spermatogenesis, in addition to its role in the processes of gamete interaction previously reported (*4*). The protein also exhibited broad specificity for carbonyl compounds, like lung carbonyl reductases that have been suggested to function in detoxification of carbonyl compounds derived from lipid peroxidation (*28*, *39*). Alternatively, P26h might act as a reductase for carbonyl compounds derived from lipid peroxidation

within the mitochondria of the cells of testis and epididymis, because the NAD^+/NADH ratio in mitochondria is generally lower than that in cytoplasm.

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