Testicular Temperature-labile Cholesteryl Ester Hydrolase

RELATIONSHIP TO ISOENZYMES FROM OTHER TISSUES, CORRELATION WITH SPERMATOGENESIS, AND INHIBITION BY PHYSIOLOGICAL CONCENTRATIONS OF DIVALENT CATIONS*

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Siewfong Wee‡ and W. McLean Grogan§

From the Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614

Temperature-labile cholesterol ester hydrolase (TLCEH) was purified 2,000-fold from rat testis cytosol using sequential ammonium sulfate precipitation, cation exchange chromatography, and isoelectric focusing chromatography. The purified enzyme, which exhibited a single silver-stained band (66 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was inhibited 89% by the elevation of the temperature from 32 to 37 °C and 65% by treatment with alkaline phosphatase. Its amino acid composition and amino-terminal sequence differed markedly from those of isoenzymes from other tissues, although 6 of 20 residues were conserved. Polyclonal antibodies raised to TLCEH exhibited no cross-immunoreactivity with cytosolic proteins from other rat tissues and inhibited 70% of testis cytosolic CEH. Western blot analysis demonstrated a high correlation between immunoreactive protein and catalytic activity in the testis during maturation of the rat, with a marked increase at the onset of spermatogenesis. TLCEH was inhibited by physiological levels of Cu²⁺ (I₅₀ = 0.60 µM) and Zn²⁺ (I₅₀ = 0.75 µM) and by Cd²⁺ (I₅₀ = 0.15 µM) but not by 0.5–5 mM Mn²⁺.

Neutral cytosolic CEH¹ is found in rat testis in a temperature-labile form (TLCEH), which is located in the Sertoli cells of the germinal epithelium, and a temperature-stable isoenzyme that is found in both Sertoli cells and interstitial cells of Leydig (1, 2). In mature rats, TLCEH accounts for 65% of cytosolic testicular CEH activity when measured at 32 °C, the scrotal temperature, but is inactive at 37 °C, the abdominal temperature. This temperature lability is not observed with the temperature-stable testicular CEH or isoenzymes from other organs (1, 2). Inasmuch as spermatogenesis is inhibited by a similar elevation in temperature (3) and by phenylmethylsulfonyl fluoride, an inhibitor of CEH (4, 5), and inhibition of spermatogenesis is often accompanied by the accumulation of cholesteryl esters (6), attention has been focused on TLCEH as a potentially important enzyme in the regulation of spermatogenesis and mediation of toxic effects on germinal cells. In support of such a role, TLCEH activity is not present in hypophysectomized rats but can be induced to normal levels by injections of follicle-stimulating hormone (2). Moreover, this enzyme is susceptible to regulation by protein kinase/phosphatase activities (7), and its activity appears in developing rat testes between 47 and 51 days (8), coinciding with puberty, an increase in testosterone synthesis (9), and the first appearance of spermatocytes (10). Whether this increased activity reflects an increase in enzyme protein or activation of enzyme already present has not been reported until now.

Further characterization of TLCEH requires purification of the protein. However, previous attempts at purification have been hampered by a loss of activity after only a few purification steps (1, 2, 7, 8). Here we describe the purification of TLCEH to apparent homogeneity, preparation of neutralizing antibodies, measurement of TLCEH protein at progressively stages of maturation, and characterization of some properties of this enzyme.

MATERIALS AND METHODS

Chemicals and Supplies—Cholesterol [1-¹⁴C]oleate (59.4 mCi/mmol) was purchased from Du Pont-New England Nuclear. All solvents and Freund's adjuvant were from Fisher Scientific. Cholic acid, EDTA, Trizma base (Tris base), bis-Tris, sodium chloride, sucrose, thiglycollate, normal goat sera, and reagents for Western blot analysis were from Sigma. Polyvinylidene difluoride membrane was from Millipore Corp. Agarose, SDS, and acrylamide were from Bio-Rad. Frozen rat testes (300–350-g rats) were from Zivic-Miller Inc. (Zelienople, PA). Ten-kg male New Zealand White rabbits were from Blue Gray Rabbitry. Polybuffer 74 was from Pharmacia LKB Biotechnology Inc. Antiserum to pancreatic CEH was provided by Dr. Linda Gallo.

Purification of CEH—As described in Wee and Grogan (8), decapsulated rat testes were homogenized with a loose fitting Teflon pestle at 4 °C in 20 mM Tris-HCl buffer, pH 7.4, 100 mM sucrose, 0.05% cholic acid, 0.1 mM EDTA, and 0.1 mM thiglycollate (1:1.5, w/v). The homogenate was centrifuged at 2,000 × g, 10,000 × g, and 104,000 × g, discarding the pellet each time. The 104,000 × g supernatant (S-104) was adjusted to 40% saturation with solid ammonium sulfate and stirred for 30 min at 4 °C. ASP was collected by centrifugation at 10,000 × g for 30 min and stored at −20 °C until needed. ASP pellets were dissolved in 750 µl of homogenizing buffer without sucrose (buffer A), desalted over a preequilibrated Sephadex G-25 column, and centrifuged for 20 min at 10,000 × g. Desalted ASP containing 30–30 mg of protein was then chromatographed on a Pharmacia Mono S HR 10/10 FPLC cation exchange column, eluted in a stepwise fashion as follows: at a flow rate of 4 ml/min 40 ml of buffer A, 72-ml linear gradient, 0–14% buffer B (buffer A with 2 M NaCl), 24 ml of 14% buffer B, 1-ml linear gradient, 14–100% buffer B, 20 ml of 100% buffer B. Eluant was collected in 8-ml fractions. Fractions 13–15, containing CEH activity, were pooled, concentrated, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme, which exhibited a single silver-stained band (66 kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was inhibited 89% by the elevation of the temperature from 32 to 37 °C and 65% by treatment with alkaline phosphatase. Its amino acid composition and amino-terminal sequence differed markedly from those of isoenzymes from other tissues, although 6 of 20 residues were conserved. Polyclonal antibodies raised to TLCEH exhibited no cross-immunoreactivity with cytosolic proteins from other rat tissues and inhibited 70% of testis cytosolic CEH. Western blot analysis demonstrated a high correlation between immunoreactive protein and catalytic activity in the testis during maturation of the rat, with a marked increase at the onset of spermatogenesis. TLCEH was inhibited by physiological levels of Cu²⁺ (I₅₀ = 0.60 µM) and Zn²⁺ (I₅₀ = 0.75 µM) and by Cd²⁺ (I₅₀ = 0.15 µM) but not by 0.5–5 mM Mn²⁺

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‡ Current address: Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121.

§ To whom all correspondence should be addressed.

The abbreviations used are: CEH, cholesteryl ester hydrolase; TLCEH, temperature-labile cholesteryl ester hydrolase; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; ASP, 40% ammonium sulfate precipitate; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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and desalted using an Amicon Centriprep 30 concentrator to a final volume of 500 μl. This preparation was further chromatographed on a Pharmacia Mono P HR 5/20 FPLC, eluted with a pH 6-5 gradient, as follows: at a flow rate of 1 ml/min 4 ml of 0.025 M bis-Tris, pH 6.4, 0.05% cholic acid, 0.1 mM EDTA, and 0.1 mM thiglycolate; 18 ml of Pharmacia Polybuffer 74, pH 5.0, diluted 1:10, with 0.05% cholic acid, 0.1 mM EDTA, and 0.1 mM thiglycolate. Fractions with CEH activity were collected, pooled, and concentrated with an Amicon Centriprep 30 concentrator, eluted over a 5-20% SDS-polyacrylamide gradient gel, according to the method of Laemmli (11), and stained by the silver staining method to assess purity. Bioread low molecular weight standards were used for gel calibration.

**Measurement of CEH Activity**—Each assay tube contained 20 μl Tris-HCl, pH 7.4, 10 mM cholic acid, 10 mM EDTA, and 10 mM thiglycolate. Aliquots of 200 μl (cation exchange) and 400 μl (chromatofocusing) from chromatography fractions were pipetted into assay tubes. The chromatofocusing fractions were adjusted to pH 7.4 with 1 M Tris base. Each tube was adjusted to 500 μl with assay buffer and incubated for 5 min at 32 °C. Cholesterol oleate (70 μM, aliquoted and stored at -20 °C) was added to each tube. After 2 h, the reaction was terminated by addition of 3.25 ml of methanol:chloroform:heptane (3.85:3.42:2.73, v/v/v) and 50 μl of 1 M NaOH and product oleate measured in the aqueous phase by liquid scintillation counting as described by Ghosh and Grogan (12). The activity was corrected by subtracting the values for control incubations in which assay buffer was substituted for column fractions. Although activity was initially detected in unadsorbed chromatofocusing fractions, CEH activity was subsequently stabilized for routine analyses by the addition of 15-25 μg of ASP. Protein was measured with the Bio-Rad microassay protein reagent using bovine serum albumin as a calibration standard.

**Protein Sequencing and Compositional Analysis**—The purification procedure was repeated many times to obtain sufficient pure CEH protein for amino-terminal sequencing and amino acid compositional analysis. After concentration and desalting, 20-30-μg aliquots of protein were electrophoresed on 5-20% SDS-polyacrylamide gels as described by Laemmli (11). The protein was electroblotted onto Millipore PVDF membrane and stained with Coomassie Blue R-250, as described by Matsudaira (13). The CEH protein band was excised and sent to the University of Illinois Biotechnology Center Genetic Engineering Facility for sequencing and amino acid analysis. The results of differentially phosphorylated forms or multimeric forms was purified 2,000-fold from rat testis cytosol using sequential ammonium sulfate precipitation, cation exchange chromatography, Chromatofocusing (Fig. 1), and isoelectric focusing chromatography (Fig. 2). Catalytic activity routinely eluted from the chromatofocusing column in more than one peak (Fig. 2), and the activity of differentially phosphorylated forms was purified from the CEH activity. The purified protein contained in fractions 14-20 exhibited specific activity (~60 nmol/h nmol/mg protein) and was subjected to electrophoresis on a 5-20% SDS-polyacrylamide gel to isolate the component of interest. The purified protein was then sent to the University of Illinois Biotechnology Center Genetic Engineering Facility for sequencing and amino acid analysis. The results of differentially phosphorylated forms or multimeric forms were compared to the known sequences of other CEHs.

**RESULTS**

**Purification of CEH**—As summarized in Table I, TLCEH was purified 9,000 fold from rat testis cytosol using sequential ammonium sulfate precipitation, cation exchange chromatography (Fig. 1), and isoelectric focusing chromatography (Fig. 2). Catalytic activity routinely eluted from the chromatofocusing column in more than one peak (Fig. 2), and the activity of differentially phosphorylated forms was purified from the CEH activity. The purified protein contained in fractions 14-20 exhibited specific activity (~60 nmol/h nmol/mg protein) and was subjected to electrophoresis on a 5-20% SDS-polyacrylamide gel to isolate the component of interest. The purified protein was then sent to the University of Illinois Biotechnology Center Genetic Engineering Facility for sequencing and amino acid analysis. The results of differentially phosphorylated forms or multimeric forms were compared to the known sequences of other CEHs.

**FIG. 1. Purification of testicular temperature-labile CEH by cation exchange chromatography.** A 40% ammonium sulfate precipitate was applied to a cation exchange FPLC column (Fig. 1), and the protein was eluted with a discontinuous NaCl gradient as shown. Each fraction was assayed for protein and CEH activity.
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Fig. 2. Purification of rat testis temperature-labile CEH by isoelectric focusing chromatography. TLCEH partially purified by 40% ammonium sulfate precipitation and cation exchange chromatography (pooled fractions 13–15) were further fractionated by chromatofocusing FPLC as described under "Materials and Methods." Individual fractions 5–30 were assayed for CEH activity. Fractions 1–4 were pooled for analysis. Shown here are a mean composite elution profile from seven purifications and a typical elution profile from a single run. Although peaks of activity shifted slightly from run to run, pooled fractions 14–20 consistently yielded a single band on SDS-PAGE (Fig. 3). This band was also observed in other fractions, accompanied by additional bands. Activity was inhibited at 37 °C in all fractions, regardless of degree of purification.

Fig. 3. SDS-PAGE of rat testis temperature-labile CEH purified by isoelectric focusing chromatography. Lane 1, purified TLCEH (2 μg) from chromatofocusing fractions 14–20; lane 2, molecular weight markers. Proteins were visualized by the silver staining method. This procedure was used as the final step in purification for amino acid analysis and sequencing for polyclonal antibodies.

μmol/h/mg of protein) comparable to that reported for other enzymes in this class (e.g. rat liver CEH, 25 μmol/h/mg of protein) (12) and yielded a single silver-stained band corresponding to 66 kDa after SDS-PAGE (Fig. 3). Pooled active fractions 14–20 from the chromatofocusing column (Fig. 2) were inhibited 89% by elevation of the temperature from 32 °C (55 μmol/h/mg of protein) to 37 °C (6 μmol/h/mg of protein), consistent with the temperature lability of this enzyme, which we have reported at other stages of purification (1, 2, 7, 8). The activity in other fractions was also inhibited at 37 °C (data not shown), indicating that TLCEH was the predominant form present in all fractions, although only fractions 14–20 exhibited a single band on SDS-PAGE.

Purified TLCEH was also inhibited 65% by preincubation with alkaline phosphatase (19 μmol/h/mg of protein versus 55 μmol/h/mg of protein, for untreated), consistent with our previous reports of protein kinase activation (7). Treatment of ASP with alkaline phosphatase prior to the purification procedure produced a similar reduction in the total TLCEH activity of chromatofocusing fractions 1–30; however, redistribution of the activity among chromatofocusing fractions and the possibility of differences in the overall recovery of enzyme complicate interpretation of these results (data not shown).

Further attempts at characterization of the highly purified enzyme were frustrated by the instability of catalytic activity following chromatofocusing. Moreover, attempts to subject the purified enzyme to gel permeation chromatography or ultrafiltration resulted in a total loss of activity (data not shown). The enzyme was not stabilized by conditions shown previously to optimize activity in less purified preparations (1, 2) or by collection into buffer containing 3 μM albumin or 20% glycerol (data not shown). However, the addition of ASP to the purified protein stabilized activity (data not shown). This permitted reliable assay of chromatofocusing fractions during purification of TLCEH for immunization, amino acid compositional analysis, and sequencing.

Inhibition by Metal Ions—Inasmuch as divalent cations are reported to inhibit liver cytosolic neutral CEH (15) and lysosomal acidic CEH (16, 17), the effects of divalent cations on TLCEH were examined. Both Cu2+ and Zn2+ inhibited the enzyme at very low concentrations (Fig. 4). Moreover, Cd2+, a well known inhibitor of spermatogenesis (6), was a very potent inhibitor of TLCEH, consistent with a possible role for this enzyme in mediating the toxic effect. In contrast, Mn2+ had no effect on activity over the range 0.5–5.0 mM (data not shown).

Immunological Characterization—Polyclonal antibodies were raised to TLCEH to measure the enzyme protein in testis at various stages of development and to compare immunological properties of this CEH with isoenzymes from other tissues. In Ouchterlony double diffusion analysis, the reaction of these antibodies with purified TLCEH and ASP from testis was consistent with monospecificity (Fig. 5). Moreover, they exhibited no cross-reactivity with similar concentrations of ASP from adrenal, serum, brain, intestine, liver, and pancreas, all of which are known to contain substantial CEH activity (not shown). In the corresponding Western blotting experiment, the antiserum reacted with TLCEH purified by chromatofocusing and with ASP from testis (Fig. 6).

![Fig. 4. Inhibition of rat testis temperature-labile CEH by divalent cations. TLCEH was assayed for 15 min with 35 μM substrate in pooled fractions 13–15 (10 μg of protein) from cation chromatography, with and without addition of the indicated cations. Experiment was repeated in triplicate with essentially the same results. Standard deviations are less than 5% for each data point. Lα concentration producing 50% inhibition of CEH activity. ▲, Cd2+; ●, Zn2+; ■, Cu2+.](image-url)
but not with equivalent amounts of ASP from the other organs (not shown). Moreover, antiserum to pancreatic CEH did not cross-react with cytosolic proteins from testis (not shown). As seen in Fig. 6, lanes B and C, additional immunoreactive bands appeared in ASP and increased with storage, suggesting the presence of degradation products. Similar multiple molecular weight forms have been reported in the most highly purified preparations of pancreatic CEH (18); however, we saw no evidence of proteolytic degradation on silver-stained polyacrylamide gels after storage of the highly purified TLCEH. As seen in Fig. 7, anti-TLCEH also inhibited 70% of testicular cytosolic CEH activity, consistent with the proportion of total CEH represented by the temperature-labile isoenzyme reported previously (1, 2). Thus, the antiserum is highly specific for the testicular TLCEH. Moreover, as we have reported previously for temperature-labile catalytic activity (1, 2), TLCEH protein is apparently localized to the testis.

 Whereas we have reported previously an increase in TLCEH activity during the induction of spermatogenesis in the rat (8), we have measured immunoreactive protein in testicular cytosol for correlation with catalytic activity at different stages of testicular development. As seen in Fig. 8, TLCEH protein closely parallels and correlates with catalytic activity (r = 0.97) from ages 14 to 365 days, rising sharply with the initiation of spermatogenesis (47–51 days) and declining somewhat in older rats. Thus, the increase in TLCEH at puberty is primarily caused by an increase in enzyme protein rather than enzyme-specific activity. Moreover, close correspondence between TLCEH protein and catalytic activity is further evidence for the specificity of these antibodies.

**Amino Acid Composition and Sequence Analysis**—As seen in Table II, the amino acid composition of purified TLCEH was similar but not identical to that of liver neutral cytosolic CEH (12). In contrast, the amino acid composition of TLCEH differed significantly from those of pancreatic CEH isoenzymes from several species. Pancreatic isoenzymes were consistently higher than the testicular isoenzyme in methionine and proline and somewhat lower in Asx. As seen in Fig. 9, the amino-terminal sequence of TLCEH was clearly different from those of pancreatic isoenzymes published by others.


**Table I**

Comparison of the amino acid composition of testicular temperature-labile CEH with compositions reported for isoenzymes from liver and pancreas

| Amino acid | Testis | Liver* | Rat pancreas* | Dog pancreas* | Human pancreas  | Rat pancreatic cDNA† |
|------------|--------|--------|--------------|--------------|----------------|----------------------|
| Ile        | 5.2    | 5.2    | 4.4          | 3.5          | 5.2            | 5.2                  |
| Val        | 8.0    | 6.0    | 6.0          | 8.4          | 6.2            | 6.2                  |
| Leu        | 9.6    | 9.9    | 8.2          | 6.8          | 9.7            | 7.3                  |
| Phe        | 3.3    | 3.0    | 4.2          | 3.3          | 3.6            | 4.6                  |
| Cys        | 0      | 0      | NR           | NR           | 0.01           | 0.01                 |
| Met        | 0      | 0.01   | 1.9          | 1.8          | 1.6            | 2.4                  |
| Ala        | 5.8    | 7.0    | 7.0          | 9.9          | 9.7            | 9.1                  |
| Gly        | 11.3   | 7.2    | NR           | 10.5         | 10.4           | 8.3                  |
| Thr        | 6.0    | 6.2    | 7.2          | 5.8          | 8.2            | 7.4                  |
| Ser        | 4.7    | 5.0    | 9.2          | 7.1          | 5.4            | 5.1                  |
| Tyr        | 2.9    | 2.6    | 4.0          | 3.6          | 3.5            | 3.9                  |
| Pro        | 3.6    | 4.0    | 8.3          | 10.7         | 13.4           | 7.4                  |
| His        | 2.4    | 2.1    | 2.3          | 1.9          | 1.6            | 2.2                  |
| Glx        | 11.9   | 12.9   | 10.2         | 7.6          | 6.0            | 8.0                  |
| Asx        | 15.2   | 17.8   | 11.8         | 12.8         | 11.1           | 11.7                 |
| Lys        | 6.0    | 6.0    | 5.8          | 4.4          | 4.8            | 5.9                  |
| Arg        | 4.0    | 4.9    | 3.5          | 3.5          | 2.8            | 2.9                  |

* Ref. 12.
† Ref. 18. NR = not reported.
‡ Calculated from rat pancreatic cDNA sequence (Ref. 22).

Fig. 9. Comparison of amino-terminal sequences of rat testis temperature-labile CEH with those of CEH isoenzymes from rat, dog, and human pancreas. Boxes indicate identical residues. Pancreatic sequences were obtained from Abouakil et al. (18).

However, 6 residues showed identity with the pancreatic isoenzymes, and an additional 9 showed conserved substitutions with regard to charge. Thus, although TLCEH is clearly distinct from other isoenzymes in these as well as other properties, there are clear indications that they belong to the same family.

**Kinetic Properties**—Whereas cholesteryl ester hydrolases have substrates that are quite insoluble and products that are both insoluble and surface active, the determination of kinetic properties is problematic. Moreover, instability ruled out kinetic studies with the highly purified TLCEH. The activity of the partially (43-fold) purified enzyme from cation exchange chromatography was nonlinear over a broad range of protein concentrations (Fig. 10). TLCEH exhibited linearity with respect to time for more than 1 h in ASP but lost activity after only 15 min in the cation exchange fractions (not shown), consistent with increasing instability with successive purification steps. Both products, oleate and cholesterol, inhibited activity of the partially purified enzyme 25-40% at 2-3 μM and 80-100% at 10-15 μM (not shown).

**Discussion**

The testicular cytosolic neutral TLCEH has been partially purified in this laboratory by a number of approaches, including gel permeation HPLC (1, 2, 7), cation exchange FPLC (8) (Fig. 1), ammonium sulfate precipitation (Table I), chromatofocusing FPLC (Fig. 2), and various combinations of these methods (Table I). Typical of all of these studies, including the current one, catalytic activity was unstable beyond two to three stages of purification. Durham and Grogan (2) reported 75-fold purification of this enzyme overall, a series of HPLC gel permeation columns with different exclusion ranges; however, additional purification steps destroyed all activity. In the current study, we have purified the enzyme 2,000-fold by sequential ammonium sulfate precipitation, cation exchange FPLC, and chromatofocusing FPLC (Table I). The activity was stabilized in fractions from the chromatofocusing column by the addition of ASP prior to the assay. Although additional purification steps resulted in a total loss of activity, the purified enzyme gave a single silver-stained band following SDS-PAGE (Fig. 3), yielded specific activity similar to that of liver cytosolic CEH (12), and exhibited temperature lability and inactivation by alkaline phosphatase (see “Results”), consistent with the known properties of TLCEH. The molecular mass (66 kDa) determined by SDS-PAGE was consistent with the 72 kDa previously estimated for the monomeric form, using retention times from gel permeation chromatography (2), and similar to the 66 kDa reported for liver CEH (12). Antibodies raised to this protein inhibited total testicular cytosolic CEH activity 70%, consistent with the fraction reported to be temperature-labile (1, 2, 8), further evidence for isolation of the TLCEH. The persistence of temperature lability and inactivation by dephosphorylation of the enzyme through all stages of purification provides confirmation that these properties are characteristic of the enzyme itself rather than contaminating factors. The characteristic temperature lability of TLCEH and its apparent susceptibility to proteolysis during storage (Fig. 6) may be related to its inherent instability, although loss of activity during purification is probably not because of contamination by protease, since there was no evidence of proteolytic degradation during storage of the highly purified enzyme. A similar susceptibility to proteolysis has been reported for a 67-kDa form of pancreatic CEH (19). Similarities to isoenzymes from other tissues, including susceptibility to inhibition by phenylmethylsulfonyl fluoride (4), suggest that TLCEH is a member of the serine esterase supergene family (5).

Although TLCEH is similar in molecular mass and specific activity to liver CEH, its amino acid composition differs from those of both liver and pancreatic CEH (Table II), and its amino-terminal sequence (Fig. 9) differs from those reported for CEH isoenzymes from other organs, which appear to have highly conserved sequences (18, 20-22). Unlike isoenzymes from other tissues (12, 23, 24), TLCEH was shown previously...
to be inhibited by relatively low concentrations of bile salts (2). Moreover, CEH isoenzymes from other tissues are not temperature-labile (2). Thus, TLCEH is unique within this class of enzymes. Inasmuch as antibodies to TLCEH did not cross-react with proteins from liver, pancreas, intestine, adrenal, or serum and antibody to pancreatic CEH did not cross-react with cytosolic proteins from testis (not shown), the isoenzyme appears to be unique to the testis. Moreover, both TLCEH activity and immunoreactive protein are increased with onset of spermatogenesis (Fig. 8), suggesting a specific role for this isoenzyme in spermatogenesis. This view is further supported by our previous report that TLCEH is induced by follicle-stimulating hormone and localized to Sertoli cells (2), which play an essential role in spermatogenesis. Moreover, TLCEH is inhibited by temperature elevation and by phenylmethylsulfonyl fluoride (4), both of which inhibit spermatogenesis, and accumulation of cholesteryl esters accompanies a number of treatments which inhibit spermatogenesis (6). Although Ghosh and Grogan (25) reported immunoreactivity between polyclonal antibodies to liver CEH and a 66-kDa protein in the testis, that protein may represent the temperature-stable component of testicular CEH (1, 2, 8) which is not inhibited by anti-TLCEH (Fig. 7). Cu$^2+$ and Zn$^2+$ were found to be highly inhibitory at 1–3 μM (Fig. 4). Although TLCEH is similar to liver neutral cytosolic CEH in this respect, Deykin and Goodman (15) used much higher concentrations of Cu$^2+$ and Zn$^2+$ (500 μM) to inhibit liver CEH. Moreover, Tanaka reported an IC$_50$ of 200–250 μM for the inhibition of lysosomal CEH by Cu$^2+$ (16) and Zn$^2+$ (17), in sharp contrast to 0.60–0.75 μM for the inhibition of TLCEH (Fig. 4). Inasmuch as 1–3 μM is 1–2 orders of magnitude below normal physiological ranges for these cations and may fall within the range of intracellular unbound Cu$^2+$ and Zn$^2+$ (26, 27), the observed inhibition by these cations may have physiological and possibly regulatory significance.

In contrast, 0.5–5 mM Mn$^2+$ had no effect on the activity of TLCEH (see “Results”). Similarly, Deykin and Goodman (15) found no effect on liver CEH with 0.5 mM Mn$^2+$, Mg$^{2+}$, Ca$^{2+}$, or Co$^{2+}$. However, Tanaka et al. (17) reported the inhibition of lysosomal CEH by Mn$^2+$, with an IC$_50$ of 2.5 mM.

Of the metal ions tested by us, Cu$^2+$ had the most pronounced inhibitory effect, suggesting a mechanism for the previously reported toxic effects of Cd$^2+$ on spermatogenesis. This toxicity is known to be accompanied by the accumulation of cholesteryl esters (6).

Bailey and Grogan (7) reported the inhibition of TLCEH in cytosol by Mg$^{2+}$, apparently because of presence of Mg$^{2+}$-dependent phosphatase activity. Although it is possible that a similar indirect mechanism mediates inhibition by Cu$^2+$ and Zn$^2+$, the inhibitory levels of Mg$^{2+}$ (0.5–10 mM) were 2–3 orders of magnitude higher than those of Cu$^2+$ and Zn$^2+$ used in the current study, and the level of purification of TLCEH was much greater in the current study. Whereas EDTA was reported earlier to stabilize TLCEH in the cytosol (1, 7), it is possible that this stabilizing effect is caused by the chelation of Cu$^2+$ and Zn$^2+$, as well as Mg$^{2+}$.

Factor(s) responsible for the stabilizing effects of ASP were not further characterized, although a wide range of potential stabilizing agents, including albumin, glycerol, sucrose, various thiol reagents, chelators, and metal ions, have been tested in these and previous studies (1, 2, 7). Whereas none of these agents has maintained activity in highly purified TLCEH, the stabilizing effect of ASP may be caused by substrate or product-binding proteins such as sterol carrier protein (28) or fatty acid-binding protein (29, 30), which are known to participate in substrate delivery and product removal in analogous reactions under physiological conditions. Both of these proteins have been found in the testis (28, 30). Moreover, fatty acid-binding protein messenger RNA was reported by others to increase 4.5-fold from days 24–70, in the developing rat (30), coinciding with the increase that we have found in TLCEH (Fig. 8). The absence of these proteins could result in the accumulation of inhibitory products. Lundberg et al. (32), while studying the effect of the substrate physical state on the activity of lysosomal acidic CEH, concluded that any accumulation of free cholesterol in the vicinity of the active site inactivated that enzyme.

Earlier studies have shown that this enzyme has very stringent substrate requirements with respect to the long chain fatty acyl moiety and mode of substrate presentation and that it is very sensitive to detergents (1, 2). The current studies provide further evidence for an unstable, temperature-labile, protein kinase-regulated enzyme that is exquisitely sensitive to environmental factors, including both products, other cytosolic proteins, and certain divalent cations, all of which could play a role in the regulation of intracellular pools of free cholesterol during spermatogenesis.

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