Preparation and Characterization of Monoclonal Antibodies Recognizing Unique Epitopes on Sexually Differentiated Rat Liver Cytochrome P-450 Isozymes†

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ABSTRACT: Cytochrome P-450 isozymes P-450$_{16\alpha}$, P-450$_{15\beta}$, and P-450$_{DEa}$ are immunochemically related, as indicated by mutual cross-reactivity with polyclonal antibody preparations. We have isolated five monoclonal antibodies to P-450$_{15\beta}$ and one antibody to P-450$_{16\alpha}$ that show selectivity for the respective antigens. High frequencies of cross-reactivity were observed, indicating a high degree of homology among P-450$_{16\alpha}$, P-450$_{15\beta}$, and P-450$_{DEa}$. All of the P-450$_{15\beta}$-specific antibodies bound to the same epitope, or closely grouped epitopes, supporting this conclusion. The specificity of each monoclonal antibody was characterized by enzyme-linked immunosorbent assay, Western immunoblotting, and antibody–Sepharose immunoadsorption of solubilized rat liver microsomes. Antibodies F22 and F23, which were apparently identical, were specific for P-450$_{15\beta}$ by these criteria. However, the apparent specificities of antibodies F3 and F20 for P-450$_{15\beta}$, and of M16 for P-450$_{16\alpha}$, were highly dependent on the analytical technique used. The five anti-P-450$_{15\beta}$ antibodies all inhibited the catalytic activity of microsomal P-450$_{15\beta}$ by a maximum of 70%. However, they also produced a similar inhibition of microsomal P-450$_{16\alpha}$ activity, indicating that even F22 and F23 have a low-affinity interaction with an epitope on P-450$_{16\alpha}$. The P-450$_{16\alpha}$-specific antibody M16 was not inhibitory. The results indicate that the apparent specificity of a monoclonal antibody for an antigen determined by, e.g., Western blotting does not allow the conclusive identification of a protein in another system, e.g., immunoprecipitation of in vitro translation reaction products. The specificities of such antibodies should be characterized under the precise conditions in which they are to be used.

The cytochrome P-450$_{16\alpha}$ and P-450$_{15\beta}$[1] isozymes of rat liver are expressed in a sexually differentiated manner (Kamataki et al., 1983; MacGeoch et al., 1984, 1985; Morgan et al., 1985a,b; Ryan et al., 1984a; Waxman, 1984). The male-specific P-450$_{16\alpha}$[2] is responsible for the steroid 16α- and 2α-hydroxylase activities of male rate liver (Morgan et al., 1985a,b; Ryan et al., 1984a; Waxman, 1984) and also catalyzes the metabolism of several drug substrates. P-450$_{15\beta}$ is a female-specific isozyme which catalyzes the 15β-hydroxylation of steroid sulfates, a reaction specific to microsomes from female rats (MacGeoch et al., 1984, 1985; Ryan et al., 1984b).

Peptide mapping studies (Waxman, 1984), NH_2-terminal analysis (Haniu et al., 1984), and immunochemical evidence (Morgan et al., 1985a; MacGeoch et al., 1984; Waxman, 1984; Bandiera et al., 1985) indicate that P-450$_{16\alpha}$ and P-450$_{15\beta}$ are highly related proteins, although differences in structure can be distinguished by the aforementioned techniques. Rabbit antibodies prepared in this laboratory to the two isozymes cross-reacted with the heterologous antigens, and also with another isozyme, P-450$_{DEa}$, purified from female rat liver (Morgan et al., 1985a; MacGeoch et al., 1984), but apparently not with any other P-450 isozyme. Immunoabsorption of the polyclonal antibodies with crude fractions of P-450 from animals of the opposite sex conferred specificity to the antibodies, such that they were monospecific in a Western blod immunoassay (MacGeoch et al., 1984, 1985; Morgan et al., 1985a,b). The antibodies were used with this assay to demonstrate that levels of the specific P-450$_{16\alpha}$ and P-450$_{15\beta}$ apoproteins are regulated reciprocally by growth hormone (MacGeoch et al., 1985; Morgan et al., 1985b). However, the immunoabsorbed antibodies were of extremely low titer, and we have since found that they are only monospecific under the conditions used for the Western blot assay, limiting their usefulness.[3] Furthermore, preparation of such antibodies consumes great amounts of antiserum, purified P-450, and time and yields only limited amounts of useful reagent.

The ability of monoclonal antibodies to recognize a single epitope on a protein provides the possibility of developing antibodies which will recognize unique sites on otherwise highly similar proteins. This approach has been used successfully for other groups of related P-450 isozymes which cross-react with conventional antisera, e.g., rat isozymes P-450c and P-450d (Thomas et al., 1984), P-450b and P-450e (Reik et al., 1985), and rabbit isozymes 1 and 3b (Reubi et al., 1984).

We present here the preparation and characterization of monoclonal antibodies recognizing unique epitopes on P-450$_{16\alpha}$ and P-450$_{15\beta}$. Differential ELISA screening was used to eliminate hybridoma colonies producing cross-reacting antibodies.

Experimental Procedures

Preparation of Microsomes. Pyrophosphate-extracted microsomes were prepared from livers of sexually mature male

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[1] Cytochrome P-450$_{16\alpha}$ is identical with the following isozymes purified in other laboratories: RLM$_5$ (Cheng & Schenkman, 1982), UT-A (Guengerich et al., 1982), 2c (Waxman, 1984), h (Ryan et al., 1984a), and male (Kamataki et al., 1983). P-450$_{15\beta}$ is equivalent to isozymes P-4502d (Waxman, 1984), i (Ryan et al., 1984a,b), female (Kamataki et al., 1983), and UT-I (Waxman et al., 1985) purified in other laboratories.

[2] Abbreviations: P-450, cytochrome P-450; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline (10 mM potassium phosphate buffer, pH 7.4, and 0.15 M KCl); BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Mab, monoclonal antibody.

[3] E. T. Morgan and J.-Å. Gustafsson, unpublished observations.

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or female Sprague-Dawley rats as described previously (Morgan et al., 1985a) and stored at −80 °C.

Preparation of Antigens. Highly purified P-450$_{16\alpha}$, P-450$_{15\beta}$, and P-450$_{DEa}$ were prepared as described previously (Morgan et al., 1985a; MacGeoch et al., 1984) and were homogeneous on SDS–polyacrylamide gel electrophoresis. P-450 PBB$_2$ was a generous gift from Dr. James Halpert, University of Arizona. "Female mix" antigen (see below) was the fraction eluted by 60 mM phosphate from the CM-Sepharose column described in the purification of P-450$_{15\beta}$ and P-450$_{DEa}$ (MacGeoch et al., 1984). The fraction contains these two isozymes as the major components, in approximately equal quantities. Female mix antigen was used to immunize mice for preparation of antibodies to P-450$_{15\beta}$, whereas purified P-450$_{16\alpha}$ was used to immunize mice for preparation of antibodies to itself.

Immunization of Mice. Several female BALB/C mice were each injected with 40 μg of P-450$_{16\alpha}$ or female mix antigen in 0.15 mL of a 1:1 emulsion of Freund's complete adjuvant containing 10^9 deactivated *Bordetella pertussis* cells; subcutaneous booster injections containing 20 μg of antigen in 0.15 mL of an emulsion of Freund's incomplete adjuvant were also given 4 and 8 weeks later. The sera of the mice were tested subsequently by ELISA for antibodies to the injected antigen. Those mice with the highest serum titers were selected by hybridoma preparation. Fusions were performed 1–6 months after the second booster injection. The mice received 20 μg of antigen intraperitoneally in PBS on each of the 3 days preceding the fusion.

ELISA. A direct ELISA (Engvall & Perlmann, 1972) was used to detect antibodies to P-450 isozymes in mouse serum and hybridoma supernatants and to characterize purified monoclonal antibodies. Microtiter plates were coated by incubation with 40 μL of a 500 ng/mL solution of female mix antigen. After blocking of nonspecific protein absorption sites with PBS containing 2% BSA, plates were incubated with the test solution diluted (where necessary) in PBS containing 0.05% Tween 20. The wells were then washed with PBS–Tween 20 and incubated with a 1 to 50 dilution of horseradish peroxidase conjugated rabbit antibodies to mouse immunoglobulins in PBS–Tween 20 and 0.5% BSA. After being washed, the antigen–antibody complex was visualized by incubation with 4 mM phenylenediamine and 2.7 mM hydrogen peroxide, and the absorbance at 492 nm was measured on a Titertek Multiscan apparatus.

Hybridoma Preparation and Screening of Clones. Fusion of BALB/c spleen cells and mouse myeloma Sp 2/0-Ag 14 cells (Shulman et al., 1978) using poly(ethylene glycol) 4000 was achieved essentially as described in this laboratory for the preparation of monoclonal antibodies to the glucocorticoid receptor (Okret et al., 1984), except that RPMI 1640 medium was substituted for Dulbecco's modified Eagle's medium. All culture media contained 8% fetal calf serum. Fusion was carried out at a spleen:myeloma cell ratio of approximately 1:1, in 1 mL of 50% (w/v) poly(ethylene glycol) 4000. The cells were then plated in microtiter wells in 0.1 mL of HAT medium (Okret et al., 1984) at a density of 5×10^5 cells/well, giving 300–400 wells per fusion.

Microtiter wells were examined for growing hybridoma clones twice weekly from the seventh day after fusion until no new clones were observed. Supernatants of clones approaching confluence were tested by ELISA for antibodies to the injected antigen (P-450$_{16\alpha}$ or female mix), and those showing a positive response were immediately tested for cross-reactivity against the heterologous antigen. Those wells giving at least a 5-fold stronger signal with the injected antigen vs. heterologous antigen were selected for cloning and further characterization.

Cloning and Production of Antibodies. Monoclonal, antibody-producing hybridomas were obtained by cloning cells twice or more at limiting dilution on a feeder layer of BALB/c macrophages (Okret et al., 1984), obtained by peritoneal lavage.

Large-scale production of antibodies was achieved by growing hybridoma clones in culture or as ascites in BALB/c mice. In the latter case, mice were injected with 3×10^5 cells, 3–10 days after an intraperitoneal injection of 0.1 mL of Freund's incomplete adjuvant–PBS emulsion (1:1). Immunoglobulins were precipitated from ascites fluid with 50% saturated ammonium sulfate and dialyzed in PBS. Immunoglobulins from hybridoma culture supernatants or ascites fluid were purified on a 1.5 × 3 cm protein A–Sepharose column. The crude fractions were applied to the column in PBS or culture medium at pH 8.3, and the purified immunoglobulins eluted in 0.1 M sodium citrate buffer, pH 3.0. The purified preparations were dialyzed in 100 volumes of PBS and stored at −20 °C.

Antibody F1, which did not bind to the protein A column, was purified on a column of rabbit anti-mouse immunoglobulins coupled to CNBr-activated Sepharose 4B according to the Pharmacia instruction manual. The rabbit anti-mouse immunoglobulins were first purified by passing them over a column of fetal calf serum proteins coupled to Sepharose 4B. Mab F1 was applied to the rabbit anti-mouse Ig column in culture medium or PBS at pH 7.4 and eluted with 0.1 M sodium citrate buffer, pH 3.0.

Isoelectric Focusing. Isoelectric focusing of the monoclonal antibodies and detection of focused peptides with horseradish peroxidase coupled protein A were performed as described previously (Okret et al., 1984).

Test for Antibody Class. The class and subclass of the heavy chain of each monoclonal antibody were determined by the Ouchterlony immunodiffusion technique, using specific antisera to mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA.

Immunoblotting. Proteins were separated by electrophoresis on SDS–polyacrylamide slab gels (Laemmli, 1970) containing 7% polyacrylamide and blotted onto 0.22-μm nitrocellulose filters (Towbin et al., 1979). The filters were incubated in PBS containing 0.05% Tween 20 and 1% BSA for 1 h before addition of monoclonal antibody to the solution. After incubation for 2 h, the filters were washed 3 times with PBS–Tween 20 and then incubated with a 1:200 dilution of horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins. The filters were washed a further 3 times in PBS–Tween 20, and the bound antibody was visualized by incubation with 2.7 mM 4-chloro-1-naphthol and 4.5 mM H_2O_2 in PBS containing 17% methanol.

Epitope Mapping. Competition of unlabeled monoclonal antibodies with labeled antibodies for binding to antigen was determined by ELISA, essentially as described by Thomas et al. (1984) except that the antibodies were labeled with biotin and detected with horseradish peroxidase–avidin (Guesdom et al., 1979). Biotinylation was performed at an antibody concentration of 1 mg/mL in 0.1 M $NaHCO_3$, pH 9.0; 0.12 volume of a 1 mg/mL solution of biotinyl-*N*-hydroxysuccinmide ester in dimethyl sulfoxide was added, and incubation proceeded for 4 h at 22 °C. The samples were then dialyzed in 200 volumes of PBS and stored at 4 °C.

ELISA plates were coated with 20 ng of the appropriate antigen and blocked with 2% BSA. After the plates were washed, unlabeled antibody (100 μg/mL) in PBS containing

0.5% BSA, or buffer alone, was added and the plate incubated for 16 h at 4 °C. The labeled antibody was added to this solution at an appropriate dilution and incubated for 2 h at 37 °C. The plate was washed, and a 1:500 dilution of avidin–horseradish peroxidase was added. After incubation for 1 h at 37 °C and washing, the plate was developed with phenylenediamine as described above.

Immunoadsorption. Monoclonal antibodies were coupled to cyanogen bromide activated Sepharose 4B at 5 mg of antibody/g of gel according to the manufacturer's instructions. Immunoadsorption of proteins from solubilized rat liver microsomes, washing, and elution of the gel were performed essentially as described by Reubi et al. (1984). Briefly, antibody–Sepharose was incubated overnight with solubilized rat liver microsomes at 4 °C. The gel was then washed 5 times with buffer containing nonionic detergent and twice with water before elution with buffer containing 2% SDS. The samples were electrophoresed on an SDS–7.5% polyacrylamide slab gel (Laemmli, 1970) and silver stained (Wray et al., 1981).

Enzyme Assays and Antibody Inhibition. Microsomal metabolism of testosterone was assayed as described previously (Morgan et al., 1985a), at a P-450 concentration of 0.05 μM. 15β-Hydroxylation of 5α-[^{3}H]androstane-3α,17β-diol 3,17-disulfate was measured in incubations containing 0.25 nmol of microsomal P-450, 2 μmol of NADPH, 6 μmol of sodium isocitrate, 15 nmol of $MnCl_2$, and 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 2 mL. Extraction and quantitation of the 15β-hydroxy metabolite were performed as described (MacGeoch et al., 1984). For determination of the effects of antibodies on the catalytic activity of microsomal P-450 isozymes, microsomes were preincubated with the antibody, or buffer only, in 0.25 mL of PBS for 30 min at room temperature before the addition of the other incubation components.

Materials. CNBr-activated Sepharose 4B, protein A, and protein A–Sepharose CL-4B were purchased from Pharmacia, Uppsala, Sweden. Freund's complete and incomplete adjuvants were from Difco, Detroit, MI. Rabbit antibodies to mouse immunoglobulins and horseradish peroxidase conjugated rabbit anti-mouse antibodies were from Dakopatts, Glostrup, Denmark. Rabbit antibodies to mouse IgG3 were from Litton Bionetics, and goat antibodies to other mouse Ig subclasses were from Meloy Laboratories, Springfield, VA. Horseradish peroxidase conjugated avidin was obtained from Vector Laboratories, Burlingame, CA, and *Bordetella pertussis* vaccine (Per/Vac) was from Wellcome, London, England. RPMI 1640 culture medium and microtiter plates for ELISA were from Flow Laboratories, Irvine, Scotland, and microwell culture plates were from Nunc, Roskilde, Denmark. Horseradish peroxidase and BSA (fraction V) were obtained from Sigma, and Tween 20 was from Kebo AB, Stockholm, Sweden. Phenylenediamine hydrochloride was from Fluka AB, Buchs, Switzerland, poly(ethylene glycol) 4000 (for gas chromatography) from Merck AG, Darmstadt, West Germany, 4-chloro-1-naphthol from Ega Chemie, Steinheim, West Germany, and biotinyl-*N*-hydroxysuccinimide ester from E. Y. Laboratories, San Mateo, CA. 5α-[^{3}H]Androstan-3α,17β-diol 3,17-disulfate and the unlabeled compound were synthesised as described previously (MacGeoch et al., 1984; Mumma et al., 1969).

RESULTS

Double-Antigen Screening of Hybridoma Colonies. Of 456 microwells resulting from a single fusion of spleen cells from a mouse immunized with female mix antigen, 60 produced antibodies which recognized the female mix antigen. Only five of these antibodies did not cross-react with P-450$_{16\alpha}$ in ELISA, and these all showed specificity for P-450$_{15\beta}$ (see below). No specific antibodies for P-450$_{DEa}$ were found.

Table I: Properties of Mabs to Sex-Specific P-450 Isozymes[a]

Mab	antibody class	binding protein A[b]	cross-reactivity with heterologous antigen[c]
F1	IgA	–	–
F3	IgG1	+	–
F10	IgG1	+	+
F13	IgG1	+	+
F20	IgG1	+	–
F22	IgG1	+	–
F23	IgG1	+	–
M3	IgG1	+	+
M7	IgG1	+	+
M8	IgM	–	+
M12	IgG1	+	+
M14	IgM	–	+
M16	IgG1	+	–

[a] The prefixes M and F designate Mabs derived from the spleens of mice immunized with P-450$_{16\alpha}$ and female mix antigen, respectively. [b] Retention on protein A–Sepharose 4B. [c] Cross-reactivity is defined here as less than 5-fold difference in ELISA absorbance using supernatants from original microwell plates.

Hybridoma clones producing specific antibodies to P-450$_{16\alpha}$ were even less frequent. Of 1224 microwells generated from 3 independent fusions of spleen cells from P-450$_{16\alpha}$-injected mice, 161 gave a positive signal with P-450$_{16\alpha}$ in ELISA. However, only three of these positive wells showed selectivity for P-450$_{16\alpha}$ in ELISA (i.e., at least a 5-fold higher absorbance), and two of the three did not maintain this specificity after cloning and recloning.

Properties of Specific Mabs. The five hybridomas producing antibodies with specificity for P-450$_{15\beta}$ and the single hybridoma producing antibody with apparent specificity for P-450$_{16\alpha}$ were cloned twice by limiting dilution, and the resulting Mabs were purified from culture supernatants or ascites fluid by chromatography on protein A–Sepharose or rabbit anti-mouse Ig–Sepharose. Several cross-reacting Mabs were also purified in this manner. The properties of the resulting Mabs are shown in Table I. The Mabs specific for P-450$_{15\beta}$ are F1, F3, F20, F22, and F23, while that showing specificity for P-450$_{16\alpha}$ is M16. All of the IgG1 antibodies were retained on the protein A–Sepharose column, while the IgA and IgM Mabs were not. No IgG2b or IgG3 antibodies were found.

The Mabs showing apparent specificity for P-450$_{15\beta}$ or P-450$_{16\alpha}$ were tested for cross-reactivity against other P-450 isozymes under a variety of conditions, as described below. However, before this was done, the anti-P-450$_{15\beta}$ Mabs were subjected to isoelectric focusing and staining with horseradish peroxidase conjugated protein A, to check that they were nonidentical. The banding patterns of Mabs F1, F3, F20, and F22 were clearly different (results not shown), indicating that they are nonidentical antibodies. However, the patterns generated by Mabs F22 and F23 were indistinguishable, suggesting that the cells producing them may be derived from a single spleen B cell which divided in vivo. This result notwithstanding, both antibodies were subjected to individual characterization.

Specificity in ELISA. The specificities of the Mabs were tested by ELISA, in which highly purified P-450 isozyme preparations were adsorbed to the microtiter plates. Figure 1 shows that Mabs F20, F22, and F23 showed a high degree of specificity for P-450$_{15\beta}$, with no detectable cross-reactivity with P-450$_{16\alpha}$, P-450$_{DEa}$, or P-450 PBB$_2$, the phenobarbital-inducible isozyme. In contrast, F1 showed considerable

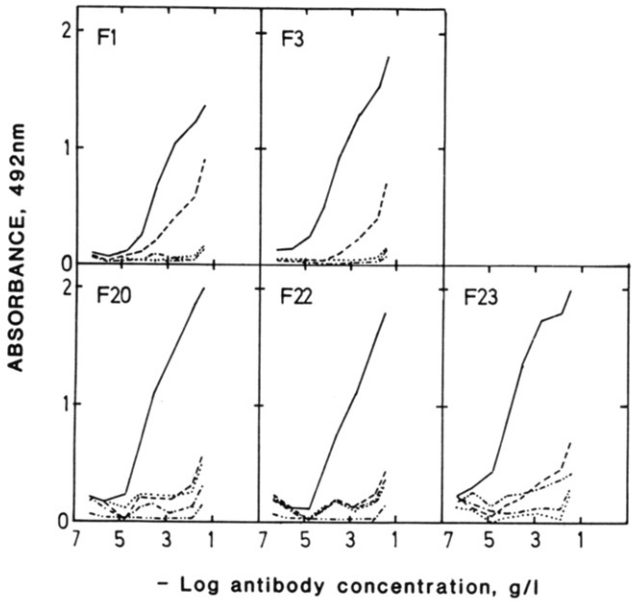

FIGURE 1: Specificity of anti-P-$450_{15\beta}$ Mabs in ELISA. Microtiter wells were coated with 40 μL of a 500 ng/mL solution of the indicated P-450 isozyme, and binding the Mabs purified from hybridoma culture supernatants was measured as described under Experimental Procedures. The Mab protein concentration was determined from the absorbance at 280 nm. The *curves* are labeled as indicated: (—) P-$450_{15\beta}$; (- - -) P-$450_{16\alpha}$; (···) P-450_{DEa}; (-·-) P-450 PBB_2; (-··-) P-$450_{15\beta}$ incubated with normal mouse serum. The immunoglobulin content of the serum was assumed to be 10 mg/mL.

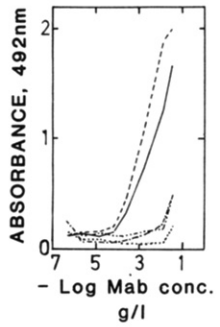

FIGURE 2: Specificity of Mab M16 in ELISA. Microtiter wells were coated with 20 ng of each P-450 isozyme. The *curves* are labeled as indicated in Figure 1: (—) P-$450_{15\beta}$; (- - -) P-$450_{16\alpha}$; (···) P-450_{DEa}; (-·-) P-450 PBB_2; (-··-) P-$450_{16\alpha}$ incubated with normal mouse serum.

cross-reactivity with P-$450_{16\alpha}$ at higher antibody concentrations, while Mab F3 showed a similar, but weaker cross-reactivity. Neither F1 nor F3 cross-reacted with P-450_{DEa} or P-450PBB_2 in the ELISA.

When the specificity of purified Mab M16 was tested in ELISA, significant cross-reactivity with P-$450_{15\beta}$ was observed (Figure 2). This was surprising in view of the previously observed, much lower reactivity of the M16 microculture supernatants with the crude, female mix antigen. In experiments not shown, we verified that (a) purified Mab M16 gave higher cross-reactivity with purified P-$450_{15\beta}$ (63%) than did the crude culture supernatant (46%) and (b) this magnitude of cross-reactivity with P-$450_{15\beta}$ was observed under conditions where neither M16 culture supernatant nor the purified Mab gave a significant signal with twice the amount of female mix antigen.

In no case did prior denaturation of the antigens by boiling in 2% SDS significantly affect the selectivities of the antibodies.

Specificity in Immunoblotting. Western immunoblotting is a method which is now being widely used to characterize

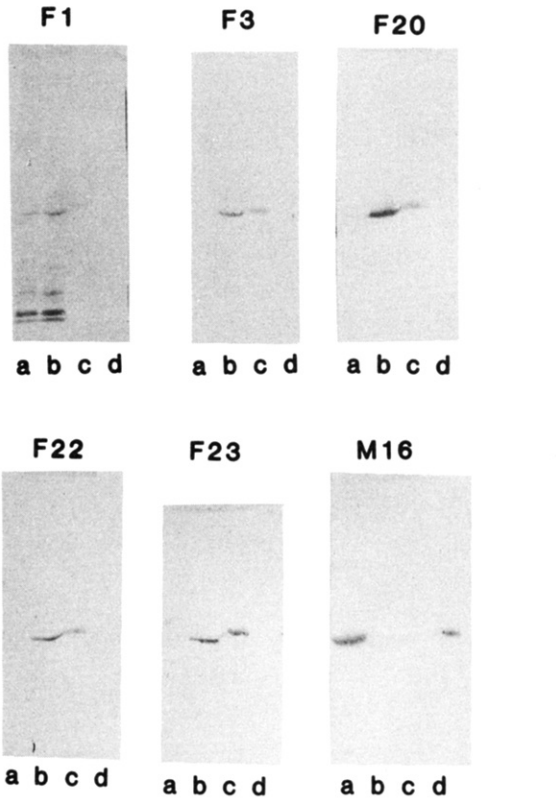

FIGURE 3: Specificity of anti-P-$450_{16\alpha}$ and anti-P-$450_{15\beta}$ Mabs in Western immunoblots. (a) Male rat liver microsomes, (b) female rat liver microsomes, (c) P-$450_{15\beta}$, and (d) P-$450_{16\alpha}$ were electrophoresed on SDS–7% polyacrylamide slab gels and blotted on nitrocellulose filters. The filters were incubated with the indicated Mabs at the following Ig concentrations and visualized as described in the text: F1, 50 mg/L; F3, 10 mg/L; F20, 5 Mg/L; F22, 5 mg/L; F23, 10 mg/L; M16, 5 mg/L. Each lane contained 1 μg of purified P-450 or 40 μg of microsomes, except for the M16 blot which received only 20 μg of microsomes.

antibody specificities. Therefore, we tested the specificities of our Mabs in this assay, in which the antigens are denatured by electrophoresis in the presence of SDS and immobilized on a hydrophobic nitrocellulose membrane. Figure 3 shows that Mabs F3, F20, F22, and F23 recognized purified P-$450_{15\beta}$ but not P-$450_{16\alpha}$ under these conditions. They also recognized P-$450_{15\beta}$ in female rat liver microsomes, whereas no bands were detected in microsomes from male rats, except in the case of F3, where a very faint band could be discerned which was of slightly higher mobility than P-$450_{15\beta}$. Mab F3 also recognized an additional (faint) band in female rat liver microsomes, whose relative mobility suggests that it may be identical with P-450_{DEa}. Mab F1 gave only faint signals on Western blots and recognized many bands in both male and female microsomes, probably due to the high antibody concentration that had to be used.

Mab M16 recognized P-$450_{16\alpha}$ with a high degree of specificity in the Western immunoblot procedure. No cross-reactivity with purified P-$450_{15\beta}$ was observed, and a single protein band was recognized in microsomes from male rats but not from females.

In subsequent experiments (not shown), we established that the different electrophoretic mobilities of purified P-$450_{16\alpha}$ and P-$450_{15\beta}$ vs. those of the microsomal proteins were due to the higher amounts of protein applied in the microsomal sample wells. Thus, for example, if purified P-$450_{15\beta}$ was mixed with male rate liver microsomes, the band detected with Mab F23 was of mobility identical with that of the band detected in microsomes from female rats.

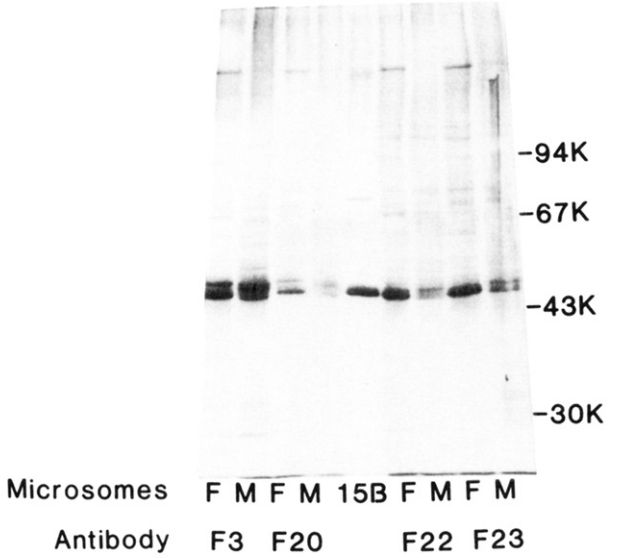

FIGURE 4: Monoclonal antibody directed immunoadsorption of solubilized rat liver microsomes. Three milligrams of solubilized microsomes from female (F) or male (M) rat liver was incubated with approximately 45 μg of the indicated Mab coupled to Sepharose 4B. After the gel was washed and adsorbed proteins were eluted as described under Experimental Procedures, 30% of the eluted products were electrophoresed on an SDS–polyacrylamide gel and silver stained. One microgram of P-450$_{15\beta}$ was applied directly to the gel without immunoadsorption.

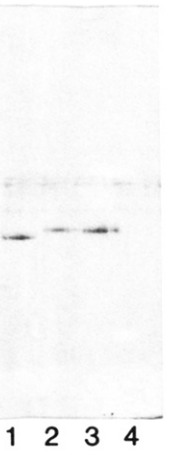

FIGURE 5: Mab M16 directed immunoadsorption of solubilized rat liver microsomes. Three milligrams of solubilized microsomes from male (lane 3) or female (lane 4) rat liver was incubated with 135 μg of Mab M16 coupled to Sepharose 4B, and the gel was washed and eluted as described. 45% of the eluted volume was applied to an SDS–7% polyacrylamide gel, electrophoresed, and silver stained. One microgram of P-450$_{15\beta}$ (lane 1) and P-450$_{16\alpha}$ (lane 2) was electrophoresed without immunoadsorption.

Specificity as Immunosorbents. The ability of the Mabs to serve as immunosorbents when coupled to Sepharose 4B was examined. Figure 4 shows an electrophoretogram of the proteins adsorbed by F3–, F20–, F22–, and F23–Sepharose after incubation with solubilized male or female rat liver microsomes. Mabs F22 and F23 bound a female microsomal protein of identical molecular weight with P-450$_{15\beta}$, and this was the major absorbed protein. The same Mabs adsorbed much smaller quantities of two other proteins from male rat liver microsomes. Mab F3 bound two major proteins in microsomes from female rats, which are assumed to be P-450$_{15\beta}$ and P-450$_{DEa}$. Mab F3 also bound two major proteins from male rat liver microsomes. On the basis of the known immunochemical relatedness of the antigens, we may speculate that the proteins adsorbed from male rat liver are P-450$_{16\alpha}$ and P-450g (Ryan et al., 1984; Bandiera et al., 1985), respectively. Mab F20–Sepharose gave similar results to F3–Sepharose, except that the interaction with Mab F20 appeared to be weaker, and more specific for the female proteins. We were unable to detect any binding of microsomal proteins to two different batches of Mab F1–Sepharose.

The immunosorbent properties of Mab M16–Sepharose are shown in Figure 5. A single protein band with molecular weight identical with that of P-450$_{16\alpha}$ was eluted from the gel incubated with microsomes from male rat liver. There was no detectable protein adsorbed from female rat liver microsomes. Thus, this adsorbent appears to be specific for P-450$_{16\alpha}$.

Epitope Mapping. Thomas et al. (1984) have used a competitive ELISA with labeled Mabs to demonstrate binding of different Mabs for P-450 c and d to spatially distinct epitopes. We used this approach to examine the binding of anti-P-450$_{15\beta}$ Mabs to the antigen. The results are shown in Table II, where the abilities of excess unlabeled Mabs to block the binding of biotin-labeled Mab are expressed as a decrease in the ELISA absorbance. If we assume that an 80% change in binding indicates binding of two Mabs to the same epitope or epitopes which are grouped sufficiently closely to cause mutual steric hindrance (Thomas et al., 1984), Table II indicates that Mabs

Table II: Mapping of Monoclonal Antibody Binding Sites on P-450$_{15\beta}$ by Competition in ELISA[a]

	% of control absorbance for biotinylated Mab				
competing antibody	F1	F3	F20	F22	F23
F1	0	16	6	0	0
F3	0	2	0	0	0
F10	43	76	69	10	21
F13	29	81	78	20	47
F20	0	4	1	0	0
F22	3	16	22	0	0
F23	7	18	14	0	0
M16	63	76	67	62	47

[a] Inhibition of biotinylated Mab binding to P-450$_{15\beta}$ by prior incubation with unlabeled Mabs was measured by ELISA as described under Experimental Procedures. The dilution of each biotinylated Mab was chosen to give approximately 50% of the maximum absorbance in ELISA, in the absence of competing antibody.

F1, F3, F20, F22, and F23 all bind to the same, or closely grouped, epitopes on P-450$_{15\beta}$, since all five Mabs block each other's binding. In contrast, antibodies F10 and F13 did not block the binding of F1, F3, and F20, although they did inhibit binding of F22 and F23 to some extent. Mab M16 did not affect the binding of any of the labeled anti-P-450$_{15\beta}$ Mabs.

Effects on Sex-Specific Enzyme Activities. Anti-P-450$_{15\beta}$ Mabs F1, F3, F20, F22, and F23 all inhibited the female-specific steroid sulfate 15β-hydroxylase activity of rat liver microsomes (Table III). Mabs F22 and F23 were the most potent inhibitors. F22 produced maximum (68%) inhibition at a Mab:microsomal P-450 molar ratio of 0.3. Mabs F1, F3, and F20 gave a similar magnitude of inhibition but required higher Mab:P-450 ratios to do so. On the basis of titration curves drawn from these data (not shown), the inhibitions of 71% and 32% produced by antibodies F23 and F3 appear to be near-maximal, while F1 and F20 might produce more potent inhibition at higher concentrations of antibody. P-450$_{15\beta}$ is the only P-450 isozyme known to catalyze 15β-hydroxylation of 5α-[^{3}H]androstane-3α,17β-diol 3,17-disulfate, and the strong inhibition of this activity by the anti-P-450$_{15\beta}$ Mabs supports our contention that P-450$_{15\beta}$ is largely, if not solely, responsible for this sex-specific microsomal enzyme activity (MacGeoch et al., 1984, 1985). In contrast, the anti-P-450$_{16\beta}$ Mab M16, or antibodies purified from ascites fluid of mice

Table III: Mab Inhibition of Microsomal Steroid Sulfate 15β-Hydroxylation[a]

antibody	Mab:P-450[b] molar ratio	% of control activity[c]
SP2/0	0.9	82
	3.1	82
F1	0.9	80
	3.1	50
F3	0.3	50
	0.9	37
	3.1	29
F20	0.3	68
	0.9	46
	3.1	30
F22	0.3	32
	0.9	38
	3.1	36
F23	0.9	38
	3.1	32
M16	0.9	94
	3.1	83

[a] 15β-Hydroxylation of 5α-[^{3}H]androstane-3α,17β-diol 3,17-disulfate by female rat liver microsomes was assayed as described in the text. [b] Assuming a molecular weight of 160 000 for each Mab. [c] The control 15β-hydroxylase activity was 0.22 nmol nmol^{-1} min^{-1} in the absence of added antibody.

injected with SP2/0-Ag 14 Myeloma cells, gave no significant inhibition (Table III).

The abilities of our Mabs to inhibit the male-specific microsomal 16α-hydroxylation of testosterone were tested. P-450$_{16\alpha}$ is the major isozyme responsible for this microsomal activity (Morgan et al., 1985a,b; Waxman, 1984). Table IV shows that while the anti-P-450$_{16\alpha}$ Mab M16 had no effect, anti-P-450$_{15\beta}$ Mabs F1, F3, F20, F22, and F23 could inhibit 16α-hydroxylase activity by up to 70%. Antibodies F1, F20, F22, and F23 required higher Mab:P-450 ratios to inhibit microsomal 16α-hydroxylase activity than to inhibit 15β-hydroxylase activity (cf. Tables III and IV), whereas F3 was equipotent in both systems. The inhibition of microsomal 16α-hydroxylase activities by these Mabs was a specific effect, since no inhibition, or even stimulation, of testosterone 6β-hydroxylation was observed (Table IV).

The fact that neither 15β- nor 16α-hydroxylase activity was fully inhibited by any of the Mabs might reflect the contribution of other isozymes to these activities or that the Mabs are not inherently fully inhibitory. In the case of P-450$_{16\alpha}$, the former possibility is unlikely, since immunoadsorbed polyclonal antibodies to P-450$_{16\alpha}$ can completely inhibit the microsomal activity (Waxman, 1984). In the case of P-450$_{15\beta}$, this question will be resolved by measuring Mab inhibition of the purified isozyme.

Table IV: Mab Inhibition of Microsomal Testosterone 16α-Hydroxylation[a]

antibody	Mab:P-450[b] molar ratio	% of control activity[c]	
		16α-hydroxylase	6β-hydroxylase
SP2/0	3.1	112	114
	9.4	93	114
F1	0.3	105	104
	0.9	103	110
	3.1	69	148
	9.4	30	135
F3	0.3	56	104
	0.9	39	101
	3.1	25	101
	9.4	31	111
F20	0.3	86	99
	0.9	65	103
	3.1	38	105
	9.4	29	101
F22	0.3	75	96
	0.9	60	99
	3.1	32	97
	9.4	40	120
F23	0.3	80	120
	0.9	62	107
	3.1	46	117
M16	0.3	108	93
	0.9	97	99
	3.1	109	109
	9.4	106	115

[a] Testosterone hydroxylase activities of male rat liver microsomes were determined as described in the text. [b] See Table III. [c] The control 16α- and 6β-hydroxylase activities were 1.33 and 2.07 nmol nmol^{-1} min^{-1}, respectively, in the absence of added antibody.

Discussion

We have prepared five Mabs to P-450$_{15\beta}$ and one Mab to P-450$_{16\alpha}$, all of which show a degree of specificity for the antigen in question. Hybridoma clones producing the specific antibodies were identified and distinguished from clones producing cross-reacting antibodies by a double-antigen screening method. By testing positive clones for cross-reactivity with heterologous antigen at an early stage, we eliminated the needless cloning of large numbers of positive colonies, only a few of which would produce specific antibodies.

As noted in the introduction, we and others have accumulated considerable evidence to suggest that P-450$_{15\beta}$ and P-450$_{16\alpha}$ share structural homology with each other and with other constitutive P-450 isozymes (Morgan et al., 1985; MacGeoch et al., 1984; Waxman, 1984; Bandiera et al., 1985). Antisera to P-450f and P-450g cross-react with P-450$_{15\beta}$ and P-450$_{16\alpha}$ (Bandiera et al., 1985), and antisera to P-450$_{15\beta}$ and P-450$_{16\alpha}$ cross-react with each other and with P-450$_{DEa}$ (Morgan et al., 1985a; MacGeoch et al., 1984; Waxman, 1984). A high degree of structural homology of the latter three isozymes is supported further by our observations here of high frequencies of cross-reacting antibody-producing hybridomas, and by the fact that even some of the (relatively) specific Mabs show a degree of cross-reactivity.

Our results indicate the importance of analyzing the properties of Mabs under different conditions. Thus, although Mabs F22 and F23 were highly specific for P-450$_{15\beta}$ in ELISA, Western blotting, and immunoadsorption, they strongly inhibited the catalytic activity of P-450$_{16\alpha}$ in male rat liver microsomes, as did Mabs F1, F3, and F20. These other Mabs also showed variable specificity depending on the analytical technique used. Mab F20 showed no significant cross-reactivity with P-450$_{16\alpha}$, P-450$_{DEa}$, or P-450 PBB$_2$ in ELISA and recognized a single protein band corresponding to P-450$_{15\beta}$ in Western blots. However, immunoadsorption of female rat liver microsomes with F20–Sepharose yielded another major protein component in addition to P-450$_{15\beta}$, probably P-450$_{DEa}$. Mab F3 also gave varying specificity, depending on the assay system. Although Mab F3–Sepharose clearly adsorbed both P-450$_{15\beta}$ and P-450$_{DEa}$ from female rat liver microsomes, and two other proteins from male rat liver microsomes, only very weak cross-reactivity with these antigens was seen using the Western blot method. Furthermore, although F3 cross-reacted significantly with P-450$_{16\alpha}$ in ELISA, no cross-reactivity with P-450$_{DEa}$ was observed. Lastly, despite strong cross-reactivity with P-450$_{15\beta}$ in ELISA, Mab M16 was highly specific for P-450$_{16\alpha}$ in both the Western blot and immunoadsorption procedures.

The above discrepancies indicate strongly that demonstration of Mab specificity in a given assay system is not sufficient evidence to conclude that the antibody will specifically recognize the antigen under a different set of experimental conditions. Thus, in our opinion, the fact that a Mab specifically

recognizes an antigen on Western blots, for example, does not allow the conclusive identification of a protein product precipitated from an in vitro translation reaction by the same Mab. This may also be true of polyclonal antibody preparations.

We have not characterized the cross-reactivities of our Mabs with all known P-450 isozymes, since they are not available in our laboratory. In particular, it would be useful to know whether any of them recognize P-450f (Bandiera et al., 1985) or PB-I (Waxman, 1984). Polyclonal antibodies to P-450f recognize both P-$450_{16\alpha}$ and P-$450_{15\beta}$, as well as P-450g. Antibodies to P-450PB-I also recognize P-$450_{16\alpha}$ and P-$450_{15\beta}$ (Waxman, 1984). Our experience in Western blotting (Figure 3 and unpublished observations) with the specific antibodies M16, F20, F22, and F23 indicates that at least these four Mabs do not recognize either P-450PB-I or P-450f under these conditions. However, cross-reactivity with these isozymes in other assays cannot yet be excluded.

Mab F1 was not an effective immunoadsorption agent when coupled to Sepharose 4B and also gave only weak signals on Western blots. This may indicate that the IgA Mab is unsuitable for such procedures because of its different physical properties or that it may be a low-affinity antibody. The relatively strong cross-reactivity with P-$450_{16\alpha}$ in ELISA, together with its ineffectiveness in the other procedures, limits the usefulness of F1.

Mabs F22 and F23 were not distinguishable by ELISA, Western blotting, inhibition of catalytic activity, or the immunoadsorption assay. Together with the identical patterns obtained from protein A directed staining of the hybridoma supernatant proteins separated by isoelectric focusing, this suggests that the clones producing F22 and F23 are derived from the same progenitor B cell and thus that the antibodies are identical.

All of the Mabs which showed selectivity for P-$450_{15\beta}$ inhibited each others binding to the antigen, whereas cross-reacting Mabs F10 and F13 and anti-P-$450_{16\alpha}$ Mab M16 were less effective. Thus, the specific Mabs all bind to the same epitope on P-$450_{15\beta}$, or epitopes not spatially distinct. Their ability to inhibit the catalytic activity of the microsomal enzyme suggests that the epitope may be located near the active site of the protein. The former finding, together with the low frequency of non-cross-reacting Mabs, may indicate that structural features of P-$450_{15\beta}$ which distinguish it from P-$450_{16\alpha}$ and P-450_{DEa} may be limited to a relatively small part of the protein. However, structural differences may also exist in nonimmunogenic parts of the protein.

The ability of the otherwise specific anti-P-$450_{15\beta}$ antibodies F22 and F23 to inhibit the catalytic activity of P-$450_{16\alpha}$ may be explained by the fact that the other procedures used to characterize the specificity of these Mabs all contained extensive wash steps after the original antibody–antigen incubation. Thus, these procedures favor detection of high-affinity interactions. However, in the catalytic assays, the Mabs are in constant contact with the antigen. Under such conditions, lower affinity antibody–antigen interactions may give rise to inhibition of catalytic activity. Our interpretion of the results from the catalytic inhibition data is that the anti-P-$450_{15\beta}$ antibodies recognize an inhibitory epitope on P-$450_{16\alpha}$, as well as on P-$450_{15\beta}$, but that the interaction with the P-$450_{15\beta}$ epitope is of higher affinity. This contention is supported by the tendency to more potent inhibition of 15β-hydroxylase than 16α-hydroxylase activities at the lower Mab:P-450 ratios. However, the latter observation could also reflect a possible difference in the specific contents of the P-$450_{16\alpha}$ and P-$450_{15\beta}$ isozymes in male and female rat liver microsomes, respectively. We have not been able to determine accurately the absolute specific contents of these isozymes using our Western blot immunoassay (Morgan et al., 1985b).

Cross-reacting Mabs F10 and F13 appeared to cause some inhibition of the binding of F22 and F23 to P-$450_{15\beta}$, indicating that the latter Mabs may bind to an epitope which is different from that of F1, F3, and F20. However, since F1, F3, and F20 all inhibited F22 and F23 binding totally, the significance of the weaker inhibition by F10 and F13 is hard to interpret, especially since different results were obtained with the otherwise identical Mabs F22 and F23.

The much stronger ELISA cross-reactivity of Mab M16 with purified P-$450_{15\beta}$ than with the female mix antigen probably is due largely to the heterogeneous nature of the latter. We routinely coated ELISA plates with female mix antigen at double the concentration of purified P-450, since P-$450_{15\beta}$ was one of the two major proteins in this preparation (the other being P-450_{DEa}). However, the female mix antigen had a higher percentage (about 60%) of non-P-450 proteins than we normally obtain in this fraction (MacGeoch et al., 1984). This contamination, together with the observed increase in cross-reactivity with P-$450_{15\beta}$ which was seen after purification of Mab M16, may account for the discrepancy. Reik et al. (1985) recently reported a critical dependence of the observed cross-reactivity of Mabs to P-450b and P-450e on the concentration of antigen used to coat the microtiter wells. These authors noted less cross-reactivity at lower antigen concentrations. This phenomenon may also account for the discrepancy observed between the apparent specificity of Mab M16 for P-$450_{16\alpha}$ in immunoblots and its cross-reactivity with P-$450_{15\beta}$ in ELISA.

The specific Mabs we have developed to P-$450_{15\beta}$ and P-$450_{16\alpha}$ will allow the development of more convenient and rapid, specific immunoassays for these proteins, facilitating future studies on the hormonal and chemical regulation of the levels of these isozymes. The inhibitory properties of the anti-P-$450_{15\beta}$ Mabs will be useful in identifying microsomal enzyme activities attributable to P-$450_{16\alpha}$ and P-$450_{15\beta}$. In this context, it will be important to establish that the Mabs do not inhibit other isozymes which might potentially catalyze a given reaction. In addition, the Mabs can provide independent corroboration of identification of P-$450_{16\alpha}$ and P-$450_{15\beta}$ in various systems, for example, in vitro translation products of mRNA selected by P-450 cDNA fragments. The Mabs will also be useful in studying structural homologies between the rat isozymes and those from other species.

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Purification and Characterization of Protein Carboxyl Methyltransferase from *Torpedo ocellata* Electric Organ

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ABSTRACT: Posttranslational modification of proteins by the enzyme protein carboxyl methyltransferase (PCM) has been associated with a variety of cellular functions. A prerequisite for the understanding of cellular mechanisms associated with PCM is the characterization of purified PCMs from different tissues. We describe here the purification and characterization of PCM from the electric organ of *Torpedo ocellata*. The enzyme was purified to homogeneity by ion-exchange chromatography and ammonium sulfate precipitation, followed by chromatography on Sephadex G-100 and hydroxylapatite columns. When visualized by silver staining, the 700-fold-purified PCM exhibited a single band on sodium dodecyl sulfate–polyacrylamide gels, corresponding to a polypeptide of M_r 29 000. The molecular weight of the nondenatured enzyme (as determined by rechromatography on Sephadex G-100 column) was also 29 000, suggesting that the enzyme is a monomer. Two isoelectric forms of PCM (pI = 6.1 and pI = 6.4) were detected in the purified enzyme preparation. The enzyme methylates various exogenous and endogenous proteins, including the acetylcholine receptor. Of the four different polypeptides of the acetylcholine receptor, the γ and β polypeptides were selectively methylated by the purified PCM. Purified *Torpedo* PCM is highly sensitive to sulfhydryl reagents. The competitive inhibitor of PCM *S*-adenosyl-L-homocysteine (AdoHcy) protected the enzyme from inactivation by sulfhydryl reagents, suggesting the existence of a cysteine residue at the active site of the enzyme. The purified PCM has a low affinity toward DEAE-cellulose and toward AdoHcy–agarose. This property, as well as the relatively high molecular weight and the marked sensitivity to sulfhydryl reagents, distinguishes between the electric organ PCM and analogous enzymes of mammalian tissues.

The enzyme protein carboxyl methyltransferase (EC 2.1.1.24) (PCM)[1] catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to free carboxyl side chains of glutamyl or aspartyl residues of proteins. This enzymatic reaction results in the formation of protein methyl esters that are subsequently hydrolyzed, either spontaneously (Axelrod & Daly, 1965) or by a protein methylesterase (Gagnon et al., 1984), to generate the methyl acceptor protein and methanol. In mammals, PCM has an ubiquitous tissue distribution [for review, see Diliberto (1982) and Clarke (1985)], and the enzyme has been purified from various organs such as the pituitary gland (Diliberto & Axelrod, 1974), the testes (Cusan et al., 1981), the brain (Aswad & Deight, 1983;

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[1] Abbreviations: PCM, protein carboxyl methyltransferase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PCMB, *p*-(chloromercuri)benzoic acid; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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