Berzofsky, J. A., & Berkower, I. J. (1976) in *Functional Immunology* (Paul, W. E., Ed.) pp 595–644, Raven Press, New York.
Fletcher, P. F., & Reichert, L. E., Jr. (1984) *Mol. Cell. Endocrinol. 34*, 39.
Grasso, P., & Crisp, T. M. (1985) *Endocrinology (Baltimore) 116*, 319.
Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114.
Heath, W. F., & Merrifield, R. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6367.
Keutman, H. T., Charlesworth, M. C., Mason, K. A., Ostrea, T., Johnson, L., & Ryan, R. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2038.
Komoriya, A., Hortsch, M., Meyers, C., Smith, M., Kanety, H., & Schlessinger, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1351.
Krstenansky, J. L., Trivedi, D., & Hruby, V. J. (1986) *Biochemistry 25*, 3853.
Krystek, S. R., Jr., Reichert, L. E., Jr., & Andersen, T. T. (1985) *Endocrinology (Baltimore) 117*, 1110.
Lerner, R. A. (1982) *Nature (London) 299*, 592.
Licht, P., Papkoff, H., Farmer, S. W., Muller, C. H., Tsui, H. W., & Crews, D. (1977) *Recent Prog. Horm. Res. 33*, 169.
Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., & Lerner, R. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4949.
Pierce, J. G., & Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465.
Reichert, L. E., Jr., Lawson, G. M., Jr., Leidenberger, F. L., & Trowbridge, C. G. (1973) *Endocrinology (Philadelphia) 93*, 938.
Rodbard, D., & Hutt, D. M. (1974) in *Symposium on RIA and Related Procedures in Medicine*, p 165, Intl. A.E.A., Vienna.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anifinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790.
Sairam, M. R., & Bhargavi, G. N. (1985) *Science (Washington, D.C.) 229*, 65.
Sairam, M. R., Seidah, N. G., & Chjretien, M. (1981) *Biochem. J. 197*, 541.
Schneyer, A. L., Sluss, P. M., Dattatreyamurty, B., & Reichert, L. E., Jr. (1986) *Endocrinology (Baltimore) 119*, 1446.
Sluss, P. M., & Reichert, L. E., Jr. (1984) *Biol. Reprod. 30*, 1091.
Sluss, P. M., Krystek, S. R., Jr., Andersen, T. T., Melson, B. E., Huston, J. S., Ridge, R., & Reichert, L. E., Jr. (1986) *Biochemistry 25*, 2644.
Sluss, P. M., Schneyer, A. L., Franke, M. A., & Reichert, L. E., Jr. (1987) *Endocrinology (Baltimore) 120*, 1477.
Todd, P. E. E., East, I. J., & Leach, S. J. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 212.
Ward, D. N. (1978) in *Structure and Function of the Gonadotropins* (McKerns, K. W., Ed.) pp 31–45, Plenum Press, New York.
Westhof, E., Altschuh, D., Moras, D., Bloomer, A. C., Mondragon, A., Klug, A., & Van Regenmortel, M. H. V. (1984) *Nature (London) 331*, 123.

Reductive Methylation of Lysine Residues in Acidic Fibroblast Growth Factor: Effect on Mitogenic Activity and Heparin Affinity†

J. Wade Harper and Roy R. Lobb*

Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received June 25, 1987; Revised Manuscript Received September 4, 1987

ABSTRACT: Reductive methylation of bovine brain derived acidic fibroblast growth factor (aFGF) with formaldehyde and sodium cyanoborohydride reduces its capacity to stimulate mitogenesis in Balb/C 3T3 cells, and this correlates with the modification of less than 3 of its 12 lysine residues. Fractionation of methylated aFGF on immobilized heparin shows that the affinity of the modified mitogen for heparin is also decreased substantially. The capacity of methylated mitogen of low heparin affinity (LA-aFGF) to stimulate mitogenesis is also reduced, and this correlates with a reduced affinity for its cell surface receptor. Structural characterization of LA-aFGF using peptide mapping and sequencing procedures demonstrates that Lys-118 is the primary site of modification. The results indicate that in aFGF, Lys-118 plays an important role in heparin binding and suggest that this residue and its local environment are involved in the interaction of aFGF with both heparin and its cell surface receptor.

Bovine brain derived acidic fibroblast growth factor (aFGF),[1] a class 1 heparin-binding growth factor (Lobb et al., 1986a,b), induces mitogenesis in a variety of mesoderm- and neuroectoderm-derived cells in vitro and is angiogenic in vivo (Thomas et al., 1984, 1985; Gospodarowicz et al., 1986; Lobb et al., 1985, 1986a; Thomas & Gimenez-Gallego, 1986). The primary structure of bovine aFGF has been determined (Esch et al., 1985a; Gimenez-Gallego et al., 1985; Strydom et al.,

† This work was supported by NIH Grant HL36373. J.W.H. was the recipient of National Research Service Award HL-07075 from the National Heart, Lung, and Blood Institute.

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Bldg., 250 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HBGF, heparin-binding growth factor; LA-aFGF, methylated aFGF of low heparin affinity; HPLC, high-performance liquid chromatography; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; MeLys, ϵ-methyllysine; Me_2Lys, ϵ-dimethyllysine; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/88/0427-0671$01.50/0

1986), and the sequence of human brain derived aFGF has also been described on the basis of both protein (Gimenez-Gallego et al., 1986; Harper et al., 1986) and cDNA sequencing (Jaye et al., 1986).

The wide range of functions associated with heparin-binding growth factors [for reviews, see Gospodarowicz et al. (1986) and Lobb et al. (1986a)] suggests that agonists or antagonists of human aFGF and related class 1 HBGF's could be of therapeutic value. The development of such clinical agents will require an understanding of the relationship between aFGF structure and function, as well as the nature and significance of the strong interaction of aFGF with heparin. The extensive homology (92%) between bovine and human aFGF suggests that the more readily available bovine mitogen might be an adequate model for structure/function studies. Therefore, chemical modification studies on bovine aFGF have been initiated to identify the role of particular amino acid residues in mitogenesis, cell receptor binding, and heparin binding.

Lysine residues have been implicated in the interaction of heparin with a number of heparin-binding proteins including platelet factor 4 (Deuel et al., 1977; Walz et al., 1977), antithrombin III (Rosenberg & Damus, 1973; Pecon & Blackburn, 1984), heparin cofactor II (Church & Griffith, 1984), and apolipoprotein E (Weisgraber et al., 1986). Therefore, our initial studies have focused on the role of lysine residues in aFGF function. The results of reductive methylation of aFGF with formaldehyde and sodium cyanoborohydride indicate that Lys-118 plays an important role in the heparin–aFGF interaction and suggest that this residue and its local environment are involved in both heparin binding and receptor binding.

Experimental Procedures

aFGF Isolation. aFGF was isolated from bovine brain (Pel-Freez) by heparin–Sepharose affinity chromatography as described (Lobb et al., 1986a). The mitogen was purified to homogeneity by cation-exchange HPLC on a Mono-S HR5/5 column (Pharmacia) either as described (Lobb et al., 1986a) or by elution with a linear gradient of sodium phosphate (0.1 M, pH 6.0, to 0.4 M, pH 7.6, in 50 min). Under the latter conditions, the mitogen elutes at ~0.2 M phosphate, pH 7.0, free of NaCl and can be iodinated directly (see below). Greater than 95% of the mitogen purified by these means is present as a mixture of two forms generally designated aFGF (M_r ~16K) (Thomas et al., 1984). These two forms differ only with respect to N-terminal heterogeneity; one form (aFGF-1) begins at Phe-1 and the other (aFGF-2) at Asn-7 (Thomas et al., 1985). This mitogen preparation stimulates half-maximal DNA synthesis in Balb/C 3T3 fibroblasts at a concentration of about 250 pg/mL (Lobb et al., 1986a). The concentration of pure aFGF was determined by amino acid analysis.

Reductive Methylation. The procedures used were adapted from Jentoft and Dearborn (1979). Typically, aFGF (2–8 μM) was incubated at 25 °C with 2 mM [^{3}H]formaldehyde (75 mCi/mmol, New England Nuclear) and 10 mM sodium cyanoborohydride (Aldrich Chemical Co.) in 0.1 M sodium phosphate, pH 6.0, and 0.35 M NaCl. At various times, aliquots were removed and quenched by diluting 15–30-fold into PBS containing 0.1% BSA for use in bioassays. Reversed-phase HPLC (C3) analysis of aliquots of the reaction mixture showed no loss of protein during reductive methylation.

Reaction mixtures were fractionated or desalted by using heparin–Sepharose chromatography and/or reversed-phase HPLC. For heparin–Sepharose chromatography, the reaction was quenched by addition of NH_4Cl (0.1 M final concentration) and the reaction mixture (0.8–3.4 mL) applied to a heparin–Sepharose column (0.2–0.5 mL) equilibrated with 10 mM Tris, pH 7.0, containing 0.2 M NaCl. The resin was washed with 10–20 column volumes each of 10 mM Tris, pH 7.0, supplemented with 0.2 M NaCl, 0.7 M NaCl, 1.2 M NaCl, or 2.0 M NaCl. The flow rate was ~1 mL/min, and 0.2–0.5-mL fractions were collected, depending on the column volume. Aliquots (10 μL) of each fraction were counted by liquid scintillation in Hydrofluor (New England Nuclear). Fractions containing radioactivity within each elution step were pooled, a portion was desalted by using reversed-phase HPLC (see below), and the yields of protein were determined by amino acid analysis. The remaining material was immediately diluted into PBS containing 0.1% BSA for bioassays and stored at −20 °C.

For desalting, reaction mixtures or heparin–Sepharose column fractions were applied to a propylsilane (C3) HPLC column (5-μm Ultrapore RPSC, 75 × 4.6 mm, Beckman Instruments) equilibrated with 0.1% TFA (solvent A). Elution was accomplished with a linear gradient of 2-propanol/acetonitrile/water (3:2:2 v/v) containing 0.1% TFA (solvent B) at a flow rate of 0.8 mL/min.

Amino Acid Analysis. Analyses were performed by using "Picotag" methodology (Waters Associates) as described (Bidlingmeyer et al., 1984; Cohen et al., 1985). Under standard conditions, the phenylthiocarbamoyl (PTC) derivative of Me_2Lys (Vega Chemical Co.) elutes from the "Picotag" HPLC column with a retention time identical with that of PTC-Thr while the PTC adduct of MeLys (Chemical Dynamics Co.) elutes 0.8 min after PTC_2-Lys and is separated from all other PTC-amino acids with base-line resolution. PTC_2-MeLys was quantitated by using its integrated peak area in conjunction with the conversion factor employed for PTC_2-Lys. Two methods were used to calculate the number of Me_2Lys residues: For method A, standard analyses were performed on hydrolyzed samples of peptides or proteins, and the HPLC eluant was collected (0.5-min fractions). Aliquots (150–250 μL) of fractions encompassing the elution positions of PTC-Me_2Lys and PTC_2-MeLys were counted by liquid scintillation and the relative counts per minute and integrated peak area for PTC_2-MeLys used to calculate the number of Me_2Lys residues present. For method B, the number of Me_2Lys residues was estimated by subtracting the number of Thr residues expected for the peptide or protein based on the sequence of aFGF (Strydom et al., 1986) from the number of residues calculated from the total integrated area for PTC-Thr plus PTC-Me_2Lys.

Peptide Mapping. Lyophilized ^{3}H-methylated aFGF (40–200 μg) was digested with *Staphylococcus aureus* protease V8 or chymotrypsin as described (Strydom et al., 1986). Peptides from protease V8 digests were fractionated by reversed-phase HPLC on an Ultrapore RPSC (C3) column employing linear gradients between solvents A and B. Chymotryptic peptide separations were performed on a Synchropak RP-P (C18) column (Synchrom, Inc., 250 × 4.5 mm) as described (Strydom et al., 1986). Where noted, peptides were rechromatographed on an Altex Ultrapore IP (C18) column (Beckman Instruments, Inc., 250 × 4 mm, 5-μm particle size) using linear gradients between solvents A and B. Peptide recoveries generally ranged from 30% to 60% after rechromatography depending on fragment size.

Hydroxylamine Fragmentation. Lyophilized ^{3}H-LA-aFGF (2.5 nmol) was dissolved in 0.1 mL of 6 M guanidine hy-

drochloride, 0.2 mL of 1.1 M NH_2OH (in 0.2 M Na_2CO_3, final pH 11) added, and the reaction mixture incubated at 35 °C for 28 h. The products were fractionated by HPLC employing a Synchropak RP-P (C18) column using linear gradients between solvents A and B.

Sequencing Studies. Automated microsequencing and identification of PTH–amino acids were performed as described (Strydom et al., 1985, 1986). Standards for PTH_2–MeLys and PTH–Me_2Lys were prepared individually in the sequencer. PTH_2–MeLys elutes in the PTH–amino acid separation system 0.5 min earlier than PTH_2–lysine and is resolved with near-base-line resolution. PTH–Me_2Lys elutes between and is resolved from PTH–Gly and PTH–Gln.

Mitogenesis Assays. Growth stimulation of confluent Balb/C 3T3 fibroblasts was assessed as described (Klagsbrun et al., 1977; Lobb & Fett, 1984). For time course studies, a mitogen concentration of 250 pg/mL was used, based on amino acid analysis. This concentration induces half-maximal stimulation in 3T3 cells (Lobb et al., 1986a), and the assay is most sensitive to changes in active mitogen concentration under these conditions. Assays on each sample were carried out in triplicate at least 3 times unless noted otherwise.

Iodination of aFGF. aFGF was iodinated according to established protocols (Friesel et al., 1986; Olwin & Hauschka, 1986) with minor modifications. Briefly, 2 μg of mitogen in ~0.2 M sodium phosphate, pH 7.0, was iodinated with Enzymobeads (Bio-Rad) at room temperature, the beads were pelletted by centrifugation, and the supernatant was applied directly to a 0.2-mL heparin–Sepharose column equilibrated with 10 mM Tris·HCl/0.6 M NaCl, pH 7.0. The column was washed extensively to remove free iodide, and the ^{125}I-aFGF was eluted with 10 mM Tris·HCl, pH 7.0, containing 2.0 M NaCl. The mitogen was stored at 4 °C in this buffer containing 0.2% gelatin and had a specific activity of ~40 μCi/μg. Consistent with other studies (Friesel et al., 1986; Olwin & Hauschka, 1986), ^{125}I-aFGF was fully active, showing half-maximal stimulation in 3T3 cells at ~0.2 ng/mL.

Radioreceptor Assays. Radioreceptor assays were performed on Balb/C 3T3 cells in 24-well cluster dishes within 48 h of reaching confluence. Assays were carried out at 4 °C in DME/0.2% gelatin (Neufield & Gospodarowicz, 1986). Binding of ^{125}I-aFGF under these conditions was maximal after 2.5 h, and this incubation time was used throughout. Consistent with other studies (Neufeld & Gospodarowicz, 1986; Olwin & Hauschka, 1986), in the presence of a 100-fold excess of unlabeled aFGF less than 15% of the input counts remained cell-associated. Scatchard analysis of the concentration dependence of the binding of ^{125}I-aFGF to confluent 3T3 cells at 4 °C indicated a single class of binding sites over the mitogen concentration range studied (20–625 pM), with an apparent dissociation constant of 1.8×10^{-10} M and 65 000 receptors/cell. These values are in reasonable agreement with those reported previously for Balb/C 3T3 cells (dissociation constant = 4×10^{-10} M and 30 000 receptors/cell; Schreiber et al., 1985). For competition assays, cells were incubated with 1 mL of DME/0.2% gelatin at 4 °C for 10 min; supernatants were removed by aspiration and replaced with a constant amount of ^{125}I-aFGF and different concentrations of either unmodified or methylated aFGF in 0.4 mL of DME/0.2% gelatin. After 2.5 h, the cells were washed 3 times with 1 mL of DME/0.2% gelatin, 4 °C; the cell-associated radioactivity was solubilized with two 0.5-mL aliquots of 1 N NaOH and counted.

Results

Effect of Reductive Methylation of aFGF on Mitogenic Activity. Incubation of aFGF (8 μM) with 2 mM [^{3}H]formaldehyde and 10 mM sodium cyanoborohydride at pH 6.0 resulted in a time-dependent decrease in its capacity to stimulate mitogenesis in Balb/C 3T3 fibroblasts (Figure 1A), with a half-time of 8 min. After incubation for 1 h, activity had decreased to 8% (Figure 1A); no further loss of activity was observed after 5 or 23 h. Neither 2 mM formaldehyde nor 10 mM sodium cyanoborohydride alone significantly inactivated the mitogen after incubation for 24 h. Figure 1B shows the concentration dependence of the mitogenic activity of aFGF reductively methylated for different time periods. The concentration of mitogen required for half-maximal stimulation of DNA synthesis increased significantly with reaction time, as expected on the basis of the data in Figure 1A. However, at sufficiently high concentrations of modified mitogen, the same maximal value of thymidine uptake was reached for each reaction time. Since the N-terminal α-amino groups of aFGF are not involved in mitogenic activity or heparin binding (see Discussion), these results indicate that the ε-amino group of at least one of the lysine residues of aFGF plays a role in mitogenesis.

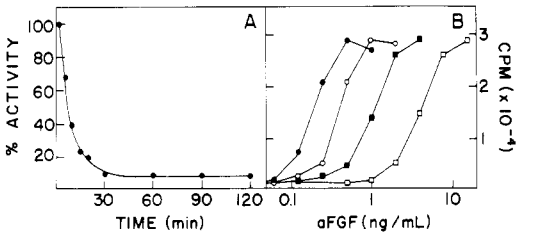

FIGURE 1: Effect of reductive methylation on the mitogenic activity of aFGF toward Balb/C 3T3 fibroblasts. (A) Time course: aFGF (8 μM) was incubated with 2 mM [^{3}H]formaldehyde and 10 mM sodium cyanoborohydride at 25 °C in 0.1 M sodium phosphate, pH 6.0, containing 0.35 M NaCl. At the indicated times, aliquots were withdrawn and quenched by dilution (30-fold) into PBS containing 0.1% BSA. Mitogenic assays (n = 6) were performed at a mitogen concentration of 250 pg/mL (see Experimental Procedures). (B) Dose response of aFGF reductively methylated for different time periods. Mitogen was modified for 0 (●), 10 (○), 20 (■), and 60 (□) min, respectively. Modification conditions were identical with those used in (A), and the mitogen concentration is plotted on a log scale. Assays were performed in triplicate.

Table I: Time Course for Reductive Methylation of Lysine Residues in aFGF

reaction time (min)	lysine residues modified[a]	% mitogenic activity[b]	distribution of residues		
			MeLys	Me_2Lys[c]	Lys
0	0	100			12[d]
10	2.0	39	1.3	0.7	10.1
20	2.8	19	1.5	1.3	8.9
60	4.5	8	2.2	2.3	6.3
300	10.7	8	1.4	9.3[e]	0.9

[a] Sum of MeLys and Me_2Lys residues. [b] Based on the time course in Figure 1A. [c] Me_2Lys residues determined by amino acid analysis using method A (see Experimental Procedures) unless otherwise noted. [d] Based on the sequence of aFGF (Figure 3). [e] Quantitation of Me_2Lys is by method B (see Experimental Procedures).

Time Dependence of Lysine Methylation. The time course for lysine methylation of aFGF was examined by incubating the protein with 2 mM [^{3}H]formaldehyde and 10 mM sodium cyanoborohydride at pH 6.0 for various times, quenching the reaction, removing excess reagents by reversed-phase HPLC, and subjecting the modified protein to amino acid analysis. Table I compares the extent of lysine modification with the mitogenic potency of modified aFGF. Although methylation of lysine residues in aFGF occurs progressively over a period of about 5 h, about three lysine residues are modified within

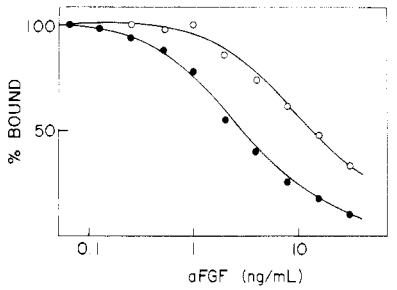

FIGURE 2: Inhibition of the binding of ^{125}I-aFGF to Balb/C 3T3 cells by either unmodified aFGF or LA-aFGF. Confluent Balb/C 3T3 cells were incubated with a fixed concentration of ^{125}I-aFGF (~4 ng/mL) at 4 °C for 2.5 h in the presence of varying concentrations of aFGF (●) or LA-aFGF (○). Cell surface associated radioactivity was then determined, and the percent ^{125}I-aFGF bound was plotted against the added mitogen concentration on a log scale. Nonspecific binding was evaluated in the presence of a 100-fold excess of aFGF. Concentrations of stock mitogen solutions were determined by amino acid analysis.

the first 20 min. Moreover, after 10 and 20 min of reaction, the mitogenic potency is reduced to 39% and 19%, respectively, compared with unmodified aFGF. These data suggest that methylation of between one and three of the most rapidly modified lysine residues is responsible for the substantial decrease in mitogenic potency.

Effect of Methylation on Heparin Binding. In order to examine the potential role of the most rapidly modified lysine residues in heparin binding, aFGF (6 μM, 370 μg) was methylated at pH 6.0 for 15 min employing [^{3}H]formaldehyde, and the products were subjected to heparin–Sepharose chromatography as described under Experimental Procedures. All of the methylated mitogen was retained on the heparin–Sepharose column after extensive washing with buffer containing 0.2 M NaCl. However, 80% of the methylated mitogen was eluted with buffer supplemented with only 0.7 M NaCl, while the remaining 20% was eluted with 1.2 M NaCl. (These values are based on a total recovery of ~ 70%.) Under these conditions, unmodified aFGF is eluted from the column with buffer containing 1.2 M NaCl, but not with buffer containing 0.7 M NaCl, and with a recovery of 60–80%.

Amino acid analysis (method A) of low heparin affinity methylated aFGF (LA-aFGF) (i.e., eluting with 0.7 M NaCl) showed the following distribution of lysine residues: MeLys, 1.8; Me_2Lys, 1.4; and Lys, 8.9. These values are in reasonable agreement with those expected on the basis of data in Table I.

Mitogenic Activity and Cell Receptor Affinity of LA-aFGF. The capacity of LA-aFGF to stimulate DNA synthesis in 3T3 cells was reduced by about 4-fold when compared with unmodified mitogen. Thus, the activity was half-maximal at 1 ng/mL while stimulation was maximal with 6–8 ng/mL LA-aFGF.

In order to determine whether the reduced mitogenic potency of LA-aFGF was due to decreased affinity for its cell surface receptor, a radioreceptor assay was developed for aFGF receptors on the surface of Balb/C 3T3 cells (see Experimental Procedures). Unlabeled aFGF competes effectively with a fixed concentration of ^{125}I-aFGF for receptor occupancy, but the ability of LA-aFGF to do so is significantly reduced (Figure 2).

Structural Characterization of LA-aFGF. The sites of methylation in LA-aFGF were identified by using a combination of peptide mapping with *S. aureus* protease V8, chymotrypsin, and hydroxylamine and peptide sequencing. The amino acid sequence of aFGF and the location of the relevant peptides are shown in Figure 3. The sites of methylation are summarized in Table II. The major site of lysine modification in LA-aFGF is Lys-118, which is almost fully modified and extensively dimethylated. The mapping and sequencing results are summarized below while a more detailed description of the results and supportive data are provided in the supplementary material (see paragraph at end of paper regarding supplementary material).

The preparation of ^{3}H-LA-aFGF used for mitogenesis and receptor assays was digested with *S. aureus* protease V8, and the resultant peptides were fractionated by reversed-phase HPLC (Figure 4). These peptides were identified on the basis of their amino acid compositions (supplementary material Tables IS and IIS). Examination of aliquots from each fraction revealed that greater than 80% of the peptide-associated radioactivity comigrated with the C-terminal peptide V-11 and with the N-terminal peptides V-10[2] and V-12

FIGURE 3: Primary structure of aFGF (Strydom et al., 1986) and positions of peptides obtained from fragmentation of ^{3}H-LA-aFGF with either protease V8 (V), chymotrypsin (C), or hydroxylamine (HY). Peptide placement is by amino acid composition. Peptides are numbered according to their elution order by reversed-phase HPLC. Peptide V-12,[2] which consists of peptide V-10 disulfide bonded to V-3, is omitted for clarity. For peptide V-10, the form beginning with Asn-7 (derived from aFGF-2) is not shown.

Table II: Distribution of Methylated Lysine Residues in ^{3}H-LA-aFGF

lysine residue	peptide[a]	% modification MeLys	% modification Me_2Lys[b]
9, 10, 12	V-10, V-12, C-23	13[c]	N.D.[c,d]
35	C-23	N.D.[d,e]	N.D.[d,e]
57	V-1, V-1a	<10[f]	N.D.[d,f]
100, 101	V-2, V-2a	<6[f,g]	N.D.[d]
105	V-11	7[h]	<1[h]
112	V-11	17[h]	<1[h]
113	V-11	6[h]	<1[h]
118	HY-2	28[h]	64[h]
128	C-7	<1[f]	N.D.[d,f]

[a] Peptides designated V, C, and HY are derived from protease V8, chymotryptic, and hydroxylamine fragmentation, respectively. Positions of these peptides within the primary structure of aFGF are shown in Figure 3, and the compositions of the peptides are provided in the supplementary material (Table IIS). [b] Unless noted otherwise, determination of Me_2Lys was by amino acid analysis (method B). [c] These values were derived from the average amino acid compositions of peptides V-10 and V-12 (supplementary material Table IIS) in combination with the analysis of peptide C-23 (see Results). The number of MeLys residues identified was 0.38 out of a total of 3 (i.e., 13%). [d] N.D., not detectable. [e] Based on the radioactivity content of peptide C-23 (Results and supplementary material Figure 2S). No detectable MeLys or Me_2Lys was found by amino acid analysis of peptide C-23 from a second digest of methylated aFGF (supplementary material Table IIIS). [f] Based on the amino acid compositions of indicated peptides (supplementary material Table IIS). [g] Less than 0.11 residue of Me-Lys and no detectable Me_2Lys out of a maximum of 2.0 lysine residues. [h] Based on the percent MeLys, Me_2Lys, and Lys identified by Edman degradation of the indicated peptide (see supplementary material Table IVS). For Me_2Lys, the detection limit was estimated to be ~2 pmol.

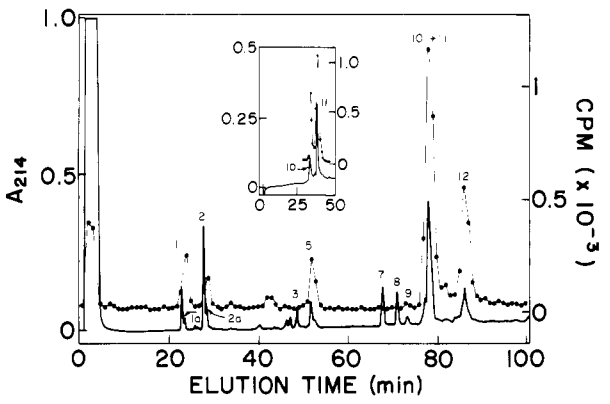

FIGURE 4: Fractionation of protease V8 peptides from ^{3}H-LA-aFGF. ^{3}H-LA-aFGF was prepared as described under Results and subjected to cleavage by protease V8. The resultant peptides were fractionated on an Ultrapore RPSC (C3) column equilibrated with solvent A and eluted with a linear gradient of 0–60% solvent B in 120 min at a flow rate of 0.8 mL/min. Peptides are numbered according to their elution position. (●) Aliquots (25 μL) from 1-min fractions were counted by liquid scintillation. (Insert) A pool of peptides V-10 and V-11 from the first fractionation was rechromatographed on an Altex Ultrapore IP (C18) column. The gradient was 25–90% solvent B in 60 min. (●) Aliquots (25 μL) of 1-min fractions were counted by liquid scintillation. Peptides V-1a and V-2a are the methylated forms of V-1 and V-2, respectively, as indicated by their composition and comigration of radioactivity.

(Figures 3 and 4). Of the 5 lysine residues in peptide V-11, 0.8 and ~0.5 residues had been converted to Me_2Lys and MeLys, respectively (supplementary material Table IIS). In contrast, the composition of peptide V-10 (and V-12) showed

[2] The presence of the two N-terminal peptides V-10 and V-12 is apparently due to disulfide bond interchange under the denaturing conditions employed (see supplementary material for additional information concerning disulfide bond interchange in aFGF).

Scheme I

$$E\text{-}\overset{+}{N}H_3 \xrightleftharpoons{CH_2=O} E\text{-}\overset{+}{N}H{=}CH_2 \xrightarrow{NaCNBH_3} E\text{-}\overset{+}{N}H_2\text{-}CH_3 \xrightarrow{\text{second cycle}} E\text{-}\overset{+}{N}H(CH_3)_2$$

that only 0.38 of the 4 lysine residues had been converted to MeLys and there was no detectable Me_2Lys (supplementary material Table IIS). Limited monomethylation (≤10%) had occurred at lysine residues in peptides V-1 and V-2 (Table II; supplementary material Table IIS).

The sites of modification near the N-terminal in ^{3}H-LA-aFGF[3] were examined further by chymotryptic peptide mapping (supplementary material Table IIS and Figure 2S). Peptide C-23 showed no incorporation of radioactivity, which ruled out Lys-35 as a site of modification. In addition, peptide C-20, which does not contain lysine, was radioactive. Its composition (supplementary material Table IIS) showed 8% of the expected value for phenylalanine, indicating that the N-terminal phenylalanine residue in LA-aFGF had been methylated.[4] At present, the distribution of monomethylation among Lys-9, -10, and -12 is not known. Although it is possible that monomethylation occurs at only one of these residues, the lack of detectable Me_2Lys would argue against this possibility.

The sites of methylation within the C-terminal peptide V-11 were initially examined by Edman degradation. The sequence of 1 nmol of V-11 through nine cycles (supplementary material Table IVS) showed that only limited monomethylation had occurred at residues 105, 112, and 113 and no PTH–Me_2Lys was detected at any of these positions (Table II). This result, in combination with the extent of modification by amino acid analysis, indicated that Lys-118 and/or Lys-128 was the major site of methylation in V-11. Modification at Lys-128 (Figure 3) was ruled out by the composition of chymotryptic peptide C-7 (supplementary material Table IIS and Figure 2S) which showed 0.95 of the expected 1.0 lysine residue and lacked radioactivity. Jointly, these data pointed to Lys-118 as the major site of methylation in LA-aFGF.

Extensive methylation of Lys-118 was confirmed by the results of Edman degradation of peptide HY-2 (0.5 nmol), obtained by hydroxylamine cleavage of ^{3}H-LA-aFGF at the Asn-114–Gly-115 bond (Strydom et al., 1986). Lysine-118 was found to be essentially fully modified, with a ratio of ε-monomethyllysine to ε-dimethyllysine residues of about 1:2 (Table II; supplementary material Table IVS). By amino acid analysis, peptide HY-2 showed 0.50 and 0.32 residues of Me_2Lys and MeLys, respectively (supplementary material Table IIS). Thus, within experimental error, all of the methylated lysine residues present in HY-2 by amino acid analysis are accounted for by methylation at Lys-118.

Discussion

Detailed information on the relationship between protein structure and function can be obtained by chemical modification of the side chains of specific amino acid residues. Such studies have been initiated with aFGF to explore the possible involvement of particular residues in mitogenesis, receptor

[3] This preparation of LA-aFGF showed 1.3 MeLys and 0.8 Me_2Lys residues by amino acid analysis (method B).

[4] Analysis of chymotryptic peptides from an additional preparation of ^{3}H-methylated aFGF that had not been fractionated on heparin–Sepharose revealed that the N-terminal α-amino groups in both aFGF-1 and aFGF-2 were >80% methylated after modification for 15 min (see supplementary material, Table IIIS).

binding, and/or heparin binding. In this study, reductive methylation with formaldehyde and sodium cyanoborohydride (Jentoft & Dearborn, 1979; Means, 1984) has been used. This modification is specific for amino groups in proteins and has been employed to evaluate the precise role of lysine residues in a variety of proteins (Weisgraber et al., 1978; Gerkin et al., 1982; Szasz et al., 1982; Shapiro & Riordan, 1983; Sherman et al., 1983). The initial product of reductive methylation is a monomethylamino group, which can undergo a second cycle of modification to give a dimethylamino group (Scheme I). The modified amino groups retain their positive charge at neutral pH since their pK values are barely affected (Means, 1984).

Reductive methylation of aFGF would be expected to occur not only at the ϵ-amino group of lysine residues but also at the N-terminal α-amino groups. However, the free α-amino groups of aFGF are not required for either mitogenesis or heparin binding since the two N-terminal forms of aFGF beginning with Phe-1 and Asn-7, as well as aFGF with a 14-residue N-terminal extension, do not differ significantly in mitogenic activity or heparin affinity (Burgess et al., 1986; Crabb et al., 1986; Jaye et al., 1986; J. W. Harper and R. R. Lobb, unpublished results). Thus, for aFGF, the functional effects of reductive methylation can be attributed entirely to modification of ϵ-amino groups of lysine residues.

Reductive methylation of aFGF leads to a rapid decrease in its mitogenic potency toward Balb/C 3T3 cells (Figure 1A,B). After incubation for 60 min, the mitogenic potency was reduced to 8% of that of the native mitogen and was not reduced further after extended incubation (5 or 23 h), even though extensive lysine methylation occurred during this period (Table I). A comparison of the mitogenic potency of modified aFGF with the extent of lysine modification suggests that the observed reduction can be attributed to modification of less than 3 of the 12 lysine residues.

The effect of reductive methylation on the ability of aFGF to bind to immobilized heparin was also examined. Modification of less than three lysine residues significantly reduced the concentration of NaCl required to elute the mitogen from heparin–Sepharose. The methylated mitogen of low heparin affinity (LA-aFGF) also showed reduced mitogenic potency which correlated with reduced affinity for the cell surface receptor. These data demonstrated that one or more of the most rapidly modified lysine residues of aFGF plays a role in the binding of aFGF to both heparin and the cell surface receptor. Structural characterization of ^{3}H-LA-aFGF showed that significant methylation was confined to Lys-118 (Table II). This residue was at least 90% modified, with 60% present as dimethyllysine. Thus, Lys-118 is strongly implicated in the interaction of aFGF with heparin.

It has been suggested that in heparin-binding proteins, clusters of basic residues are important for the protein–heparin interaction (Deuel et al., 1977; Schwarzbauer et al., 1983; Weisgraber et al., 1986). We noted the presence of two segments of the primary structure of aFGF containing clusters of basic residues which might play a role in heparin binding (Strydom et al., 1986). A basic cluster (lysines-9, -10, and -12) is found near the amino terminus. In addition, the region from residue 100 to residue 122 is highly basic and contains a total of 10 lysine, arginine, and histidine residues, including 2 Lys-Lys sequences at positions 100–101 and 112–113 (Figure 3). The most rapidly modified lysine residue in aFGF is indeed within this region. However, the two Lys-Lys sequences found at positions 100–101 and 112–113 are not methylated significantly in LA-aFGF (Table II). The data indicate that

```
                110                                                 120
Bovine aFGF     Gly-Leu-Lys-Lys-Asn-Gly-Arg-Ser-Lys-Leu-Gly-Pro-Arg
Human aFGF      Gly-Leu-Lys-Lys-Asn-Gly-Ser-Cys-Lys-Arg-Gly-Pro-Arg

                119                                                 129
Bovine bFGF     Ala-Leu-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys
Human bFGF      Ala-Leu-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Ser-Lys
```

FIGURE 5: Amino acid sequences of bovine aFGF and human aFGF in the region of Lys-118 (boxed) and the homologous segments of bovine and human bFGF. Sequences are numbered according to Strydom et al. (1986) and Esch et al. (1985b).

these residues are not as reactive as Lys-118 but do not necessarily rule out a potential role for these, or other, lysine residues in either heparin or receptor binding. Indeed, it is likely that the strong affinity of aFGF for heparin reflects multiple interactions involving basic amino acids. Nevertheless, Lys-118 is clearly in a microenvironment that favors its rapid modification as compared to other lysine residues in aFGF, and methylation of Lys-118 alone is sufficient to substantially reduce the affinity of aFGF for heparin and its cell surface receptor.

The primary structures of human and bovine aFGF are 92% identical (Gimenez-Gallego et al., 1986; Harper et al., 1986). Of the 11 nonidentical residues in the 2 proteins, 3 are located in the immediate vicinity of Lys-118 (Figure 5). However, this lysine residue is conserved. Thus, on the basis of homology, Lys-118 in human aFGF would also be implicated in mitogenesis and heparin binding.

The sequences of bovine and human basic FGF's (Gospodarowicz et al., 1984; Esch et al., 1985b; Abraham et al., 1986), which are class 2 HBGF's (Lobb et al., 1986a,b), and bovine aFGF are about 55% identical. The residues in bovine and human bFGF corresponding to Lys-118 of aFGF are lysines also (Figure 5). While it is tempting to speculate that this lysine residue in bFGF's plays a role in heparin binding, numerous substitutions are found in this region of the two classes of HBGF which may influence the functional role of this residue.

Secondary structure analysis of aFGF by the method of Chou and Fasman (1978) predicts that Lys-118 is contained within a β-turn region encompassing residues 112–123. In general, the side chains of residues contained within such loop structures form part of the protein surface. This prediction is consistent with a direct role for Lys-118 and its local environment in heparin binding. Despite the presence of several amino acid substitutions in the homologous regions of human aFGF as well as human and bovine bFGF (Figure 5), a loop structure is also predicted for these mitogens. The functional role of residues in this segment of HBGF's warrants further study.

The finding that lysine modification in LA-aFGF is limited almost exclusively to Lys-118 indicates that the observed effects on heparin binding, receptor affinity, and mitogenic potency are likely attributable to methylation of only this residue. This suggests that the binding of aFGF to heparin may be required for maximal mitogenic potency. If this is the case, then either heparin or related glycosaminoglycans associated with the aFGF cell surface receptor are responsible for mitogen binding and signal transduction, or a ternary complex between mitogen, receptor, and a heparin-like molecule is required for maximal induction of mitogenesis. For example, recent studies on apolipoprotein E show that the heparin-binding and receptor-binding domains in this protein are found within the same linear sequence of about 10 amino acids (Weisgraber et al., 1986). Furthermore, the binding of both antithrombin III and thrombin to heparin via a ternary complex is a prerequisite for the acceleration of the antithrombin–thrombin reaction (Danielson et al., 1986).

The results of this study show that Lys-118 plays a role in both heparin binding and receptor binding and raise the possibility that the binding of aFGF to heparin-like molecules may be necessary for maximal mitogenic potency. If this is indeed the case, then the interaction of aFGF with heparin is of physiologic significance, and therefore of relevance to the design and therapeutic use of agonists or antagonists of class 1 HBGF's. With the availability of the gene for this mitogen (Jaye et al., 1986), oligonucleotide-directed mutagenesis of Lys-118 and other residues in this region of the protein should provide additional insight into the relationship between structure and function in this class of heparin-binding growth factors.

ACKNOWLEDGMENTS

We thank Dr. Bert L. Vallee for continued advice and support, Dr. James F. Riordan for helpful discussions, Dr. Daniel J. Strydom for performing the sequencing studies, and Nazik Sarkissian and Sandra Vaters for excellent technical assistance.

SUPPLEMENTARY MATERIAL AVAILABLE

Tables IS–VS containing amino acid compositions and sequence data of protease V8, chymotryptic and hydroxylamine peptides from modified aFGF, Figures 1S–3S showing chromatographic separations of protease V8 and chymotryptic peptides from native or modified aFGF, and Figure 4S showing chromatographic separations of methylated aFGF, a detailed description of characterization of sites of methylation in LA-aFGF, a description of the effect of guanidine hydrochloride on disulfide interchange in aFGF, and a description of cation-exchange HPLC methods for purification of methylated aFGF (27 pages). Ordering information is given on any current masthead page.

Registry No. aFGF, 106096-92-8; Lys, 56-87-1; heparin, 9005-49-6.

REFERENCES

Abraham, J. A., Whang, J. L., Tumulo, A., Mergia, A., Friedman, J., Gospodarowicz, D., & Fiddes, J. C. (1986) *EMBO J. 5*, 2523–2528.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Burgess, W. H., Mehlman, T., Marshak, D. R., Fraser, B. A., & Maciag, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7216–7220.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Church, F. C., & Griffith, M. J. (1984) *Biochem. Biophys. Res. Commun. 124*, 745–751.

Cohen, S. A., Tarvin, T. L., Anderson, D. B., Bidlingmeyer, B. A., & Strydom, D. J. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1211.

Crabb, J. W., Armes, L. G., Carr, S. A., Johnson, C. M., Roberts, G. D., Bordoli, R. S., & McKeegan, W. L. (1986) *Biochemistry 25*, 4988–4993.

Danielsson, A., Raub, E., Lindahl, U., & Bjork, I. (1986) *J. Biol. Chem. 261*, 15467–15473.

Deuel, T. F., Keim, P. S., Farmer, M., & Heinrikson, R. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2256–2258.

Esch, F., Ueno, N., Baird, A., Hills, F., Denoroy, L., Ling, N., Gospodarowicz, D., & Guillemin, R. (1985a) *Biochem. Biophys. Res. Commun. 133*, 554–562.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Böhlen, P., & Guillemin, R. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6507–6511.

Friesel, R., Burgess, W. H., Mehlman, T., & Maciag, T. (1986) *J. Biol. Chem. 261*, 7581–7584.

Gerkin, T. A., Jentoft, J. E., Jentoft, N., & Dearborn, D. G. (1982) *J. Biol. Chem. 257*, 2894–2900.

Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., & Thomas, K. (1985) *Science (Washington, D.C.) 230*, 1385–1388.

Gimenez-Gallego, G., Conn, G., Hatcher, V. B., & Thomas, K. A. (1986) *Biochem. Biophys. Res. Commun. 138*, 611–617.

Gospodarowicz, D., Cheng, J., Lui, G.-M., Baird, A., & Böhlen, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6963–6967.

Gospodarowicz, D., Neufeld, G., & Schweigerer, L. (1986) *Cell Differ. 19*, 1–17.

Harper, J. W., Strydom, D. J., & Lobb, R. R. (1986) *Biochemistry 25*, 4097–4103.

Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I.-M., Ravera, N. W., O'Brien, S. J., Modi, W. S., Maciag, T., & Drohan, W. N. (1986) *Science (Washington, D.C.) 233*, 541–544.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.

Klagsbrun, M., Langer, R., Levenson, R., Smith, S., & Lillehei, C. (1977) *Exp. Cell Res. 105*, 99–108.

Lobb, R. R., & Fett, J. W. (1984) *Biochemistry 23*, 6295–6299.

Lobb, R. R., Alderman, E. M., & Fett, J. W. (1985) *Biochemistry 24*, 4969–4973.

Lobb, R. R., Harper, J. W., & Fett, J. W. (1986a) *Anal. Biochem. 154*, 1–14.

Lobb, R. R., Sasse, J., Sullivan, R., Shing, Y., D'Amore, P., Jacobs, J., & Klagsbrun, M. (1986b) *J. Biol. Chem. 261*, 1924–1928.

Means, G. E. (1984) *J. Protein Chem. 3*, 121–130.

Neufeld, G., & Gospodarowicz, D. (1986) *J. Biol. Chem. 261*, 5631–5637.

Olwin, B. B., & Hauschka, S. D. (1986) *Biochemistry 25*, 3487–3492.

Pecon, J. M., & Blackburn, M. N. (1984) *J. Biol. Chem. 259*, 935–938.

Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490–6505.

Schreiber, A. B., Kenney, J., Kowalski, W. J., Friesel, R., Mehlman, T., & Maciag, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6138–6142.

Schwarzbauer, J. E., Tamkun, J. W., Lemischka, I. R., & Hynes, R. O. (1983) *Cell (Cambridge, Mass.) 35*, 421–431.

Shapiro, R., & Riordan, J. F. (1983) *Biochemistry 22*, 5315–5321.

Sherman, G., Rosenberry, T. L., & Sternlicht, H. (1983) *J. Biol. Chem. 258*, 2148–2156.

Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry, 24*, 5486–5494.

Strydom, D. J., Harper, J. W., & Lobb, R. R. (1986) *Biochemistry 25*, 945–951.

Szasz, J., Burns, R., & Sternlicht, H. (1982) *J. Biol. Chem. 257*, 3697–3704.

Thomas, K. A., & Gimenez-Gallego, G. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 81–84.

Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 357–361.

Thomas, K. A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rodkey, J., & Fitzpatrick, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6409-6413.
Walz, D. A., Wu, V. Y., de Lamo, R., Dene, H., & McCoy, L. E. (1977) *Thromb. Res. 11*, 893-898.
Weisgraber, K. H., Innerarity, T. L., & Mahley, R. W. (1978) *J. Biol. Chem. 253*, 9053-9062.
Weisgraber, K. H., Rall, S. C., Jr., Mahley, R. W., Milne, R. W., Marcel, Y. L., & Sparrow, J. T. (1986) *J. Biol. Chem. 261*, 2068-2076.

Cytochrome P-450$_{C\text{-}M/F}$, a New Constitutive Form of Microsomal Cytochrome P-450 in Male and Female Rat Liver with Estrogen 2- and 16α-Hydroxylase Activity†

Osamu Sugita,‡ Shigeru Sassa,*,‡ Shinichi Miyairi,‡ Jack Fishman,‡ Ichiro Kubota,§ Teruhisa Noguchi,§ and Attallah Kappas‡

The Rockefeller University Hospital, New York, New York 10021, and Suntory Institute for Biomedical Research, Osaka, Japan

Received July 21, 1987; Revised Manuscript Received September 24, 1987

ABSTRACT: A new cytochrome P-450 isozyme, P-450$_{C\text{-}M/F}$, has been purified from untreated rat liver microsomes. The purified preparation was electrophoretically homogeneous and contained 12-15 nmol of P450/mg of protein and had a minimum molecular weight of 48 500. The NH_2-terminal amino acid sequence of P-450$_{C\text{-}M/F}$ was different from that of other P-450's. Immunoblot analysis of microsomes demonstrated that P-450$_{C\text{-}M/F}$ was present in the liver of untreated male as well as female rats. Treatment of rats with phenobarbital, 3-methylcholanthrene, or β-naphthoflavone did not induce P-450$_{C\text{-}M/F}$. Cytochrome P-450$_{C\text{-}M/F}$ exhibited little activities of 7-ethoxycoumarin and 7-ethoxyresorufin O-deethylation or hydroxylation of arylhydrocarbon, testosterone, androstenedione, and progesterone. In contrast, it was highly active in N-demethylation of ethylmorphine and benzphetamine and in 2- and 16α-hydroxylation of estrogens, particularly that of estradiol. These studies establish that cytochrome P-450$_{C\text{-}M/F}$ is constitutively present in both male and female rats and suggest that it may be involved in the oxidative metabolism of estradiol, particularly in the formation of estriol, the uterotropic metabolite of estradiol.

Cytochrome P-450 is a collective term for a group of microsomal hemeproteins that serve as the terminal oxidases in the mixed-function oxidase system (Cooper et al., 1965). This multicomponent system plays a vital role in the oxidation of a variety of foreign chemicals such as drugs (Conney, 1967; Lu et al., 1970), carcinogens (Thorgeirsson et al., 1973), and hydrocarbons (Orrenius & Ernster, 1974) as well as diverse endogenous substances such as fatty acids (Wada et al., 1968; Bjorkhem & Danielson, 1970), steroids (Kuntzman et al., 1964; Lu & Levin, 1974) and vitamin D (Hansson et al., 1981). A number of studies have been carried out to elucidate the multiplicity of this cytochrome species. These studies, most of which have been indirect in nature, include spectral characteristics, catalytic activity, substrate specificity, modulation of enzyme activity by specific inhibitors, and electrophoretic mobility. Comparisons have also been made between untreated control and experimental animals in sex, age, genetic background, species, maintenance conditions or treatment of animals with chemicals known to specifically induce or decrease cytochrome P-450 content in the liver [see review in Conney (1967)].

Recently, more definitive studies have been carried out by purifying cytochrome P-450 isozymes from microsomes and by studying their physicochemical properties; these have provided clear evidence for a multiplicity of this cytochrome species. Most of these studies, however, have been confined largely to the major forms of cytochrome P-450 that are induced in the liver of animals treated with xenobiotics such as phenobarbital (Guengerich et al., 1982) or 3-methylcholanthrene (Wood et al., 1983). To date, only a small number of cytochrome P-450 isozymes have been purified from livers of untreated animals (Schenkman et al., 1982; Kamataki et al., 1983; Ryan et al., 1984). These native forms of cytochrome P-450 are of great interest in view of the fact that they may be involved in the metabolism of endogenous substrates such as vitamin D (Hansson et al., 1981), testosterone (Ryan et al., 1982b; Cheng & Schenkman, 1983; Harada & Negishi, 1984), progesterone (Johnson et al., 1983; Cheng & Schenkman, 1984), estradiol (Johnson et al., 1983; Cheng & Schenkman, 1984), prostaglandins (Okita et al., 1981; Vatsis et al., 1982), fatty acids (Gibson et al., 1980; Tamburini et al., 1984), and cholesterol (Waxman, 1986). In this manner, such P-450 isozymes may play a significant role in regulation of the metabolism of a variety of natural compounds important to normal cellular metabolism. Some of these native P-450 isozymes are also known to be inducible by treatment of animals with xenobiotics.

We report in this paper a new cytochrome P-450 species isolated from untreated male and female rat liver that is not inducible by treatment of animals with phenobarbital, 3-methylcholanthrene, or β-naphthoflavone. Since this newly identified P-450 isozyme is present constitutively in hepatic microsomes of both sexes, we propose the term "cytochrome P-450$_{C\text{-}M/F}$" for this molecule.

† This work was supported in part by grants from the USPHS (ES-01055 and DK-32890) and the Suntory Fund for Biomedical Research.
* Author to whom correspondence should be addressed.
‡ Rockefeller University Hospital.
§ Suntory Institute for Biomedical Research.

0006-2960/88/0427-0678$01.50/0