Journal of Protein Chemistry, Vol. 9, No. 6, 1990

Recombinant Human Prorenin from CHO Cells: Expression and Purification

Thomas F. Holzman,[1,4] Christine C. Chung,[1] Rohinton Edalji,[1] David A. Egan,[1] Earl J. Gubbins,[2] Annemarie Rueter,[2] Gail Howard,[2] Lana K. Yang,[2] Terry M. Pederson,[2] Grant A. Krafft,[3] and Gary T. Wang[3]

Received July 27, 1990

The gene for human preprorenin was obtained from total RNA prepared from primary human chorion cells. An expression vector was constructed containing an SV40 early promoter, a human preprorenin cDNA, bovine growth hormone poly-A addition signal, and a dihydrofolate reductase (dhfr) expression cassette. This vector was inserted into the DXB-11 Chinese hamster ovary (CHO) cell line. The recombinant protein was exported by CHO cells into the tissue culture media. At harvest the prorenin levels ranged from ~1–5 mg/L. For prorenin isolation the cell culture supernatants were processed by filtration, concentration, dialysis, and batch extraction. Preparative-scale isolation of prorenin was accomplished using blue-dye chromatography and size-exclusion chromatography. The isolated prorenin yielded a single SDS-gel band with Mr ~40,000. The proprotein was characterized with respect to N-terminal sequence and N-linked sugar composition. Trypsin-activated renin prepared from the proprotein was characterized with respect to N-terminal sequence and *p*H-activity profile. Enzyme activity was measured with a newly developed fluorogenic peptide substrate containing the P_6–P_3' sequence of human angiotensinogen.

KEY WORDS: Prorenin; renin; N-terminal sequence; fluorogenic substrate; cDNA cloning.

1. INTRODUCTION

A thorough understanding of the protein biochemistry of prorenin and renin and the mechanism of action of renin is essential to the design and characterization of renin inhibitors as antihypertensive agents. To this end recombinant DNA technology was used to express human prorenin in CHO (Chinese hamster ovary) cells. Renin is a member of the aspartyl proteinase family of proteins. It is produced, *in vivo*, as the proenzyme, prorenin. Prorenin is a protein of 383 residues (Imai *et al.*, 1983) containing three disulfide bonds and two N-linked glycosylation sites (Imai *et al.*, 1983; Green *et al.*, 1990). Mature renin catalyzes the cleavage of the N-terminal decapeptide from angiotensinogen to form angiotensin I. Angiotensin I is subsequently converted to the octapeptide angiotensin II, which functions as a vasoconstrictor and Na^+ regulator. Renin has an alkaline *p*H optimum relative to other aspartyl proteinases; it has been proposed that the enzyme could function through a substrate-directed catalytic process in which the P_2 His of the angiotensinogen substrate forms a hydrogen bond with the renin active center Asp 226 (Green *et al.*, 1990). Certain workers have described the cloning, expression, and analytical-scale purification of human renin from CHO cells

[1] Protein Biochemistry, Pharmaceutical Discovery Research, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064.
[2] Molecular Biology, Pharmaceutical Discovery Research, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064.
[3] Probe Molecular Design, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, Illinois 60064.
[4] To whom correspondence should be addressed.

0277-8033/90/1200-0663$06.00/0

(Poorman *et al.*, 1986; Ishizuka *et al.*, 1988). Other workers have provided preliminary descriptions of certain steps of semi-preparative purifications from a CHO expression system (Carilli *et al.*, 1988) used to produce renin for X-ray structural analysis (Sielecki *et al.*, 1989). Recently, the cloning and expression of prorenin in a mouse mammary tumor cell line has been reported (Green *et al.*, 1990). Based on cost, time, or material quality, none of the expression and purification techniques in these various reports were judged to be suitable for the repeated preparation of large quantities (100 mg–1 g scale) of prorenin/renin. Routine, reliable, expression and purification procedures are required for a thorough physical/chemical analysis of the protein and its interactions with various inhibitors by such techniques as X-ray crystallography and isotope-edited NMR (Otting *et al.*, 1986; Bax and Weiss, 1987; Fesik *et al.*, 1988) and heteronuclear three-dimensional NMR techniques (Fesik and Zuiderweg, 1988; Marion *et al.*, 1989; Fesik and Zuiderweg, 1990). In this work we describe our independent efforts in the cloning, expression, and purification of human prorenin, and our initial observations relating to the protein chemical characterization of the proenzyme and trypsin-activated mature renin.

2. MATERIALS AND METHODS

2.1. Molecular Biology Reagents

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I, and klenow fragment were obtained from Gibco-BRL, Boehringer-Mannheim, or New England BioLabs. Calf intestine alkaline phosphatase was obtained from Boehringer-Mannheim. Molony murine leukemia virus (M-MLV) reverse transcriptase, EcoRI methylase, and HB101 frozen competent cells were obtained from Gibco-BRL. Oligo-dT and Oligo-dT cellulose were obtained from Pharmacia. Endoglycosidases were obtained from New England Nuclear. Polyethylene glycol 8000 (PEG) was obtained from the Sigma.

2.2. Construction and Isolation of a Human Preprorenin cDNA

The sequentially constructed plasmids for preparation of the PsvPprBgh construct are presented in Fig. 1. Total RNA was prepared from primary human chorion cells secreting high levels of prorenin by the guanidine isothiocyanate/cesium chloride method and poly(A)$^+$ RNA was isolated by two cycles of chromatography on oligo-dT cellulose. First strand cDNA was synthesized using M-MLV reverse transcriptase. Second strand cDNA was synthesized as described by Gubler and Hoffman (1983). The cDNA was then methylated using EcoRI methylase. The cDNA was blunt-end ligated to phosphatase-treated, HincII-digested pUC19 plasmid DNA by T4 DNA ligase, in the presence of 15% PEG (Zimmerman and Pheiffer, 1983). The resultant multimers were digested with EcoRI (which cleaved within the pUC19 multicloning site). The DNA was then circularized using T4 DNA ligase and transformed into *E. coli* employing selection for ampicillin resistance. Approximately 50,000 transformants were grown on nitrocellulose membranes on LB-ampicillin plates, replica plated, and screened using two different ^{32}P-labeled oligonucleotides complementary to the known human renin cDNA sequence (GGTACAGGAGCCCCAGAGCA and TTGGGAATTCTCGGAATCTC, corresponding to bases 86–105 and 731–750, respectively) (Imai *et al.*, 1983). Positive colonies were picked, streaked onto agar plates, and reprobed to isolate individual clones. The clones were then characterized by restriction site mapping and DNA sequence analysis of the cDNA termini. pEG1 was found to be missing 15 bases on the 5′ end and to be complete on the 3′ end, which includes the complete preprorenin coding sequences (Imai *et al.*, 1983).

The human renin cDNA plasmid pEG1 was digested with XbaI and HindIII, liberating the complete renin cDNA sequence. This was ligated into XbaI/HindIII-cleaved pBGH, a plasmid containing a synthetic bovine growth hormone poly-A addition site (GenBank entry BOVGH, bases 1946–2167) inserted into pUC18 (Fig. 1, upper panel). The latter was then digested with HindIII and partially digested with EcoRI. The fragment containing the renin cDNA and the bovine growth hormone poly-A site was isolated by agarose gel electrophoresis. The vector pSV2dhfr was digested with HindII and PvuII, and the fragment containing the SV40 early promoter was isolated by agarose gel electrophoresis. Another aliquot of pSV2dhfr was digested with BamHI, then treated with DNA polymerase I klenow fragment to fill in the cohesive ends. The DNA was then digested with EcoRI, treated with calf intestinal phosphatase, and the large vector fragment was isolated by gel electrophoresis. The three isolated fragments were

then treated with DNA ligase to yield the renin expression vector PsvPprBgh.

2.3. CHO Cell Expression

The above-designed plasmid was transfected into dhfr$^-$ CHO cells (DXB-11 from Laurence Chasin, Columbia University) via the $CaPO_4$ precipitation method (Wigler *et al.*, 1979). The selection medium was Hams F-12 minus hypoxanthine, thymidine, and glycine with L-glutamine (Minus Medium formula #81-0264AJ, Gibco) with 10% dialyzed fetal bovine calf serum (FBS) (Gibco). Serum was dialyzed in 12–14,000 molecular weight cut-off (MWCO) tubing in seven changes (volume dialysis ratio = 3.3 : 1) of D-phosphate-buffered saline (D-PBS) (Gibco), filtered twice (0.2 μm filter), and stored at −20°C until used. Amplification was accomplished via resistance to increasing methotrexate (Aldrich) concentrations. The transfected CHO cell population was first acclimated to growth medium with 150 nM methotrexate 1 week beyond outgrowth followed by growth medium with 300 nM methotrexate 1 week beyond outgrowth and then cloned by limiting dilution. The selected clone demonstrating the greatest prorenin expression was then stepped up to 600 nM methotrexate and finally 1 μM methotrexate 1 week beyond outgrowth. CHO cells were then subcultured twice weekly at a 1 : 2 split ratio via trypsinization [0.25% in Hanks balanced salt solution (HBSS)] in vented 75 cm^2 or 225 cm^2 culture flasks (Costar) or 1 L roller bottles (850 cm^2 surface area, 300 ml media per bottle) (Corning).

2.4. Prorenin Purification

Certain separation methods used were variants of those devised by Egan *et al.* (1988) for the purification of prorenin from human chorion and activation to renin. In particular, Prep Superose 12 (Pharmacia) was substituted for Ultrogel AcA-44 (LKB-Pharmacia) and the blue-dye chromatography step, for selective binding of prorenin, was retained. The affinity column step for isolation of activated renin, using the Boc-Phe-His-Leu^R-Val-Ile-His ligand, was eliminated. The residual "nascent" renin (below) was removed using either a commercial pepstatin affinity resin (Sigma) or an affinity resin (Holzman *et al.*, 1990) based on the Abbott renin inhibitor A-64662 (Kleinert et al., 1988).

Table I. Predicted N-terminal Sequence of Human Preprorenin, Prorenin, and Renin

NH_2-M-D-G-W-R-R-M-P-R-W-
−66 −60
Preprorenin

G-L-L-L-L-L-W-G-S-C-
−50

T-F-G-L-P-T-D-T-T-T-
−40
Prorenin

F-K-R-I-F-L-K-R-M-P-
−30

S-I-R-E-S-L-K-E-R-G-
−20

V-D-M-A-R-L-G-P-E-W-
−10

S-Q-P-M-K-R-L-T-L-G-
−1 1
Renin

N-T-T-S-S-V-I-L-T-N-
10

Preprorenin begins at Met −66, prorenin begins at Leu −43, and renin begins at Leu 1, after the dibasic Lys-Arg cleavage site, sequence based on Imai *et al.* (1983).

2.4.1. Prorenin Production and Preparation of CHO Cell Supernatants

Prorenin was produced in minus medium with 0.5% dialyzed FBS without methotrexate but with penicillin (20 U/ml) and streptomycin (20 μg/ml). The production medium was harvested every 3–4 days and collected in 1 L bottles following 0.45 μm filtration. Phenyl methyl sulfonyl fluoride (PMSF) was added to 10 μg/ml and the pooled supernatants were stored at 4°C with sodium azide added (0.004%, final) until subsequently processed in larger 20–30 L batches, usually after 1–2 weeks. Assays indicated there were small amounts of renin activity (5–10% of total after activation) observable before processing (below). This material likely arises either from further proteolytic processing beyond the protein pre-pro region (Table I) during export of the protein from the cell or from partial proteolysis in the cell culture medium after export. For descriptive purposes we term this material "nascent" renin.

2.4.2. Step 1: Concentration and Buffer Exchange

The purification process was initiated at room temperature (~22°C) by pooling 20–30 L of CHO cell supernatant in 20-L carboys followed by the stirred

addition of stock ethylenediamine tetraacetic acid (EDTA) (100 mM, *p*H 7.9) to 1 mM final and solid PMSF to 100 μg/ml. After the PMSF was dispersed (~30 min) the pooled CHO cell supernatant was passed through a 0.45-μm cartridge filter (Gelman Sciences) and concentrated using a Minisette tangential flow concentrator (Pharmacia) equipped with a set of five 10 kD Omega-type membranes (4.5 ft^2 total surface area). The entire pooled volume was typically concentrated to 300–500 ml in 3–5 hr. After concentration, the solution buffer was exchanged with the same apparatus using consecutive dilutions to 1 L with 6 L of 20 mM Tris, *p*H 7.9 (buffer A) (~96–98% buffer exchange in ~2 hr).

2.4.3. Step 2: Batch Extraction

After buffer exchange, aliquots of DEAE cellulose (hydroxide form, Whatman DE-52), preequilibrated in buffer A, were added at room temperature (~22°C) to the concentrated, buffer-exchanged sample. The adsorption of protein on the ion-exchange resin was monitored by the decrease in 280 nM absorbance of the sample. It was usually possible to capture +95% of the total 280 nm absorbance by the addition of 70–110 g of suction-dried ion-exchange resin (35–40 A_{280} U/g of resin). The resin was then transferred to an 11-cm Buchner funnel and washed with three to four 100-ml aliquots of buffer A to remove unbound protein. Protein was then eluted by batch extraction with three to four 100 ml volumes each of the following step buffers: buffer A with 60 mM NaCl and buffer A with 80 mM NaCl.

2.4.4. Step 3: Affinity Chromatography to Remove Nascent Renin Activity.

Nascent renin was captured by passing the pooled activity from the batch extraction step over a pepstatin affinity column or an A-64662 affinity column (both 2.5 × 9 cm, ~44 ml). The sample application, column washing, and renin elution were performed at flow rates of ~100 ml/hr at room temperature (~22°C). The breakthrough from the column, containing the prorenin, was subjected to blue-dye chromatography (below). After capture of the breakthrough from the affinity column, the column was washed with 4–5 column volumes of 50 mM Tris, 100 mM NaCl, *p*H 7.5. Nonspecifically bound protein was removed by washing with 2–3 column volumes of 1.0 M NaCl, 25 mM acetate, *p*H 5.5. Bound nascent renin was eluted in a single peak by *p*H shift to 9.5 with 0.1 M 2-[*N*-cyclohexylamino]-ethanesulfonic acid (CHES). After use, the affinity columns were washed with 5.4 M guanidine HCl and stored in 1 : 1 ethanol : water.

2.4.5. Step 4: Blue-Dye Chromatography

The breakthrough from the pepstatin column was applied to a blue-dye column (Biorad) (2.5 × 20 cm, ~100 ml) using a Pharmacia FPLC maintained at 4°C in a cold room. After washing with 300 ml of 50 mM Tris, 100 mM NaCl, *p*H 7.9, the prorenin was eluted in the same buffer with a 1000 ml linear gradient to 1.0 M NaCl. Application, washing, and elution steps were all performed at a flow rate of 1.0 ml/min.

2.4.6. Step 5: Size Exclusion Chromatography

Pooled activity from blue-dye chromatography was concentrated ~20-fold using an Amicon YM-10 membrane on an Amicon CEC1 tangential flow concentrator. The concentrated sample was chromatographed at 1.0 ml/min on a Prep Superose 12 (Pharmacia) column (5 × 95 cm, ~1.9 L) preequilibrated in 25 mM Tris, 50 mM NaCl, *p*H 7.5, 1 mM EDTA, using a Pharmacia FPLC maintained at 4°C in a cold room.

2.5. Enzyme Assays

Activity measurements were performed using a new fluorogenic peptide substrate for renin (Holzman *et al.*, 1990; Wang *et al.*, 1990). The substrate, DABCYL-IHPFHLVIHT-EDANS [4-(4-diamethylaminophenylazo) benzoic acid-IHPFHLVIHT-5-[(2-aminoethyl)-amino]naphthalene-1-sulfonic acid] (Wang *et al.*, 1990), has an excitation maximum at ~340 nm and an emission maximum at ~490 nm when cleaved by renin. Prior to assay prorenin was activated with TPCK-trypsin (Worthington Biochemicals) using the protocol of Egan *et al.* (1988). Initial kinetic rates were measured in 0.10 M phosphate, *p*H 6.7, with a Shimadzu RF-5000U spectrofluorimeter using a total assay volume of 115 μl and a final fluorogenic substrate concentration of 20 μM (Holzman *et al.*, 1990; Wang *et al.*, 1990). For assay of multiple samples, the generation of fluorescence was measured at single, fixed time-points using a Perkin-Elmer LS-50 Luminescence Spectrophotometer with a 96-well microtiter plate attachment. A sample volume of 200 μl/well was used with a final fluororgenic substrate concentration of 20 μM.

2.6. Gel Electrophoretic Analyses

Gel electrophoretic analyses were performed using a Pharmacia Phast gel system and Pharmacia precast gels for gradient sodium dodecyl sulfate (SDS) electrophoretic analysis. Standard Pharmacia instrument control programs were used for automated electrophoresis, staining, and destaining of all gels.

2.7. N-terminal Sequence and N-Linked Sugar Analysis

Prior to N-terminal sequence analysis, protein samples were subjected to HPLC on a Vydac C4 column (4.6 × 300 mm) using a linear gradient from H_2O/0.1% trifluoracetic acid (TFA) to acetonitrile (ACN)/0.1% TFA as described in Figs. 3–5. After sample collection, the protein was dried in a Savant Speed-Vac. Samples for sequencing were resuspended in 1 : 2 : 7 TFA, formic acid, H_2O and immediately applied to a glass-fiber filter support treated with polybrene and precycled on the sequencer. Sequencing was accomplished using an Applied Biosystems model 471A Gas/Pulsed Liquid protein sequencer and standard sequencing programs. Chromatographic data from the integral sequencer HPLC was acquired using a Beckman HPLC system equipped with a model 406 analog-to-digital converter. Protein N-linked sugar composition was determined after a 20-hr sample hydrolysis in 4 N HCl, 90°C, followed by chromatographic analysis on a Beckman model 6300 amino acid analyzer using a standard Beckman separation program protocol.

3. RESULTS AND DISCUSSION

3.1. Cloning and Expression of Prorenin in CHO Cells

Upon $CaPO_4$ transfection, both PsvPprBgh (Fig. 1) and a control plasmid, pSV2dhfr, produced striking changes in the morphology and doubling times of the CHO cells. For both, the cells appeared highly transformed, and the cell doubling times were markedly reduced. Three-day culture supernatants from cells transfected with PsvPprBgh showed prorenin activity on 1–2 mg/L, whereas culture supernatants of cells transfected with pSV2dhfr showed no detectable renin activity.

The PsvPprBgh-transfected cell pools were cultured in the presence of increasing levels of methotrexate in order to increase the levels of renin expression *via* gene amplification. The cells were cultured for 1 week at a level of 150 nM methotrexate, followed by a 1 week at 300 nM methotrexate. The cells appeared to tolerate these increases without distress. At this point the cell pools were cloned by limiting dilution into microtiter plates. Individual clones were expanded to T-75 flasks, and 3-day culture supernatants were assayed for total renin activity. One clone (IV-2G), of approximately 100 clones assayed, gave values of approximately 15 mg/L total renin activity. Clone IV-2G was selected for further work. As this clone was grown in roller bottles for large-scale expression, its rapid growth rate tended to exhaust the medium (resulting in cell death) if left for longer than 3 days. It required splitting every 3 days. It was found that by switching from F12/10% serum to F12/0.5% serum, after the cells had reached confluence, the cells could be kept healthy and productive for as long as 6 weeks with twice weekly media changes. However, while the prorenin production could be as high as 15 mg/L in small flasks, in roller bottles the best that could be obtained was only 4–5 mg/L.

Clone IV-2G was adapted to growth in higher methotrexate concentrations in an attempt to increase its expression level. Methotrexate was sequentially increased to 600, 1000, 3000, and finally 5000 nM. At the levels of 1000–5000 nM methotrexate the cells were severely stressed and took some weeks to adapt. However, when these cells were grown out into roller bottles there was no significant improvement in their levels of renin expression. Thus, production runs for prorenin were maintained at the lower methotrexate levels.

3.2. Purification and Protein Chemical Characterization of Prorenin

When CHO cells were grown in low levels of added serum (0.5% serum) it was possible to purify prorenin from cell supernatants using two simple preparative column separations (see Materials and Methods). The elution profile for the final size exclusion column is shown in Fig. 2. Based on gel electrophoresis we estimate the pooled prorenin is >95% pure at this stage (Fig. 2, inset).

Sequence analysis of the first 15 residues of purified recombinant protein revealed N-terminal processing of the preprorenin construct at Leu −43 with a small amount of the second cycle proline present (Fig. 3). Although prorenin or renin derived from kidney or chorion are glycosylated (Imai *et al.*, 1983; Egan *et al.*, 1988), the composition and

sequence of the attached oligosaccharides are unknown. The natural prorenin/renin, as well as the recombinant protein construct, have two potential N-linked glycosylation sites (Asn 5, Asn 75). Analysis of the recombinant protein for amino sugar composition revealed the presence of only glucosamine (≤5 mol/mol protein) and low levels of sialic acid (S. Rao, personal communication) (< ~0.2% w:w). These data indicate the CHO-cell-derived recombinant protein is not heavily glycosylated.

Activation of the recombinant prorenin by trypsin using the conditions of Egan *et al.* (1988) produced mature enzyme with a specific activity of ~1000 Goldblatt units (GU)/mg. For comparison, Egan *et al.* (1988) observed suitably purified and activated human chorionic renin had a specific activity of ~1100 GU/mg. With initial-rate assay methodology and the new fluorogenic substrate for renin (see Materials and Methods), the mature protein exhibited a specific activity of ~40 arbitrary fluorescence units (FU)/min-μg at *p*H 6.7, 37°C (Holzman *et al.*, 1990). For a typical 20–30 L pool of culture media, yields of total renin (both as nascent and prorenin) ranged from 1–5 mg/L. N-terminal sequence analysis of the trypsin-activated mature renin indicated the presence of a single amino terminal Leu residue but two amino terminal sequences (Fig. 4). One site was consistent with trypsin cleavage at Arg −11, the other with trypsin cleavage at Arg −1. Due to the complexity of the Edman chemistry it is not possible to precisely quantitate sequence data for exact levels of the two sequences. However, the data suggested the two N-termini were present in roughly equal proportions. Based on this observation we conclude that it is

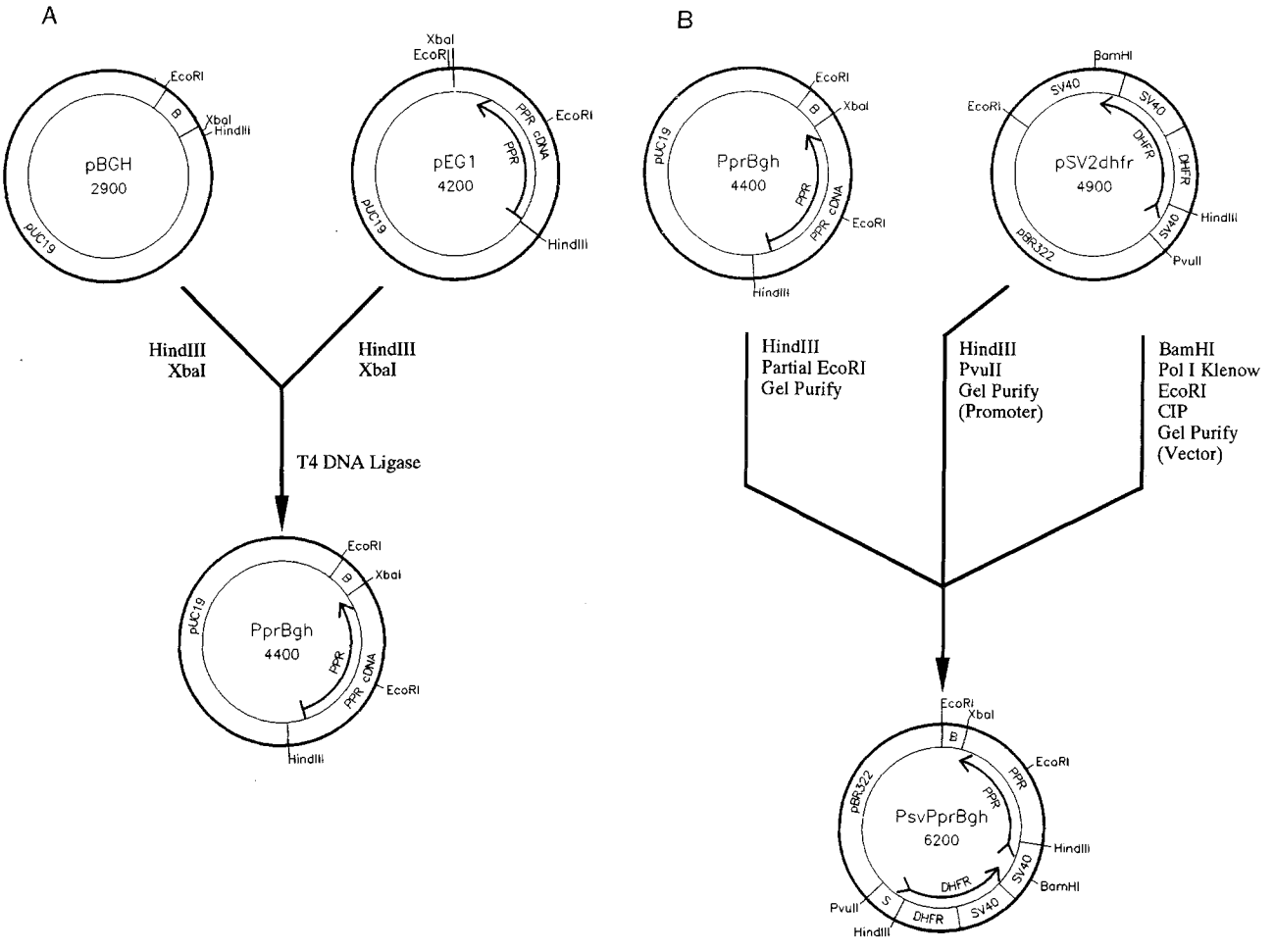

Fig. 1. Schematic construction sequence for a plasmid coding for human prorenin. (A) Construction of a gene cassette consisting of a human renin cDNA linked to a bovine growth hormone poly-A addition site. (B) Construction of the human prorenin expression plasmid PsvPprBgh.

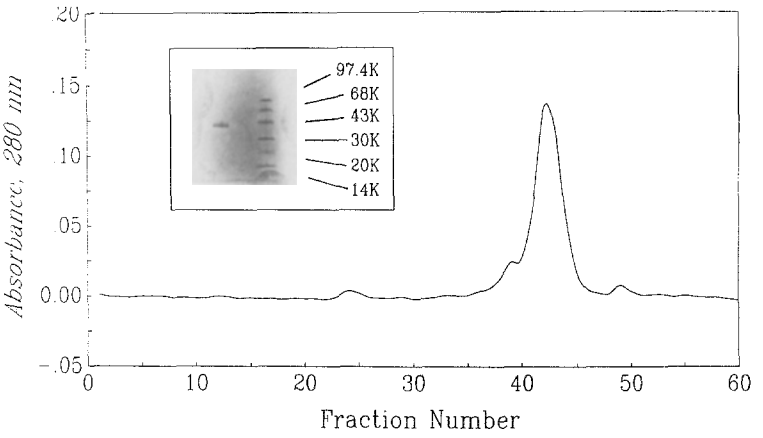

Fig. 2. Size exclusion chromatography of prorenin on superose 12. (Inset) SDS gel of pooled 20 ml fractions 41–47. The gel was an 8–25% gradient Pharmacia Phast gel with a 4 μl comb loading volume for samples and standards. The gel was electrophoresed and stained with Coomaisse blue using standard SDS gel protocols from Pharmacia. (SDS Gel Lanes) Right lane, Pharmacia MW standards: phosphorylase B, 97.4K; bovine serum albumin, 68K; ovalbumin, 43K; carbonic anhydrase, 30K; trypsin inhibitor, 20K; alpha lactalbumin, 14K; left lane, purified prorenin. Based on the stained SDS bands, we estimate the purified prorenin is ~95+% pure.

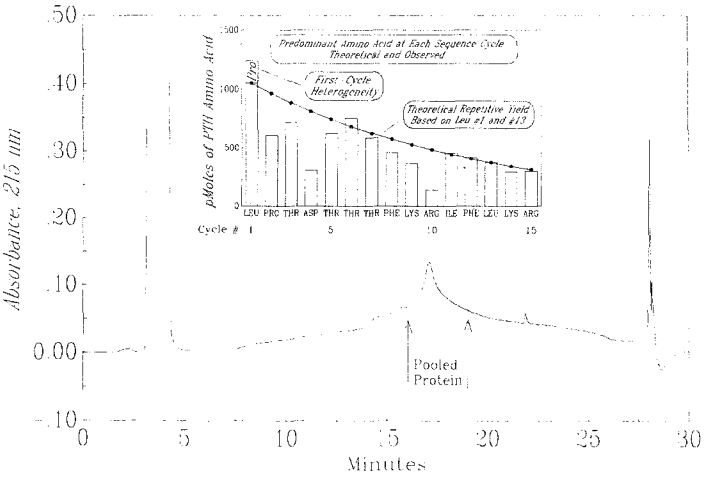

Fig. 3. Reverse-phase HPLC and N-terminal sequence analysis of recombinant prorenin. The purified prorenin was analyzed by chromatography on a Vydac C4 reverse-phase column (4.5×300 mm). The column was developed with a 20-min linear gradient (starting at 2 min after injection at $t=0$) from H_2O/0.1% TFA to ACN/0.1% TFA. The pooled protein from the prorenin peak was dried on a Savant speedvac and subjected to 15 cycles of N-terminal sequence analysis (inset). Based on Leu residues 1 and 13 the repetitive yield was calculated to be 94%. The small amount of amino terminal proline observed (<15%) was consistent with partial removal of the first-cycle amino terminal Leu.

possible to have a significant portion of the enzyme population possessing an extra amino terminal segment and still maintain high specific activity. A corollary of this conclusion is that it is sufficient to remove prorenin residues −43 to −12 to produce an enzymatically active molecule. This interpretation is consistent with the recent report on the activation of prorenin after mutational alteration of Arg residues in the prosequence (Yamauchi *et al.*, 1990) and with the N-terminal heterogeneity observed for nascent CHO cell renin isolated by affinity chromatography on a pepstatin column (below).

The active nascent renin recovered from processed culture media by affinity chromatography (see Materials and Methods) was also analyzed for N-terminal sequence (Fig. 5). This material showed clear evidence of multiple amino terminal sequences. The sequence data was consistent with at least five start sites (Fig. 5, inset) at residues −11, −6, 9, 13, and 32. These data demonstrate that a unique amino terminal sequence is not required for the protein to bind to a pepstatin affinity resin. Thus, the nature and geometries of enzyme-active center interactions with the immobilized pepstatin inhibitor must be, to a certain extent, degenerate. That is, the active center appears to maintain sufficient integrity to bind to the pepstatin affinity ligand even when proteolytic clipping removes as much as the first 31 residues of the normal protein amino terminus. In addition, the sequence data suggests that clipping of the proseg-ment after Arg −12 is sufficient to permit binding to the pepstatin affinity ligand, and that at least the form of the protein beginning at Leu −11 is enzymatically active (above). Although the absence of observed sequence for residues −12 to −43 for affinity-purified nascent renin does not constitute proof, it suggests

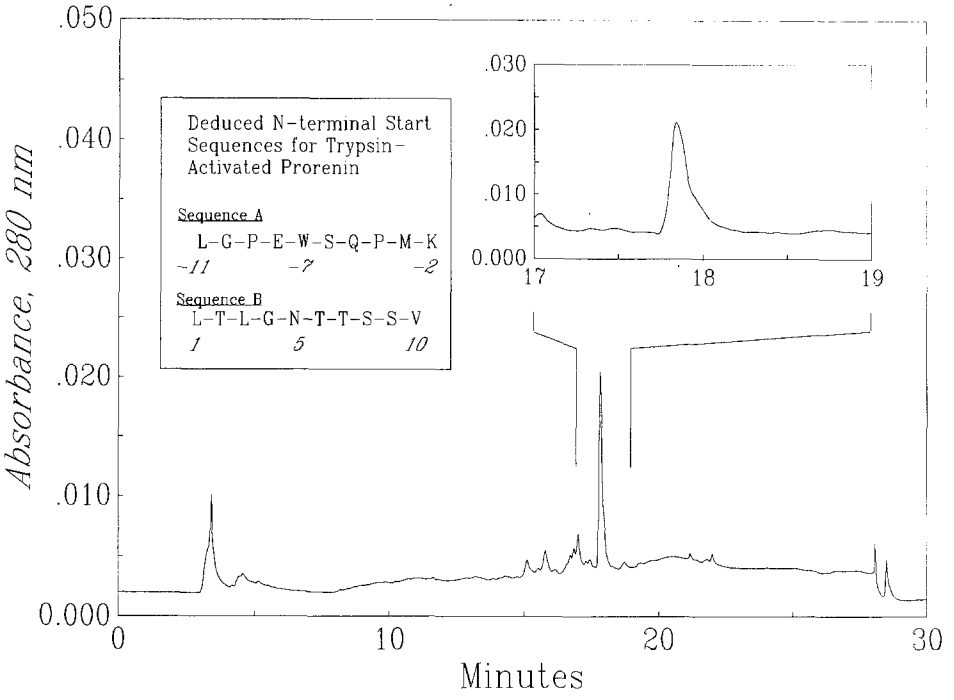

Fig. 4. Reverse-phase HPLC and N-terminal sequence analysis of mature renin from trypsin-activated prorenin. The purified renin was analyzed by chromatography on a Vydac C4 reverse-phase column (4.5 × 300 mm). The column was developed with a 20-min linear gradient (starting at 2 min after injection at $t = 0$) from H_2O/0.1% TFA to ACN/0.1% TFA. The pooled protein from the renin peak (~17.8 min, inset) was dried on a Savant speedvac and subjected to 10 cycles of N-terminal sequence analysis (inset). The first cycle of Edman degradation yielded only Leu. The deduced sequence consistent with the remaining cycles indicated the presence of two amino termini, one starting at Leu −11 the other at Leu 1.

that the presence of residues beyond Arg −12 can act to block enzyme binding to the pepstatin affinity ligand.

3.3. Activation of Recombinant Prorenin to Renin and the *p*H-Activity Profile of the Mature Enzyme

In the angiotensinogen assay the cleavage of human angiotensinogen by renin to form angiotensin I is measured by antibody-mediated binding competition with radiolabeled angiotensin I (Fig. 6). The recombinant mature renin exhibits the normal *p*H-activity profile expected for natural renin (Galen *et al.*, 1979; Slater *et al.*, 1981). Although the assay may be performed with commercial radioimmunoassay kits using automated pipetters (Egan *et al.*, 1988), it is still labor intensive and is awkward to use for enzyme kinetic measurements. By comparison, the fluorogenic substrate assay is simple to perform and ideal for enzyme kinetic measurements (Wang *et al.*, 1990) and fixed-time measurements using a fluorescence-based titer plate reader (G. T. Wang *et al.*, manuscript in preparation). The assay relies on the rate of generation of a fluorescent signal from cleavage of a fluorophore-containing peptide. In the intact peptide the intervening peptide sequence acts to spatially constrain the distance between a fluorophore (EDANS) at the peptide C-terminus and a chromophore (DABCYL) at the peptide N-terminus. The fluorophore and chromophore are chosen to have an appropriate overlap integral between the emission dipole of the fluorophore and the absorption dipole of the chromophore. This dipole-dipole coupling of the donor-acceptor pair permits nonradiative, or Förster, energy transfer to occur upon excitation of the fluorophore (Lackowicz, 1983). Cleavage of the peptide results in an immediate increase in the average distance between donor-acceptor pairs. Since the rate of energy transfer falls off as function of $1/r^6$, upon cleavage by renin fluorophore quenching is eliminated and a fluores-

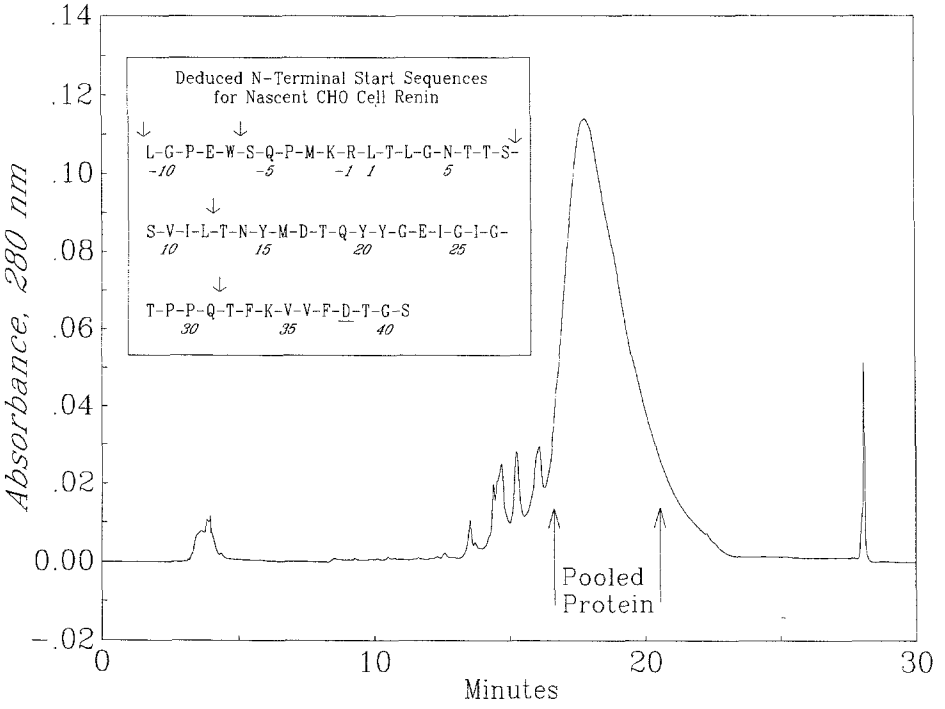

Fig. 5. Reverse-phase HPLC and N-terminal sequence analysis of nascent renin isolated by pepstatin affinity chromatography from cell culture media. The purified renin was analyzed by chromatography from cell culture media. The purified renin was analyzed by chromatography on a Vydac C4 reverse-phase column (4.5 × 300 mm). The column was developed with a 20-min linear gradient (starting at 2 min after injection at $t = 0$) from H_2O/0.1% TFA to ACN/0.1% TFA. The pooled protein from the nascent renin peak was dried on a Savant speedvac and subjected to 10 cycles of N-terminal sequence analysis (inset). The first cycle of Edman degradation yielded Leu, Thr, and Ser. The deduced sequence consistent with the remaining cycles indicated the presence of at least five amino termini inset, vertical arrows).

cence signal is observed. For assay of renin, the fluorogenic substrate contains the P_6-P_3' sequence of human angiotensinogen normally cleaved by renin (Holzman *et al.*, 1990; Wang *et al.*, 1990). The *p*H-activity profiles for mature renin assayed with angiotensinogen and the fluorogenic substrate (DABCYL-IHPFHLVIHT-EDANS) are presented in Fig. 6. These data demonstrate that, while the recombinant renin has a normal *p*H-activity profile with human angiotensinogen (a *p*H optimum at *p*H ~5.4), the activity profile is dramatically shifted by ~2.5 *p*H units to *p*H ~8.0 with the fluorogenic substrate.

The substrate specificity of a recombinant renin has recently been examined using two tetradecapeptides derived from porcine angiotensinogen (Green *et al.*, 1990). One peptide contained the normal procine P_2-site His and the other a P_2 Gln. The normal His derivative exhibited a maximum velocity at *p*H ~7.0, while the Gln derivative exhibited maximum velocity at *p*H ~5.5 (Green *et al.*, 1990). The authors propose a hydrogen bond is formed between the tetradecapeptide (or angiotensinogen) P_2 His and renin Asp-226. The data presented by Green *et al.* (1990) are taken to indicate that renin hydrolysis of angiotensinogen proceeds by virtue of *substrate-directed* catalysis. It is proposed that a specific hydrogen bond between substrate and enzyme is central to the catalytic process and is primarily responsible for the alkaline-shifted *p*H-activity profile of renin relative to other aspartyl proteinases (Green *et al.*, 1990). It is important to note, however, that the P′ residues in the porcine angiotensinogen sequence are different than those in human angiotensinogen. In particular, the P_1' sequence for human angiotensinogen contained in our fluorogenic substrate (Val-Ile-His, P_1'-P_3') differs in spatial volume at each residue and hydrogen bonding capability at the P_3' His in comparison to the porcine P_1' sequence (Leu-Val-Tyr). The data supporting the arguments of Green *et al.* (1990)

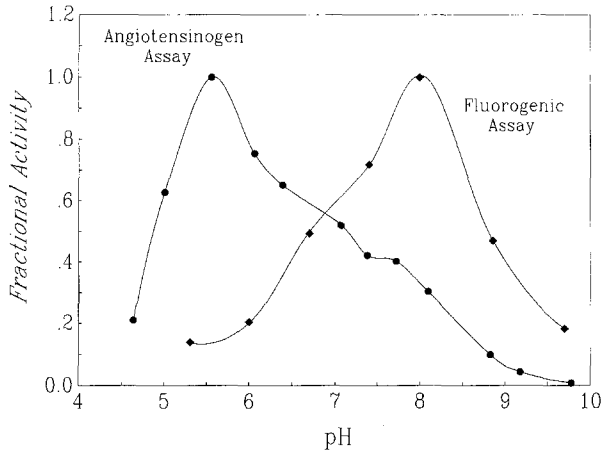

Fig. 6. *p*H-activity profiles of CHO renin using fluorogenic and angiotensinogen assays. The mature CHO renin was prepared as described under Materials and Methods. The buffers for the fluorogenic assay were as follows. *p*H 9.78: 100 mM CHES, 3 mM EDTA; *p*H 8.85: 1 part *p*H 9.7, 1 part *p*H 8.09; *p*H 8.09: 125 mM Tris, 3 mM EDTA; *p*H 7.4: 125 mM HEPES, 3 mM EDTA; *p*H 6.0: 167 mM maleic acid, 3 mM EDTA; *p*H 4.6: 125 mM succinic acid, 3 mM EDTA; *p*H 6.7: 1 part *p*H 7.40, 1 part *p*H 6.00; *p*H 5.30: 1 part *p*H 6.0, 1 part *p*H 4.63. Fluorogenic assays were performed in 5-mm fluorescence cuvettes using 200 μl of the appropriate buffer, 25 μl renin (~5 nM, final), and 25 μl of the fluorogenic substrate in dimethyl sulfoxide (DMSO) (25 μM, final) with excitation and emission monochomator settings as described in Materials and Methods. Initial rates of cleavage of the fluorogenic substrate were measured in arbitrary fluorescence units per minute. The buffers for the angiotensinogen assay were as follows. *p*H 9.78: 100 mM CHES, 3 mM EDTA; *p*H 9.18: 1 part *p*H 8.00, 2 parts *p*H 9.78; *p*H 8.85: 1 part *p*H 9.78, 1 part *p*H 8.0; *p*H 8.0: 125 mM Tris, 3 mM EDTA; *p*H 7.72, 1 part *p*H 7.4, 1 part *p*H 8.0; *p*H 7.4: 125 mM HEPES, 3 mM EDTA; *p*H 7.07: 1 part *p*H 6.00, 2 parts *p*H 7.4; *p*H 6.39: 2 parts *p*H 6.0, 1 part *p*H 7.4; *p*H 6.00: 167 mM maleic acid, 3 mM EDTA; *p*H 5.56: 1 part *p*H 4.63, 2 parts *p*H 6.0; *p*H 5.01: 2 parts *p*H 4.63, 1 part *p*H 6.0; *p*H 4.63: 125 mM succinic acid, 3 mM EDTA. The angiotensinogen assay was performed as previously described (Egan *et al.*, 1988). Rates were measured at fixed time-points as nanograms angiotensin I generated per hour. The rates for each assay were normalized to the maximum value observed for each type of *p*H-activity profile assay.

are interesting. However, the extent of the alkaline shift in the *p*H-activity profile we observe, with the fluorogenic substrate based on the human angitensinogen sequence, suggests there may be additional interactions with the P′ residues, other than an enzyme-substrate hydrogen bond at the P_2 His, which contribute to an alkaline renin catalytic profile relative to other aspartyl proteinases.

ACKNOWLEDGMENTS

We thank Sally Dorwin for amino sugar analysis, Dr. Suba Rao, of PPD Analytical Research, for sialic acid content analyses, and Dr. Herman Stein for useful discussions.

REFERENCES

Bax, A., and Weiss, M. A. (1987). *J. Mag. Reson.* **71**, 571-575.
Carilli, C. T., Wallace, L. C., Smith, L. M., Wong, M. A., and Lewicki, J. A. (1988). *J. Chromatog.* **444**, 203-208.
Egan, D. A., Grzegorczyk, V., Tricarico, K. A., Rueter, A., Holleman, W. A., and Marcotte, P. A. (1988), *Biochem. Biophys. Acta* **965**, 68-75.
Fesik, S. W., Gampe, R. T., and Rockway, T. W. (1987). *J. Mag. Reson.* **74**, 366-371.
Fesik, S. W., Luly, J. R., Erickson, J. W., and Abad-Zapatero, C. (1988). *Biochemistry* **27**, 8297-8301.
Fesik, S. W., and Zuiderweg, E. R. P. (1988). *J. Mag. Reson.* **78**, 588-593.
Fesik, S. W., and Zuiderweg, E. R. P. (1990). *Q. Rev. Biophys.* **23**, 97-131.
Galen, F. X., Devaux, C., Guyenne, T., Menard, J., and Corvol, P. (1979). *J. Biol. Chem.* **254**, 4848-4855.
Green, D. W., Aykent, S., Gierse, J. K., and Zupec, M. E. (1990). *Biochemistry* **29**, 3126-3133.
Gubler, U., and Hoffman, B. J. (1983). *Gene* **25**, 263-269.
Holzman, T. F., Chung, C. C., Egan, D. A., Gubbins, E. G., Rueter, A., Yang, L., Pederson, T., Krafft, G. A., and Wang, G. T. (1990). *Biophys. J.* **57**, 38a.
Imai, T., Miyazaki, H., Hirose, S., Hori, H., Hayashi, T., Kageyama, R., Ohkubo, H., Nakanishi, S., and Murakami, K. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 7405-7409.
Ishizuka, Y., Saito, M., Hori, H., Poorman, R. A., Yoshida, S., and Murakami, K. (1988). *Biochem. Biophys. Res. Commun.* **152**, 849-856.
Kleinert, H. D., Martin, D., Chekal, M. A., Kadam, J., Luly, J. R., Plattner, J. J., Perun, T. J., and Luther, R. R. (1988). *Hypertension* **11**, 613-619.
Lackowicz, J. R. (1983). *Principles of Fluorescence Spectroscopy*, Plenum, New York, pp. 305-327.
Marion, D., Kay, L. E., Sparks, S. W., Torchia, D. A., and Bax, A. (1989). *J. Am. Chem. Soc.* **111**, 1515-1517.
Otting, G., Senn, H., Wagner, G., and Wüthrich, K. (1986). *J. Mag. Reson.* **70**, 500-505.
Poorman, R. A., Palermo, D. P., Post, L. E., Murakami, K., Kinner, J. H., Smith, C. W., Reardon, I., and Heinrikson, R. L. (1986). *Proteins: Structure, Function, Genetics* **1**, 139-145.
Sielecki, A. R., Hayakawa, K., Fujinaga, M., Murphy, M. E. P., Fraser, M., Muir, A. K., Carilli, C. T., Lewicki, J. A., Baxter, J. D., and James, M. N. G. (1989). *Science* **243**, 1346-1351.
Slater, E. E., and Strout, H. V. (1981). *J. Biol. Chem.* **256**, 8164-8171.
Wang, G. T., Chung, C. C., Holzman, T. F., and Krafft, G. A. (1990). *Biophys. J.* **57**, 39a.
Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1979). *Cell* **14**, 725-731.
Yamauchi, T., Nagahama, M., Watanabe, T., Ishizuka, Y., Hori, H., and Murakami, K. (1990). *J. Biochem.* **107**, 27-31.
Zimmerman, S. B., and Pheiffer, B. H. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 5852-5856.