Wong, G., Kawajiri, K., Kato, Y., Harada, N., & Negishi, M. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 2176.
Yabusaki, T., Shimizu, M., Murakami, H., Nakamura, K., Oeda, K., & Ohkawa, H. (1984) *Nucleic Acids Res. 12*, 2929–2938.
Yoshioka, H., Norohashi, K., Sogawa, K., Miyata, T., Kawajiri, K., Hirose, T., Inayama, S., Fujii-Kuriyama, Y., & Omura, T. (1987) *J. Biol. Chem. 262*, 1706–1711.
Young, R. A., & Davis, R. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.

Characterization and Purification of a Protease in Serum That Cleaves Proatrial Natriuretic Factor (ProANF) to Its Circulating Forms†

Jerome B. Zisfein, Robert M. Graham, Stephen V. Dreskin, Gary M. Wildey, Alan J. Fischman, and Charles J. Homcy*

Cellular and Molecular Research Laboratory, Cardiac Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

Received April 15, 1987; Revised Manuscript Received June 30, 1987

ABSTRACT: Atrial natriuretic factor (ANF) is synthesized and stored in atrial cardiocytes as a 17-kilodalton (kDa), 126 amino acid polypeptide, proANF, but circulates as smaller, 24 and 28 amino acid peptide fragments of the carboxy terminus of proANF. It has previously been shown that proANF is secreted intact from cultured atrial cardiocytes and can be cleaved by a serum protease to smaller, 3-kDa peptides believed to be the circulating forms. This report describes the purification and characterization of this proANF-cleaving protease from rat serum. The cleavages both of ^{35}S-labeled proANF derived from rat atrial cell cultures, as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/autoradiography, and of a synthetic *p*-nitroanilide-containing substrate were used as assays for the detection of enzyme activity. ProANF-cleaving activity was found in rat serum, with no such activity detectable in rat plasma. Cleavage in serum was not dependent on the presence of platelets or other cellular elements. Complete inhibition of proANF cleavage was obtained with the protease inhibitors benzamidine, leupeptin, phenylmethanesulfonyl fluoride, and diisopropyl fluorophosphate (DFP) but not with aprotinin, soybean trypsin inhibitor, pepstatin, or hirudin. Unlike the vitamin K dependent plasma proteins, the proANF-cleaving protease did not adsorb to barium sulfate. With the sequential application of ion-exchange, hydroxylapatite, lectin affinity, and gel filtration chromatography, a 5000–6000-fold purification of the enzyme from rat serum was achieved. Fractionation of either whole serum or the purified enzyme by gel filtration chromatography revealed a single peak of activity corresponding to a protein with a Stokes radius of 45 Å. The p*I* of the enzyme was found to be approximately 5.6. Incubation of the purified enzyme with [^{3}H]DFP followed by SDS–PAGE and autoradiography revealed a specifically labeled 38-kDa peptide, the substrate binding subunit. Analysis by high-performance liquid chromatography of the 3-kDa products resulting from the cleavage of ^{35}S-labeled proANF by the purified enzyme revealed, as previously described with whole serum, two radiolabeled peptides which coeluted with the 28 and 24 amino acid C-terminal peptides. Moreover, a time-dependent increase in the abundance of the latter peptide was found. These observations imply a precursor–product relationship, with the initial cleavage of proANF to the 28 amino acid peptide, which is then cleaved to the 24 amino acid peptide. These studies indicate that the majority of proANF cleavage activity found in rat serum is represented by that of a distinct serine protease whose properties are different from a variety of well-characterized proteases, such as kallikrein, plasmin, and the vitamin K dependent plasma proteins. The role of this protease in the in vivo processing of proANF remains to be defined.

Atrial natriuretic factor (ANF) is synthesized in atrial cardiocytes as a 152 amino acid "preprohormone" (Seidman et al., 1984; Yamanaka et al., 1984; Maki et al., 1984) and is stored in atrial granules as a 126 amino acid polypeptide, proANF (Kangawa et al., 1984; Glembotski et al., 1985; Vuolteenaho et al., 1985; Zisfein et al., 1986a; Miyota et al., 1985). Analysis of immunoreactive and bioactive ANF in blood has revealed that the major circulating forms are the 24 and 28 amino acid peptides, corresponding to the carboxy-terminal fragments of proANF (Sugarawa et al., 1985; Yamaji et al., 1985; Schwartz et al., 1985; Thibault et al., 1985). There is some evidence that cleavage of proANF to these smaller peptides is necessary for full biological activity (Currie et al., 1984a,b). The site of cleavage of proANF to these smaller forms is unknown.

Recently, we have demonstrated by intrinsic labeling studies that proANF, identified as a peptide of approximately 17 kilodaltons (kDa) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), is both stored and secreted by rat atrial cardiocytes in culture (Bloch et al., 1985; Zisfein et al., 1986b). Incubation of the 17-kDa proANF derived from these cells with rat serum results in cleavage to two major fragments: a cysteine-containing 3-kDa peptide and a 14-kDa methionine-containing peptide (Bloch et al., 1987). High-performance liquid chromatography (HPLC) and radiosequence analysis of the 3-kDa fragment revealed the presence of two major peptide products corresponding to the 28 and 24 amino acid carboxy-terminal ANF peptides (Bloch et al.,

† This work was supported by a grant from the National Institutes of Health (HL-35642-01) and by a grant from the R. J. R. Nabisco Co. R.M.G. is an Established Investigator, American Heart Association (AHA 82-240).

* Address correspondence to this author.

0006-2960/87/0426-8690$01.50/0

1987). Similar findings have been reported by other investigators using gel filtration and radioimmunoassay techniques (Glembotski & Gibson, 1985). We now report on the characterization and purification of the protease in rat serum responsible for this apparently specific endolytic cleavage of proANF.

Materials and Methods

Materials

The chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginyl-*p*-nitroanilide dihydrochloride was obtained from Helena Laboratories. Ion-exchange and gel filtration resins were obtained from Pharmacia Co. Leupeptin was obtained from Transformation Research. Other protease inhibitors, factors II and VII deficient plasma, Russell's viper venom, and lectin resins were from Sigma Co. [^{35}S]Cysteine (>600 Ci/mmol) and [^{3}H]DFP (4 Ci/mmol) were obtained from Amersham. Rat atriopeptin III (24 amino acid carboxy-terminal fragment of proANF) and rat ANP (28 amino acid carboxy-terminal peptide) were obtained from Peninsula Laboratories. Liquiscint and Autofluor were obtained from National Diagnostics. SDS–polyacrylamide gels were run on a Hoefer SE 600 electrophoresis apparatus and peptides identified by silver staining (Bio-Rad). Quantitative densitometry was performed on autoradiographs using an LKB 22202 laser densitometer and a Hewlett Packard integrator. HPLC analysis (Beckman-Altex) of ^{35}S-labeled proANF cleavage peptides was performed on a Vydac C-18 (4.6 × 250 mm) analytical column with a Hewlett Packard 8450A spectrophotometer. Fractions were counted in a Beckman LS 1800 β counter at an efficiency of 50%.

Methods

Cleavage Assays. The ability of whole serum or various purified fractions to cleave proANF was evaluated by two separate assays. The first assay is specific, but poorly quantitative. This assay involves the direct evaluation of cleavage of intrinsically labeled proANF by serum or various purified fractions. Neonatal rat atrial cardiocytes were isolated and cultured as previously described (Bloch et al., 1985). On the fifth day in culture, intrinsic labeling was carried out with [^{35}S]cysteine. Confluent, adherent cardiocytes in a 100 × 20 mm culture dish were incubated for 30 min with 6 mL of cysteine-deficient RPMI media, followed by the same media containing 100 μCi of [^{35}S]cysteine for 3 h. We have previously demonstrated that this procedure leads to the accumulation in the culture media of ^{35}S-proANF, with the appearance of little, if any, lower molecular weight products (Bloch et al., 1985). The media were then harvested and either used immediately or frozen at −70 °C for use within 1 week. Cleavage of ^{35}S-proANF was effected by incubating 300 μL of ^{35}S-proANF-containing media with 300 μL of sample in a 1.5-mL plastic microfuge tube at 37 °C for 30 min. Immunoprecipitation of ANF with rabbit antisera directed to the C-terminal 24 amino acid peptide of proANF (rat atriopeptin III), which cross-reacts 100% with the 28 amino acid C-terminal peptide, and subsequent analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography were then carried out as previously described (Bloch et al., 1985). Cleavage activity was qualitatively assessed by conversion of the 17-kDa proANF to the 3-kDa peptide (Figure 1A). The 14-kDa amino-terminal fragment that also results from this cleavage reaction is not routinely identified in this assay but is readily demonstrable if a similar protocol is employed using [^{35}S]methionine-labeled proANF and immunoprecipitation carried out with an antiserum directed to a site located near the N-terminus (Bloch et al., 1987).

A second assay was used to more quantitatively assess cleavage activity. This colorimetric assay employed a nitroanilide-derivatized tripeptide, H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride, as substrate (Lottenberg et al., 1981) (Figure 1B). The substrate was dissolved in 150 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.3, at a concentration of 1 mM. The sample solution (50 μL) was added to the substrate solution (450 μL) in a quartz cuvette, and the absorbance at 405 nm was monitored over 2 min. The change in absorbance with time (ΔA/min) was used as a measure of cleavage activity in the sample solution.

Serum and Plasma Preparation. Serum was prepared by allowing blood, collected from 250–350-g Sprague-Dawley rats after decapitation, to clot in a glass tube. The sample was then centrifuged at 2000*g* for 20 min, and the serum was aspirated and pooled for assay or purification. Plasma was prepared from blood collected through a heparinized indwelling catheter in the carotid artery of a pentobarbital-anesthetized rat. The blood was collected into a glass tube containing either heparin (100 units/mL) or ethylenediaminetetraacetic acid (EDTA) (25 mM) and centrifuged, and the plasma was then aspirated for use in assays. Serum was also prepared from both platelet-poor and platelet-rich plasma. Platelet-rich plasma was prepared from heparinized rat blood collected as described above but centrifuged at 140*g* for 20 min at room temperature to pellet only the red and white blood cells. After collection of the platelet-containing supernatant, protamine sulfate (1 mg/100 units of heparin) was added, and clotting was allowed to proceed overnight at 4 °C. The clot was removed after centrifugation at 2000*g* for 20 min. Serum derived from platelet-poor plasma was prepared by centrifugation of heparinized blood at 2000*g* followed by addition of protamine sulfate to the plasma supernatant and subsequent clot removal.

Purification Procedure. Fractions collected during all purification steps were analyzed for absorbance at 280 nm as well as for proANF cleavage activity using both assays described above. All buffers contained 0.02% sodium azide.

Rat serum (60 mL) was dialyzed against 10 mM sodium phosphate buffer, pH 7. The sample was then applied to a 3 × 75 cm DEAE-cellulose column equilibrated in the same buffer at 4 °C. Elution was obtained at a flow rate of 50 mL/h with a linear gradient of 10–100 mM sodium phosphate, pH 7. The fractions (8 mL) containing the peak of the cleavage activity were then pooled, concentrated 4-fold on an Amicon membrane concentrator (PM-10 membrane), and applied to a 10 × 30 mm hydroxylapatite column equilibrated with 40 mM sodium phosphate, pH 7. The fractions (4 mL) containing the cleavage activity were eluted with a linear gradient of 40–200 mM sodium phosphate, pH 7, pooled, and applied to a 10 × 30 mm column of concanavalin A–Sepharose 4B equilibrated with 100 mM sodium phosphate, pH 7. Following a 200-mL wash with a buffer of 100 mM sodium phosphate and 500 mM sodium chloride, pH 7, elution was obtained with the same buffer containing 200 mM methyl α-D-mannoside. The fractions (4 mL) containing activity were then concentrated to 10 mL, applied to a 2.5 × 100 cm AcA 34 Ultrogel column equilibrated with a buffer of 100 mM sodium phosphate and 50 mM sodium chloride, pH 7, and eluted with the same buffer at a flow rate of 30 mL/h. Solutions containing known protein standards were fractionated under identical conditions on the same column, and their Stokes radii were plotted as a function of K_{av}, and this plot was used to determine the Stokes radius of the enzyme. The

active fractions (4 mL) resulting from AcA 34 fractionation were then pooled and applied to a 5 × 10 mm wheat germ lectin–Sepharose 6 MB column as a final concentration and purification step. The column was washed with 25 mL of a buffer containing 100 mM sodium phosphate and 500 mM sodium chloride, pH 7, and elution was obtained with the same buffer containing 200 mM *N*-acetylglucosamine with collection of 1-mL fractions.

Protein concentrations were determined by the method of Lowry et al. (1951).

Barium Sulfate Adsorption. Barium sulfate ($BaSO_4$) adsorption with rat serum was performed as previously described for plasma (Osterud & Flengsrud, 1975). Briefly 100 mg of $BaSO_4$ was added to 1 mL of rat serum and agitated for 1 h at 4 °C. Following centrifugation at 3000*g* for 15 min, the supernatant was aspirated, the precipitate was washed twice with 1 mL of 0.2 M sodium chloride, and elution was attempted with 1 mL of 0.15 M sodium citrate followed by another 0.5 mL of the same solution. Supernatants from each step were assayed for protease activity by using the chromogenic substrate method described above. As a positive control, a similar adsorption procedure was performed on citrated (0.35%) rat plasma with subsequent assay of prothrombin activity in the supernatants and eluates by the method of Hjort et al. (1955), using factors II and VII deficient plasma, 0.025 M calcium chloride, and Russell's viper venom in cephalin. The sodium citrate concentration of all samples was made 0.15 M followed by 1:10 dilution of samples prior to assay.

Protease Inhibitor Analysis. Aprotinin, benzamidine, leupeptin, diisopropyl fluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), pepstatin, and hirudin were tested for their ability to inhibit the cleavage of proANF by serum. [^{35}S]Cysteine–proANF was incubated with rat serum as described above in the absence and presence of increasing concentrations of protease inhibitor, immunoprecipitated, and analyzed by SDS–PAGE/autoradiography as described above.

SDS–PAGE. The purified proANF-cleaving enzyme was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% and 12% polyacrylamide according to the method of Laemmli (1970) and subsequent silver staining (Bio-Rad). Molecular weight standards (BRL) were simultaneously analyzed.

[^{3}H]DFP Labeling. Samples (60 μL) of the purified proANF-cleaving enzyme in 100 mM sodium phosphate buffer were preincubated in the presence or absence of unlabeled diisopropyl fluorophosphate (DFP) at a concentration of 10 mM overnight at 4 °C. To each sample, 20 μL of [^{3}H]DFP (100 μCi) was then added, to a final concentration of 250 μM, and incubation was continued for 4 h at room temperature. An equal volume of SDS–PAGE sample buffer containing 10% β-mercaptoethanol was then added to each sample, and SDS–PAGE was carried out in 10% polyacrylamide gels. The gel slabs were then incubated in Autofluor for 1 h prior to autoradiography on Kodak XAR-5 film for 10 days at −70 °C.

Analysis of ProANF Cleavage Products. ^{35}S-Labeled proANF derived from atrial cardiocytes was purified from culture media on a 100 × 3 cm G-75 Sephadex column. The protein was eluted with a buffer of 100 mM sodium phosphate and 50 mM sodium chloride, pH 7, at a flow rate of 40 mL/h at 4 °C. Fractions were analyzed by immunoprecipitation followed by SDS–PAGE/autoradiography as described above. The purest fractions were then pooled for use in the following experiment.

Purified ^{35}S-proANF (0.5 mL) was incubated with proANF-cleaving enzyme (0.25 mL) and purified as described above with an activity of 0.20 ΔA unit/min, as determined by the chromogenic substrate cleavage assay, for various times from 0 to 30 min at 37 °C. The reaction was stopped by the addition of 10 μL of 50 mM PMSF (see Protease Inhibitor Analysis) and cooling to 4 °C. ANF contained in the samples was then immunoprecipitated with ANF-directed antisera as described above and analyzed both by SDS–PAGE/autoradiography and by reverse-phase high-performance liquid chromatography (HPLC) as described below. Individual autoradiographic bands were quantitatively analyzed with an LKB 22202 laser densitometer and a Hewlett Packard peak integrator. For analysis by HPLC, immunoprecipitates were vortexed vigorously after the addition of 10 μL of 3 M acetic acid and then centrifuged in a microfuge for 10 min. A 16-μL aliquot of the resulting supernatant, containing approximately 10 000 cpm, was then added to 2 μL of 0.05% trifluoroacetic acid (TFA)/water containing 2 μg each of rat atriopeptin III (24 amino acid C-terminal fragment of proANF) and rat ANP (28 amino acid C-terminal fragment) and analyzed by HPLC using a Vydac C-18 analytical column. A nonlinear elution gradient was constructed between solvent A (0.05% TFA/water) and solvent B (0.05% TFA/acetonitrile) at a flow rate of 1 mL/min to separate the 24 and 28 amino acid peptides. The elution gradient consisted of 0% solvent B for the first 5 min, 0–20% over the next 5 min, 20–50% over the next 20 min, and 50–80% over the final 5 min. Absorbance at 214 nm was monitored, and fractions were collected that coincided precisely with the elution times of the unlabeled synthetic 28 and 24 amino acid peptides. Fractions were also collected before and after the elution of these peptides, and 0.5-mL aliquots of all fractions were counted in a β counter after the addition of 10 mL of Liquiscint scintillation fluid.

Results

Cleavage Activity in Plasma and Serum. Incubation of ^{35}S-proANF with rat serum resulted in nearly complete cleavage of the 17-kDa proANF to the 3-kDa ANF peptide(s) within 5 min (Figure 1A, lane marked "serum"). However, no cleavage was observed following a 30-min incubation with plasma prepared from either heparinized or EDTA-treated blood (data not shown). In contrast, the addition of heparin (100 units/mL) or EDTA (25 mM) to serum did not inhibit its cleavage activity.

Neither platelet-enriched nor platelet-poor plasma was found to contain proANF cleavage activity. However, serum derived from these plasma preparations did cleave proANF.

Protease Inhibitors. Serum was incubated with ^{35}S-proANF in the absence and presence of increasing concentrations of various protease inhibitors. ProANF cleavage activity was then assessed by SDS–PAGE and autoradiography (Figure 2). Complete inhibition of proANF cleavage was obtained with benzamidine (1 mM), leupeptin (20 μg/mL), PMSF (0.5 mM), and DFP (1 mM). No inhibition was observed with aprotinin (1000 KIU/mL), soybean trypsin inhibitor (40 μg/mL), pepstatin (40 μg/mL), or hirudin (200 μg/mL).

$BaSO_4$ Adsorption. $BaSO_4$ adsorption with rat serum was performed as previously described in the purification of the vitamin K dependent plasma proteins (Osterud & Flengsrud, 1975). The proANF cleaving protease was found not to bind to $BaSO_4$. As a positive control, rat plasma prothrombin was found to readily adsorb to $BaSO_4$, from which it could be eluted with sodium citrate.

Purification of ProANF Cleaving Enzyme from Serum. The results of the sequential steps in the purification of the

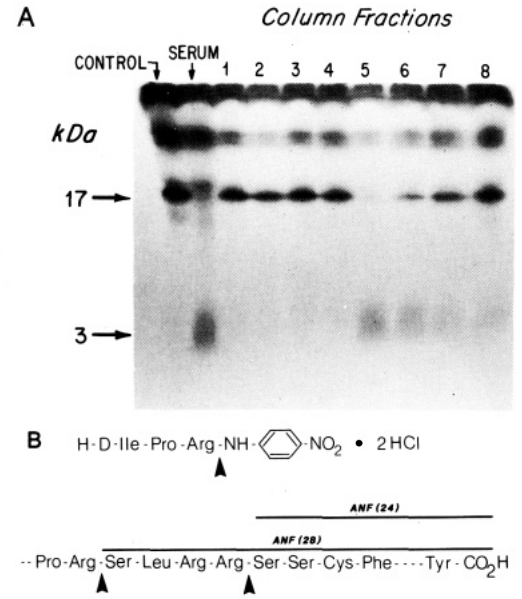

FIGURE 1: Assays used in assessment of proANF cleavage activity. (A) Cleavage of the 17-kDa [^{35}S]cysteine-labeled proANF ("control") to the 3-kDa peptide(s) by serum and by sequential fractions (lanes 1–8) in the DEAE-cellulose purification step following immunoprecipitation wth ANF-directed antisera and SDS-PAGE/autoradiography in 17% polyacrylamide gels. Lane marked "5" corresponds to fraction 150 in Figure 3A. Higher molecular weight bands (>17K) have been shown in previous experiments to be nonspecific immunoprecipitation products (Bloch et al., 1985, 1987). (B) Sequence of the chromogenic substrate used to quantitatively assess proANF cleavage activity is compared to that of the carboxy-terminal sequence of proANF. The cleavage site of this substrate (arrow) which results in a color change is similar to the two cleavage sites (arrows) of proANF leading to the formation of the 28 and 24 amino acid peptides, the major C-terminal cleavage fragments.

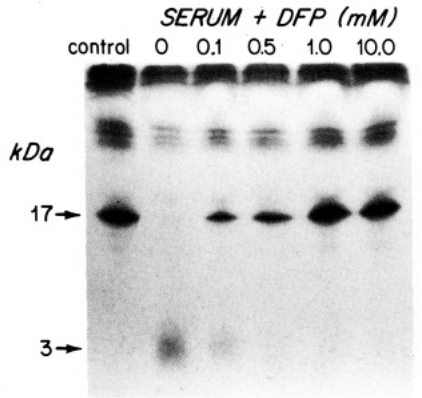

FIGURE 2: Concentration-dependent inhibition of proANF cleavage activity in serum by DFP. [^{35}S]ProANF (lane marked "control") was incubated with serum in the absence and presence of increasing concentrations of DFP (lanes 2–6), followed by immunoprecipitation with ANF-directed antibodies and SDS–PAGE/autoradiography. Similar experiments were performed in determining the ability of other protease inhibitors to inhibit proANF cleavage (see text).

proANF cleaving enzyme from rat serum are shown in Figure 3 and are summarized in Table I. A 5000–6000-fold purification of this enzyme from serum was achieved. The enzyme activity in serum was found to be relatively stable for several weeks at 4 °C and for months at −20 °C. However, activity of fractions from later purification steps declined rapidly (over several days) if not frozen at −70 °C. The enzyme was found to bind specifically to both concanavalin A (Con A) and wheat germ lectin, indicating that it is a glycoprotein (Figure 3C,D). Size analysis by AcA 34 gel filtration chromatography revealed a Stokes radius of 45 Å (Figure 3E). Preparative isoelectric

Table I: Purification of ProANF Cleaving Protease from Rat Serum

fraction	protein concn (mg/mL)	act. [ΔA min^{-1} (0.05 mL)$^{-1}$]	sp act. (activity/mg)	purification (x-fold)
serum	80.00	0.200	0.05	1
DEAE-cellulose	10.50	1.260	2.40	47
hydroxylapatite	0.900	0.196	4.35	83
Con A I	0.066	0.106	32.1	610
Con A II	0.045	0.166	73.8	1140
AcA 34	0.007	0.080	228.6	4395
wheat germ lectin	0.031	0.420	270.9	5250

focusing performed on a purified enzyme preparation (DEAE-cellulose pool) demonstrated an isoelectric point of 5.5–6.0 (data not shown).

Analysis of fractions resulting from the last purification step by SDS–PAGE in the absence of reducing agents demonstrated several broad bands of approximately 35–96 kDa (Figure 4A). Similar analysis under reducing conditions revealed three major bands of approximately 50, 38, and 19 kDa (Figure 4B).

[^{3}H]DFP Labeling. Incubation of a purified enzyme preparation with [^{3}H]DFP resulted in the labeling of a single 38-kDa protein as assessed by SDS–PAGE and autoradiography, performed under reducing conditions. This labeling was blocked by preincubation with unlabeled DFP (Figure 5).

Analysis of ProANF Cleavage Products. Results of HPLC and SDS–PAGE/autoradiography performed on the immunoprecipitated products resulting from the cleavage of proANF by purified enzyme and the HPLC fractionation of the 28 and 24 amino acid synthetic ANF peptides are shown in Figures 6 and 7. Incubation of the 17-kDa ^{35}S-proANF with the purified enzyme for as little as 1 min resulted in almost complete cleavage to the 3-kDa peptide(s) (Figure 6). Following 30 min of incubation, the 3-kDa band is clearly present, with a barely detectable 17-kDa band. Analysis of the autoradiograph in Figure 6 by quantitative densitometry revealed that following 1-, 5-, 15-, and 30-min incubation with the protease, the 3-kDa band represented 68%, 72%, 84%, and 95%, respectively, of the sums of the 3- and 17-kDa bands at each consecutive time point. Furthermore, when the sums of the 3- and 17-kDa bands at each time point were compared to the 17-kDa band at time 0, they were found to represent 106%, 90%, 84%, and 62% consecutively of the 17-kDa band at time 0. This temporal pattern of early quantitative recovery and subsequent loss of [^{35}S]cysteine-labeled peptides with prolonged incubations suggests that, initially, the 17-kDa peptide is cleaved to form the 3-kDa peptide(s), which over time may be slowly degraded to smaller, unrecognized forms.

Figure 7A–D demonstrates the HPLC elution patterns of the labeled immunoprecipitates. A comparison to the elution patterns of the synthetic 28 and 24 amino acid C-terminal ANF peptides (Figure 7E) revealed the presence of both the 28 and 24 amino acid peptides in the ^{35}S-labeled immunoprecipitates, as evidenced by their coelution with the corresponding unlabeled synthetic peptides. Following a 1-min incubation with the purified protease, the radiolabeled 28 and 24 amino acid products were detected at a ratio of approximately 2:1 (Figure 7B). This ratio progressively declined with longer incubations such that following 30 min (Figure 7D), the ratio was reversed, suggesting that proANF is cleaved initially to the 28 amino acid C-terminal peptide, which, in turn, is cleaved to the smaller 24 amino acid peptide.

Discussion

Since the initial report of a natriuretic substance in atrial

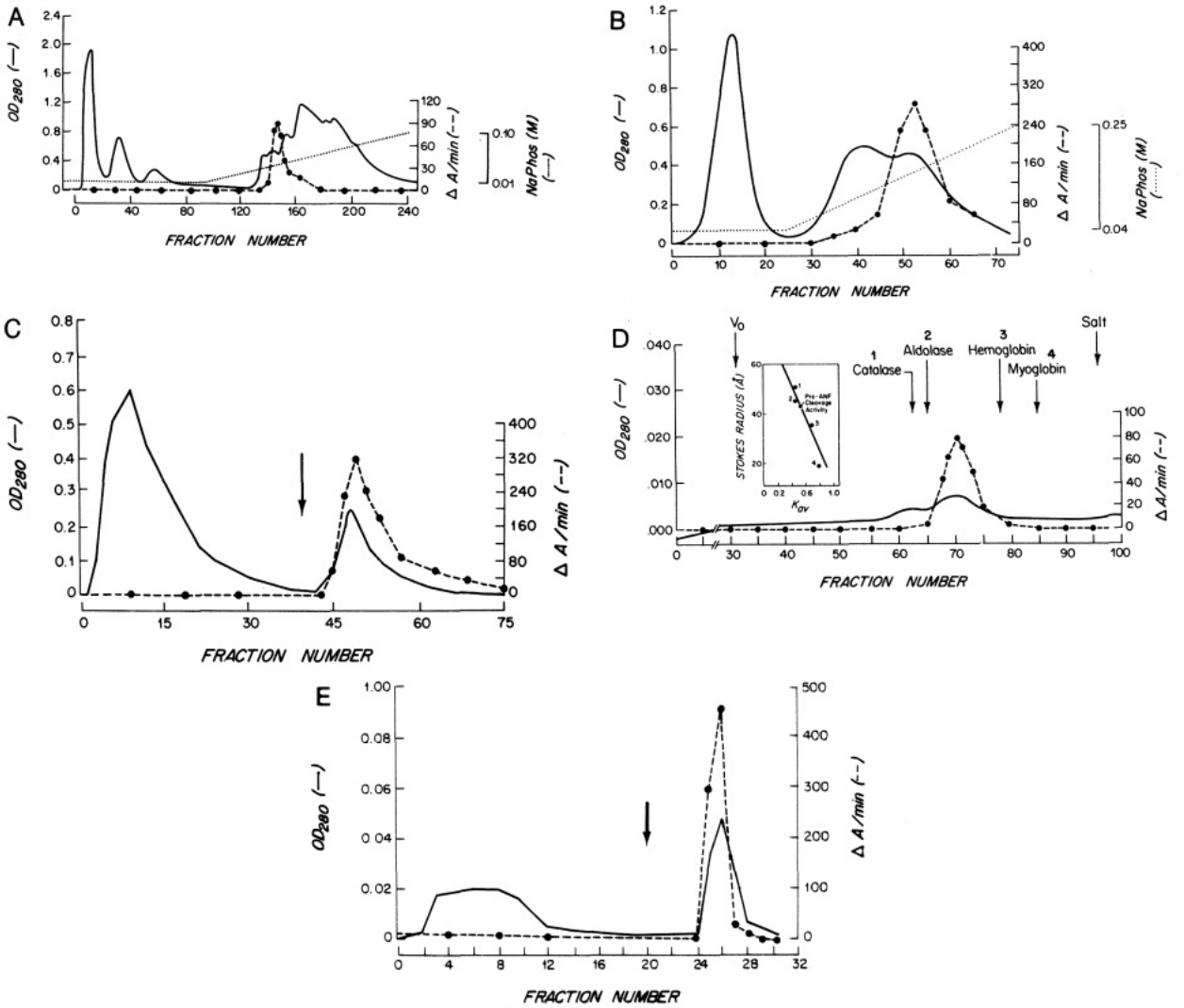

FIGURE 3: Sequential steps in the purification of proANF cleaving protease from rat serum. Representative chromatographs from DEAE-cellulose (A), hydroxylapatite (B), concanavalin A–Sepharose (C), AcA 34 Ultragel (D), and wheat germ lectin–Sepharose (E) purification steps. Absorbance at 280 nm (solid line) and proANF cleavage activity, as assessed by the chromogenic assay (dashed line) (ΔA/min × 10^3), are plotted against fraction number. Sodium phosphate gradients are indicated (dotted line) in the DEAE and hydroxylapatite steps, and arrows indicate the beginning of elution in the two lectin steps and the elution pattern of protein standards in the AcA 34 Ultragel step. Fraction volumes are as indicated under Methods. Figure 3D inset: Stokes radius is plotted against K_{av} of standards (1–4) and proANF cleaving enzyme (asterisk), determining the size of the enzyme.

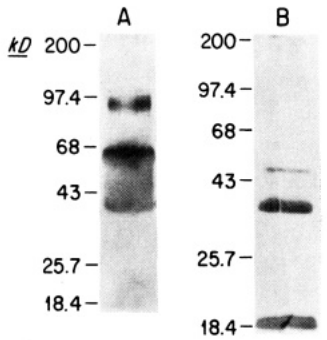

FIGURE 4: SDS–PAGE of purified proANF cleaving protease. Protein (0.1 μg) was analyzed in a 10% polyacrylamide gel in the absence of β-mercaptoethanol (A) and in a 12% gel in the presence of β-mercaptoethanol (B) followed by silver staining. The position of the molecular weight standards, myosin (200 000), phosphorylase B (97 400), bovine albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 000), and β-lactoglobulin (18 000), is shown.

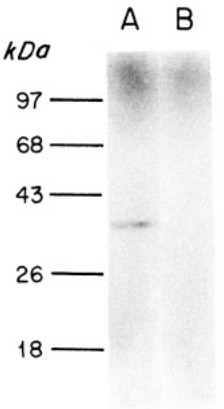

FIGURE 5: [^{3}H]DFP labeling of purified proANF cleaving protease. A purified preparation of proANF cleaving protease was incubated with [^{3}H]DFP in the absence (A) and presence (B) of unlabeled DFP (10 mM) and then analyzed in a 12% polyacrylamide gel in the presence of β-mercaptoethanol followed by autoradiography.

tissue by deBold and colleagues (deBold et al., 1981), there has been much progress in our understanding of the biosynthesis and processing of ANF in atrial cardiocytes. On the basis of the cloned gene sequence, it is known that in the rodent, ANF is translated as a 152 amino acid "preprohormone" with a 24 amino acid amino-terminal hydrophobic "signal peptide" (Seidman et al., 1984; Yamanaka

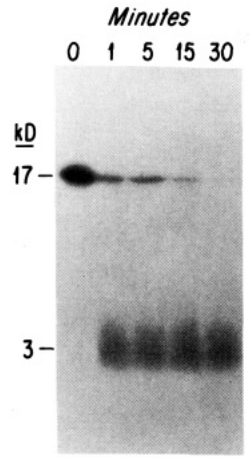

FIGURE 6: Analysis of ^{35}S-proANF cleavage products. SDS–PAGE/autoradiographs of immunoprecipitated ANF obtained after 0, 1, 5, 15, and 30 min of incubation of [^{35}S]cysteine-labeled proANF with purified cleaving enzyme.

et al., 1984; Maki et al., 1984). Like that of other peptide hormones (Habener et al., 1981; Tager et al., 1981), the signal peptide is presumably cleaved cotranslationally, since pre-proANF has never been isolated from atrial tissue, nor can it be identified using antibodies directed to the signal peptide cleavage site (Zisfein et al., 1986a). Analysis of ANF stored in atrial granules by antibodies (Zisfein et al., 1986a), gel filtration chromatography (Glembotski et al., 1985; Vuolteenaho et al., 1985; Miyota et al., 1985), HPLC (Kangawa et al., 1984; Miyota et al., 1985), and sequence analysis (Kangawa et al., 1984) have demonstrated the 126 amino acid proANF to be the major storage form. Similar analysis of ANF in plasma has revealed that the major circulating forms are the 28 and 24 amino acid carboxy-terminal fragments of proANF, which have been reported to be present in ratios varying from 1:1 to 9:1 (Sugarawa et al., 1985; Yamaji et al., 1985; Schwartz et al., 1985; Thibault et al., 1985). There is also some evidence that cleavage of proANF to these smaller fragments is necessary for full biological activity (Currie et al., 1984a,b). Whether cleavage of proANF in vivo to these smaller forms occurs before, during, or after secretion from the atriocyte remains unclear.

Intrinsic labeling studies in our laboratory (Bloch et al., 1985, 1987; Zisfein et al., 1986b) and similar studies by other investigators (Glembotski & Gibson, 1985) have demonstrated that atrial cardiocytes in culture store and secrete the intact proANF molecule. Additionally, we have demonstrated that serum contains an enzyme that appears to effect a specific endolytic cleavage of proANF to yield both the 28 and 24 amino acid carboxy-terminal peptides (Bloch et al., 1985, 1987). However, since whole serum was used in these studies, it was unclear whether both peptides resulted from the cleavage of proANF by one or by several different enzymes. Others have suggested that a platelet factor may be responsible for this cleavage (Trippodo et al., 1985).

In the present study, this serum protease has been purified and its molecular characteristics investigated. ProANF cleavage activity was not present in plasma prepared with either heparin or EDTA. Neither of these substances, however, inhibited the activity of the protease in serum. This enzyme does not seem to originate from platelets or any of the other cellular blood elements, since cleavage activity was clearly present in serum prepared from cell-free and platelet-poor plasma. These findings imply the existence of an inactive precursor of this protease in plasma that is activated during clotting in a manner analogous to the activation of other

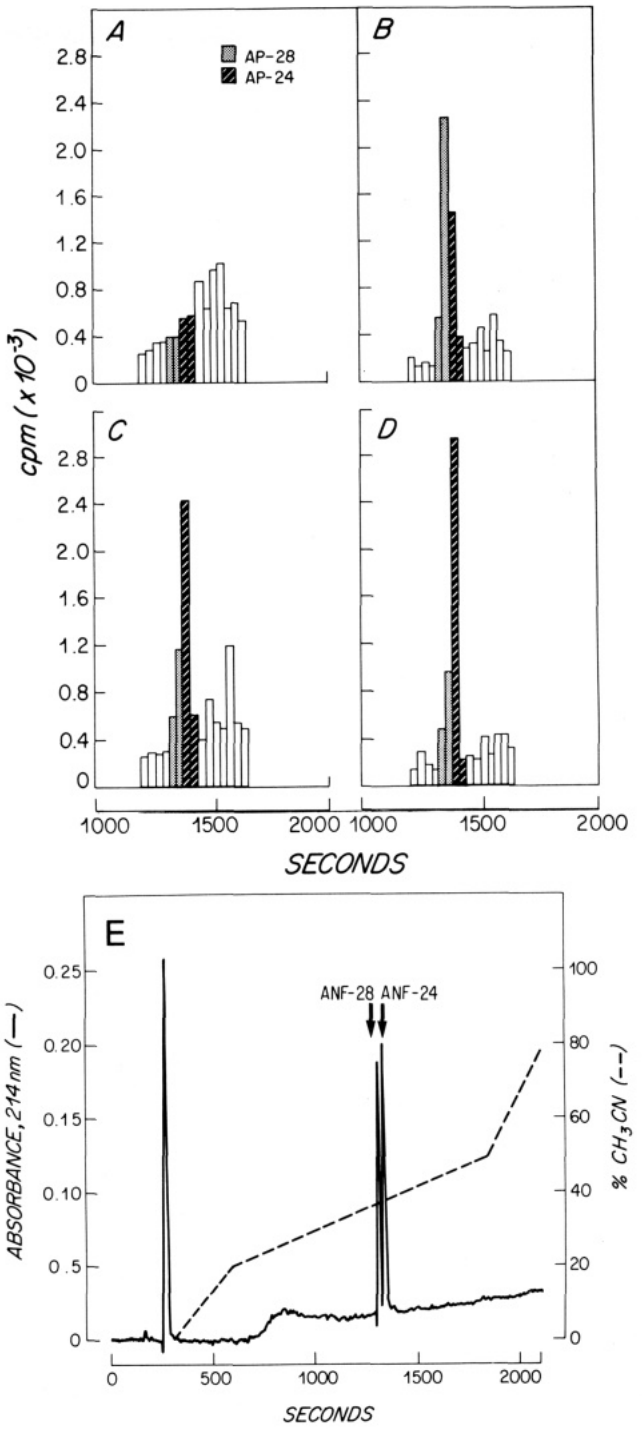

FIGURE 7: Reverse-phase HPLC analysis of ^{35}S-proANF cleavage products. Plots are shown for samples following 0-, 1-, 5-, and 30-min incubation of [^{35}S]cysteine-labeled proANF with purified cleaving enzyme (panels A–D, respectively). Elution conditions were similar to those shown in (E). Radioactivity (cpm) of fractions (0.5 mL) collected between 1200 and 1500 s is plotted. Solid bars indicate fractions coeluting with synthetic 28 amino acid ANF, and hatched bars indicate fractions coeluting with 24 amino acid ANF. Reverse-phase HPLC elution patterns of synthetic 28 and 24 amino acid rat ANF (2 μg of each) are shown in (E). The solid line denotes the absorbance at 214 nm, and the dashed line indicates the H_2O/acetonitrile gradient.

plasma proteases. Furthermore, since plasma prepared with EDTA or heparin does not cleave proANF, *activation* of the cleaving enzyme either is calcium dependent and antithrombin III inhibited or requires the presence of another calcium-dependent antithrombin III dependent enzyme.

Since plasma does not cleave proANF to the smaller cir-

culating forms, it is possible that this enzyme is not responsible for the in vivo conversion of proANF to the active C-terminal fragment(s). However, differences clearly exist between in vitro plasma preparations and circulating blood; most notable is the delicate equilibrium that exists in circulating blood between thrombosis and thrombolysis. The constant, low-level, spontaneous activation of clotting enzymes is held in check by inactivators and inhibitors of these enzymes. In contrast, collection of plasma requires anticoagulation by calcium chelators, such as EDTA, or by agents, such as heparin, that promote the action of antithrombin III. These agents prevent the low-level, spontaneous activation of many blood proteases. Thus, it is likely that many of these proteases, and also possibly the proANF cleaving enzyme, are more active in circulating blood than under the artificial conditions imposed by the preparation of plasma. Alternatively, it is possible that the activity of the circulating proANF cleaving enzyme is enhanced by some other factor related to the presence of endothelial cells, similar to the interaction of thrombin and thrombomodulin (Clouse & Comp, 1986).

Following six purification steps, a greater than 5000-fold purification of this serum protease was achieved. It is a glycosylated protein, as indicated by its affinity for at least two plant lectins. On the basis of size-exclusion gel filtration chromatography, it has a Stokes radius of 45 Å, corresponding to a globular protein with a molecular weight of approximately 130K. Analysis by SDS–PAGE demonstrated several poorly resolving bands of approximately 35–96 kDa, which under reducing conditions are more clearly resolved into three major bands of approximately 50, 38, and 19 kDa. Incubation of a purified preparation of this protease with [^{3}H]DFP resulted in the specific labeling of a single, 38-kDa protein, as analyzed by SDS–PAGE under reducing conditions. This finding suggests that the activated proANF cleaving protease is at least a dimer, with a major substrate binding subunit of 38 kDa. The inability to obtain sharp resolution in the absence of reducing agents was observed consistently with several purified preparations and cannot be readily explained, although glycosylation of the peptide subunits may be a contributing factor. Alternatively, it is possible that one or several of these bands represent contaminants. When compared to its elution pattern by AcA gel chromatography, which demonstrates a single species of approximately 130 kDa, it appears that the 38-kDa binding subunit is disulfide linked to at least one other subunit, similar to the structures of many other plasma proteases.

Like most serine proteases (Barrett & McDonald, 1980), the proANF cleaving enzyme is inhibited by benzamidine, PMSF, and DFP. However, unlike plasmin and kallikrein, it is not inhibited by high concentrations of aprotinin, and unlike thrombin and factor IXa, it is not inhibited by hirudin.

Adsorption of the proANF cleaving protease with $BaSO_4$ was attempted, as previously described in the purification of the γ-carboxylated glutamic acid (Gla)-containing vitamin K dependent plasma proteases (Osterud & Flengsrud, 1975). The proANF cleaving protease did not bind to $BaSO_4$, whereas prothrombin, employed as a positive control, was quantitatively adsorbed. This makes it unlikely to be any of the activated circulating Gla-containing proteins (factors VIIa, IXa, and Xa; proteins C, S, Z, and M), all of which retain their Gla domains after activation (with the exception of thrombin, which has been excluded based on its inhibitor profile).

Incubation of the purified serum protease with ^{35}S-labeled proANF resulted in the specific cleavage of the high molecular weight precursor form of ANF to smaller fragments that coelute on HPLC with the 28 and 24 amino acid carboxy-terminal peptides, both of which are biologically active (Schwartz et al., 1985; Flynn et al., 1983; Thibault et al., 1984; Garcia et al., 1985, 1986) and have been demonstrated to circulate in vivo (Schwartz et al., 1985; Thibault et al., 1985). Amino-terminal radiosequence analysis of these smaller peptides has identified the 28 and the 24 amino acid carboxy-terminal ANF peptides as the major cleavage products (Bloch et al., 1987). It is of interest that the two cleavage sites involved in the generation of these peptides are identical in their Arg-Ser sequence and are both recognized by the same enzyme. It is also of note that cleavage to the 28 amino acid peptide seems to precede further cleavage to the 24 amino acid peptide, indicating a preference of the enzyme for the first site. This may reflect the fact that a proline residue, often present at turns in secondary protein structure, precedes the first cleavage site, thus promoting its accessibility.

Whether the proANF cleaving protease in serum plays a physiologic role in determining the availability of the biologically active circulating forms of the hormone remains to be defined. Similarly, whether this is a unique, previously undescribed enzyme or, alternatively, one of many known serum proteases with other additional substrate specificities is as yet unclear. It should be noted, however, that the characteristics of this protease, including its size, p*I*, inhibitor profile, and inability to bind to $BaSO_4$, do not match those of any of the commonly known serum proteases, such as plasmin, kallikrein, or any of the family of vitamin K dependent plasma proteins. Furthermore, it performs a highly specific and sequential endolytic cleavage of proANF to the two smaller peptides found in the circulation. Large-scale purification and the availability of either partial sequence data that can be used to isolate a cDNA for the enzyme or complete sequence analysis will allow determination of the precise structure of this enzyme and its possible similarity to other known proteases.

ACKNOWLEDGMENTS

We gratefully thank D. Rollins for excellent secretarial assistance, Drs. G. Matsueda, M. Margolies, K. Bloch, and J. Seidman for valuable discussions, and an anonymous reviewer for his/her helpful comments.

REFERENCES

Barrett, A. J., & McDonald, J. K. (1980) in *Mammalian Proteases: A Glossary and Bibliography*, Academic Press, London and New York.

Bloch, K. D., Scott, J. A., Zisfein, J. B., Fallon, J. T., Margolies, M. N., Seidman, C. E., Matsueda, G. R., Homcy, C. J., Graham, R. M., & Seidman, J. G. (1985) *Science (Washington, D.C.) 230*, 1168–1171.

Bloch, K. D., Zisfein, J. B., Margolies, M. N., Homcy, C. J., Seidman, J. G., & Graham, R. M. (1987) *Am. J. Physiol. 252*, E147–E151.

Clouse, L. H., & Comp, P. C. (1986) *N. Engl. J. Med. 314*, 1298–1304.

Currie, M. G., Geller, D. M., Chao, J., Margolius, H. S., & Needleman, P. (1984a) *Biochem. Biophys. Res. Commun. 120*, 461–466.

Currie, M. G., Geller, D. M., Cole, B. R., & Needleman, P. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1230–1233.

deBold, A. J., Borenstein, H. B., & Veress, A. T. (1981) *Life Sci. 28*, 89–94.

Flynn, T. G., deBold, M. L., & deBold, A. J. (1983) *Biochem. Biophys. Res. Commun. 117*, 859–865.

Garcia, R., Thibault, G., Seidah, N. G., Lazure, C., Cantin, M., Genest, J., & Chretien, M. (1985) *Biochem. Biophys.*

Res. Commun. 126, 178–184.
Garcia, R., Cantin, M., DeLean, A., Genest, J., Godin, G., Gutkowska, J., Schiffrin, E. L., & Thibault, G. (1986) *Biochem. Biophys. Res. Commun. 135*, 987–993.
Glembotski, C. C., & Gibson, T. R. (1985) *Biochem. Biophys. Res. Commun. 132*, 1008–1017.
Glembotski, C. C., Wildey, G. M., & Gibson, T. R. (1985) *Biochem. Biophys. Res. Commun. 129*, 671–678.
Habener, J. F., Kronenberg, H. M., & Potts, J. T. (1981) *Methods Cell Biol. 23*, 51–71.
Hjort, P., Rapapport, S., & Owren, P. A. (1955) *J. Lab. Clin. Med. 46*, 89–93.
Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazato, H., Fukada, A., Minamino, N., & Matsuo, H. (1984) *Nature (London) 312*, 152–155.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lottenberg, R., Christensen, U., Jackson, C. M., & Coleman, P. L. (1981) *Methods Enzymol. 80*, 341–364.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 58*, 265–275.
Maki, M., Takayanagi, R., Misono, K. S., Paudey, K. N., Tibbetts, C., & Inagami, T. (1984) *Nature (London) 309*, 722–724.
Miyota, A., Kangawa, K., Tashimori, T., Hatch, T., & Matsuo, H. (1985) *Biochem. Biophys. Res. Commun. 129*, 248–255.
Osterud, B., & Flengsrud, R. (1975) *Biochem. J. 145*, 469–474.
Schwartz, D., Geller, D. M., Manning, P. T., Seigel, N. R., Fok, K. R., Smith, C. E., & Needleman, P. (1985) *Science (Washington, D.C.) 229*, 397–400.
Seidman, C. E., Duby, A. D., Choi, E., Graham, R. M., Haber, E., Homcy, C. J., Smith, J. A., & Seidman, J. G. (1984) *Science (Washington, D.C.) 225*, 324–326.
Sugarawa, A., Nakao, K., Morii, N., Sakamoto, M., Suda, M., Shimokura, M., Kiso, Y., Kihara, M., Yamori, Y., Nishimura, K., Soneda, J., Ban, T., & Imura, H. (1985) *Biochem. Biophys. Res. Commun. 129*, 439–446.
Tager, H. S., Steiner, D. F., & Patzelt, C. (1981) *Methods Cell Biol. 23*, 73–88.
Thibault, G., Garcia, R., Carrier, F., Seidah, N. G., Lazure, C., Cantin, M., & Genest, J. (1984) *Biochem. Biophys. Res. Commun. 125*, 938–946.
Thibault, G., Lazure, C., Schiffrin, E. L., Gutkowska, J., Chartier, L., Garcia, R., Seidah, N. G., Chretien, M., Genest, J., & Cantin, M. (1985) *Biochem. Biophys. Res. Commun. 130*, 981–986.
Trippodo, N. C., Januszewicz, A., Pegram, B. L., Cole, F. E., Kahashi, N., Kardon, M. B., MacPhee, A. A., & Frohlich, E. D. (1985) *Hypertension 7*, 905–912.
Vuolteenaho, O., Arjamaa, O., & Ling, L. (1985) *Biochem. Biophys. Res. Commun. 129*, 82–88.
Yamaji, T., Ishibashi, M., & Takaku, F. (1985) *J. Clin. Invest. 76*, 1705–1709.
Yamanaka, M., Greenberg, B., Johnson, C., Seilhammer, J., Brewer, M., Friedmann, T., Miller, J., Atlas, S., Laragh, J., Lewicki, J., & Fiddes, J. (1984) *Nature (London) 309*, 719–722.
Zisfein, J. B., Matsueda, G. R., Fallon, J. T., Bloch, K. D., Seidman, C. E., Seidman, J. G., Homcy, C. J., & Graham, R. M. (1986a) *J. Mol. Cell. Cardiol. 18*, 917–929.
Zisfein, J. B., Bloch, K. D., Sylvestre, D., Margolies, M. N., Seidman, J. G., Homcy, C. J., & Graham, R. M. (1986b) *Circulation 74*, II–462.

Hinging of Rabbit Myosin Rod†

M. E. Rodgers* and W. F. Harrington
Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218
Received November 3, 1986; Revised Manuscript Received August 24, 1987

ABSTRACT: The question of hinging in myosin rod from rabbit skeletal muscle has been reexamined. Elastic light scattering and optical rotation have been used to measure the radius of gyration and fraction helix, respectively, as a function of temperature for myosin rod, light meromyosin (LMM), and long subfragment 2 (long S-2). The radius of gyration vs temperature profile of myosin rod is shifted with respect to the optical rotation melting curve by about −5 °C. Similar studies on both LMM and long S-2 show virtually superimposable profiles. To correlate changes in the secondary structure with the overall conformation, plots of radius of gyration vs fraction helix are presented for each myosin subfragment. Myosin rod exhibits a marked decrease in the radius of gyration from 43 nm to ~35 nm, while the fraction helix remains at nearly 100%. LMM and long S-2 did not show this behavior. Rather, a decrease in the radius of gyration of these fragments occurred with comparable changes in fraction helix. These results are interpreted in terms of hinging of the myosin rod within the LMM/S-2 junction.

It is generally believed that force generation and shortening in a contracting muscle are a result of the cyclic attachment and detachment of cross-bridges between neighboring thick and thin filaments concomitant with the hydrolysis of ATP. The ultimate goal of research on muscle contraction is to understand the events of the cross-bridge cycle at the molecular level. Most of the current models of contraction which attempt to provide a molecular mechanism include two flexible regions in the myosin molecule: one between each myosin head (subfragment 1) and the coiled-coil α-helical rod and a second located near the center of the rod. The swivellike joint between myosin subfragment 1 (S-1)[1] and the rod of myosin provides

† Supported by National Institutes of Health Grant AM-04349 (to W.F.H.) and by a Muscular Dystrophy Association postdoctoral fellowship (to M.E.R.). This is Contribution No. 1386 from the McCollum-Pratt Institute of The Johns Hopkins University.
* Address correspondence to this author at the Department of Biology, The Johns Hopkins University.

0006-2960/87/0426-8697$01.50/0