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Purification and Characterization of a Ca^{2+}-Dependent Prothrombin Activator, Multactivase, from the Venom of *Echis multisquamatus*[1]

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We previously found a novel Ca^{2+}-dependent prothrombin activator, designated as carinactivase-1, in *Echis carinatus leucogaster* venom [D. Yamada, F. Sekiya, and T. Morita (1996) *J. Biol. Chem.* 271, 5200-5207]. Of the Viperidae snake venoms examined, the *Echis multisquamatus* venom had the strongest carinactivase-like activity. We isolated and characterized the carinactivase-like prothrombin activator in *E. multisquamatus* venom. From 50 mg of *E. multisquamatus* venom, we isolated 2.3 mg of a Ca^{2+}-dependent prothrombin activator designated as multactivase. Unlike other *Echis* snake venoms, the *E. multisquamatus* venom contained no ecarin-like Ca^{2+}-independent prothrombin activator. The structure and function of multactivase are similar to those of carinactivase. Multactivase is composed of a catalytic subunit with metalloprotease activity and a regulatory subunit comprising two homologous polypeptides bound by S-S bridge(s), and it activates prothrombin *via* recognition of the Ca^{2+}-bound conformation of its Gla domain. We developed a chromogenic assay involving multactivase for normal prothrombin activity in plasma from individuals orally administered anticoagulants. The normal prothrombin activity, as a percentage, measured with multactivase was highly correlated with the prothrombin time. Multactivase is useful for the simple quantification of normal prothrombin in plasma from warfarin-treated individuals.

Key words: calcium, prothrombin time, prothrombin activator, venom metalloprotease, warfarin.

Prothrombin is one of the most important coagulation factors in a vitamin K-dependent manner. Prothrombin is physiologically converted to α-thrombin by the prothrombinase complex which consists of the physiological prothrombin activator, factor Xa, the cofactor, factor Va, anionic phospholipids, and Ca^{2+} ions (*1*). Some snake venoms have also been reported to contain different types of prothrombin activators (*2*). These activators have been classified into three groups, and prothrombin-activating metalloproteases belong to group 1 (*3*). The best-known example of a group 1 activator is ecarin, which is the Ca^{2+}-independent prothrombin activator from *Echis carinatus* venom (*4*). The isolation and characterization of a novel prothrombin activator, designated as carinactivase-1 (CA-1), in the venom of *Echis carinatus leucogaster* revealed the existence of a novel group of prothrombin activators tentatively named group 1B, while the previously identified group 1 proteases were renamed group 1A proteases (*5*). CA-1 is a metalloprotease composed of a group 1A-like catalytic subunit and a C-type lectin-like regulatory subunit that comprises hetero-dimeric polypeptides linked by disulfide bond(s). The enzyme primarily recognizes the Ca^{2+}-bound conformation of the Gla domain of prothrombin *via* the regulatory subunit, and the catalytic subunit catalyzes the subsequent conversion of prothrombin to active thrombin. Considering the activation mechanism for this activator, CA-1 was shown to be useful for the assessment of normal prothrombin levels in plasma from vitamin K antagonist-treated individuals (*5*). To find a snake venom containing a carinactivase-like enzyme with stronger activity, we searched for Ca^{2+}-dependent prothrombin-activating activity in the venoms of Viperidae snakes (*6*). All the venoms of *Echis* snakes had carinactivase-like activity, and that of *Echis multisquamatus* venom was the strongest among those examined. Interestingly, ecarin-like Ca^{2+}-independent activity was not detected in this venom. In this study, we isolated and characterized a carinactivase-like enzyme designated as multactivase from *E. multisquamatus* venom.

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Abbreviations: CA-1, carinactivase-1; PAGE, polyacrylamide gel electrophoresis; Gla, γ-carboxyglutamic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin; PT, prothrombin time; RVV-X, factor X activating enzyme from Russell's viper venom; IX/X-bp, factor IX/factor X-binding protein; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Materials—The lyophilized venom of *E. multisquamatus* used for the isolation of multactivase was purchased from Latoxan (Rosans, France). The gels for chromatography, *i.e.* Superdex 200 pg, Q-Sepharose High Performance, and Blue Sepharose CL-6B, were from Pharmacia Biotech (Uppsala, Sweden). Bovine serum albumin (essentially fatty acid-free, ELISA grade) and phospholipids were from Sigma Chemical (St. Louis, MO, USA). *t*-Butoxycarbonyl-Val-Pro-Arg-*p*-nitroanilide (Boc-Val-Pro-Arg-*p*NA), the chromogenic substrate for thrombin, was purchased from Seikagaku Kogyo (Tokyo). Plasma samples from warfarin-treated patients were generous gifts from Drs. Kazunori Iwade and Masaya Kitamura of Tokyo Women's College of Medicine.

Proteins—Prothrombin was prepared from bovine plasma by the method of Hashimoto *et al.* (7). Fragment 1 was obtained from bovine prothrombin by the method of Morita *et al.* (8). All proteins were homogeneous, as judged on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Chromogenic Assay for Prothrombin Activation—Two microliter aliquots of samples appropriately diluted with Tris-buffered saline (TBS) with 140 mM NaCl in 20 mM Tris-HCl, pH 7.5, containing 1 mg/ml BSA (TBS/BSA), were added to 88 μl of 0.1 μM (or as otherwise indicated) prothrombin in the same buffer in the presence and absence of 3 mM $CaCl_2$, and then incubated at 37°C for 20 min. Then, 10 μl of 4 mM *t*-butoxycarbonyl-Val-Pro-Arg-*p*-nitroanilide was added. The amount of thrombin generated was determined by measuring the initial velocity of *p*-nitroaniline liberation at 405 nm with a kinetic plate reader (Well Reader, Seikagaku Kogyo). If necessary, multactivase was inactivated with EDTA prior to the addition of the chromogenic substrate.

SDS-Polyacrylamide Gel Electrophoresis and Determination of the Molecular Weight—SDS-PAGE of multactivase was carried out on 15% polyacrylamide gels containing 1% SDS in Tris-glycine buffer as described by Laemmli (9). The gels were stained with Coomassie Brilliant Blue R-250 (Sigma). The molecular weight of multactivase was determined by SDS-PAGE under non-reducing conditions (the catalytic subunit and regulatory subunit) and reducing conditions (all chains). After SDS-PAGE with multactivase, the molecular weight was calculated by interpolation of a linear semi-logarithmic plot of relative molecular mass *vs.* distance of migration, with the following proteins as standards: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Determination of the Protein Concentration—Twenty microliters of samples containing multactivase were added to 200 μl of fivefold diluted reagent (BCA protein assay kit from Pierce) and then incubated for 5 min at room temperature. The multactivase concentration was determined by measuring the absorbance at 595 nm with various concentrations of BSA as a standard.

Isolation of Multactivase—Fifty milligrams of crude venom of *E. multisquamatus* was dissolved in 1 ml of 50 mM Tris-HCl, pH 8.0, and insoluble materials were removed by centrifugation. The supernatant was loaded onto a Superdex 200 pg (1.6×60 cm) column for gel filtration with the same buffer at the flow rate of 1 ml/min. One-milliliter fractions were collected, and prothrombin activator activity was measured as described above. The active fractions were pooled and then applied to a column of Q-Sepharose High Performance (1.6×20 cm) preequilibrated with 50 mM Tris-HCl, pH 8.0, and then chromatography was performed at the flow rate of 2 ml/min. After washing (approximately 1 column volume), the bound proteins were eluted with a linear gradient of NaCl in the same buffer (0–0.4 M; 70 ml each). Two-milliliter fractions were collected, and the fractions containing prothrombin activator activity were pooled. After dialysis against 50 mM Tris-HCl, pH 8.0, the pooled fractions were separated on a column of Blue Sepharose CL-6B (1.0×20 cm) pre-equilibrated with the same buffer without NaCl at the flow rate of 0.5 ml/min, and eluted with a linear gradient of NaCl in the same buffer (0–1.0 M NaCl; 70 ml each). Multactivase was completely isolated at this step. All chromatography steps were performed at 4°C with a fast protein liquid chromatography system (Pharmacia). The enzyme obtained was stored at either 4°C or −80°C, and was stable for 2 weeks at 4°C and for months at −80°C.

Protein Sequencing—Purified multactivase was subjected to SDS-PAGE under reducing conditions and then electroblotted onto a poly(vinylidene difluoride) membrane (Millipore) by the method of Hirano (10). The bands transferred onto a poly(vinylidene difluoride) membrane were stained with Amido Black and then cut out. After treatment with 20 mg/ml dithiothreitol in 4 M urea at 50°C for 2 h, the sections of membrane containing the bands of multactivase were incubated at room temperature for 30 min in the presence of 100 mg/ml iodoacetamide. The bands of reduced *S*-acetamidomethylated polypeptides were subjected to analysis with an Applied Biosystems protein sequencer (model 473A).

Quantification of Normal Prothrombin with Multactivase and E. multisquamatus Venom—Normal and abnormal plasma were mixed in the indicated ratios. Ten microliter aliquots of mixed plasma diluted tenfold with TBS/BSA were blended with 10 μl of 30 mM $CaCl_2$, 10 μl of 2.5 mM Boc-Val-Pro-Arg-*p*NA, and 60 μl of TBS/BSA. The samples were incubated for 2 min at 37°C. After the addition of 10 μl of 2.5 nM multactivase or 1 μg/ml of *E. multisquamatus* venom, the amount of normal prothrombin in mixed plasma was determined by measuring the rate of *p*-nitroaniline liberation by multactivase-generated thrombin at 405 nm with a kinetic plate reader (Well Reader, Seikagaku Kogyo).

Plasma samples from patients receiving oral anticoagulant therapy were also examined as described above, with various concentrations of normal plasma (Nycomed Pharma AS., Oslo, Norway) as a standard. These normal prothrombin level data were compared with the prothrombin time data by least-squares linear regression analysis.

RESULTS

Isolation of Multactivase from Echis multisquamatus Venom—Gel filtration on a column of Superdex 200 pg separated the crude venom (50 mg) into five peaks, as found on monitoring at 280 nm. Analysis of prothrombin

activator activity in the presence and absence of Ca^{2+} ions revealed Ca^{2+}-dependent activity in the first peak (Fig. 1A). In contrast to in the case of *E. carinatus leucogaster* venom (*5*), this activity was hardly detected in the absence of Ca^{2+} ions. In the second step involving Q-Sepharose High Performance column chromatography, two peaks were observed on the basis of the absorbance at 280 nm, and the fractions containing the second peak could activate prothrombin in the presence of Ca^{2+} ions (Fig. 1B). A Ca^{2+}-dependent prothrombin activator was isolated from these active fractions by chromatography on a Blue Sepharose CL-6B column (Fig. 1C). This activator was designated as multactivase, following the recommended principles for the nomenclature of exogenous hemostatic factors of the International Society on Thrombosis and Haemostasis (*11*). In a typical purification, we obtained 2.3 mg of multactivase from 50 mg of crude venom. Interestingly, we did not detect an ecarin-like Ca^{2+}-independent prothrombin activator in any chromatographic fraction of this venom. The results of SDS-PAGE of the purified multactivase are shown Fig. 1D compared with CA-1. Two bands (62 kDa plus 24 kDa) were obtained under non-reducing conditions, and three bands (62, 16, and 15 kDa) under reducing conditions, similar to in the case of CA-1. However, the heavy chain of multactivase did not give two bands, like that of CA-1 did. These polypeptides could not be subsequently separated by any chromatographic procedure under nondenaturing conditions. However, gel filtration on Superdex 200 pg under denaturing conditions with 4 M guanidine hydrochloride separated these polypeptides, and showed them to have molecular weights of 62 and 24 kDa (data not shown).

N-Terminal Amino Acid Sequence of Each Polypeptide Comprising Multactivase—The N-terminal amino acid sequences of the individual polypeptide chains comprising multactivase are shown in Fig. 2. The 62-kDa chain was highly homologous to those of CA-1 (*5*) and ecarin (*12*). These sequences were not only similar to one another (Fig. 2A), but also resembled those of various metalloproteases of the replolysin family derived from snake venoms (*13*), which function as hemorrhagic factors, factor X activators, and prothrombin activators. The two polypeptides (16 and 15 kDa) which constituted the 24-kDa subunit of multactivase were highly homologous to each other (Fig. 2B). Their sequences were not only similar to those of CA-1, but also showed homology to those of C-type lectin-like proteins from the venoms of Viperidae snakes, *e.g.* coagulation factor IX/factor X-binding protein (*14-16*), platelet

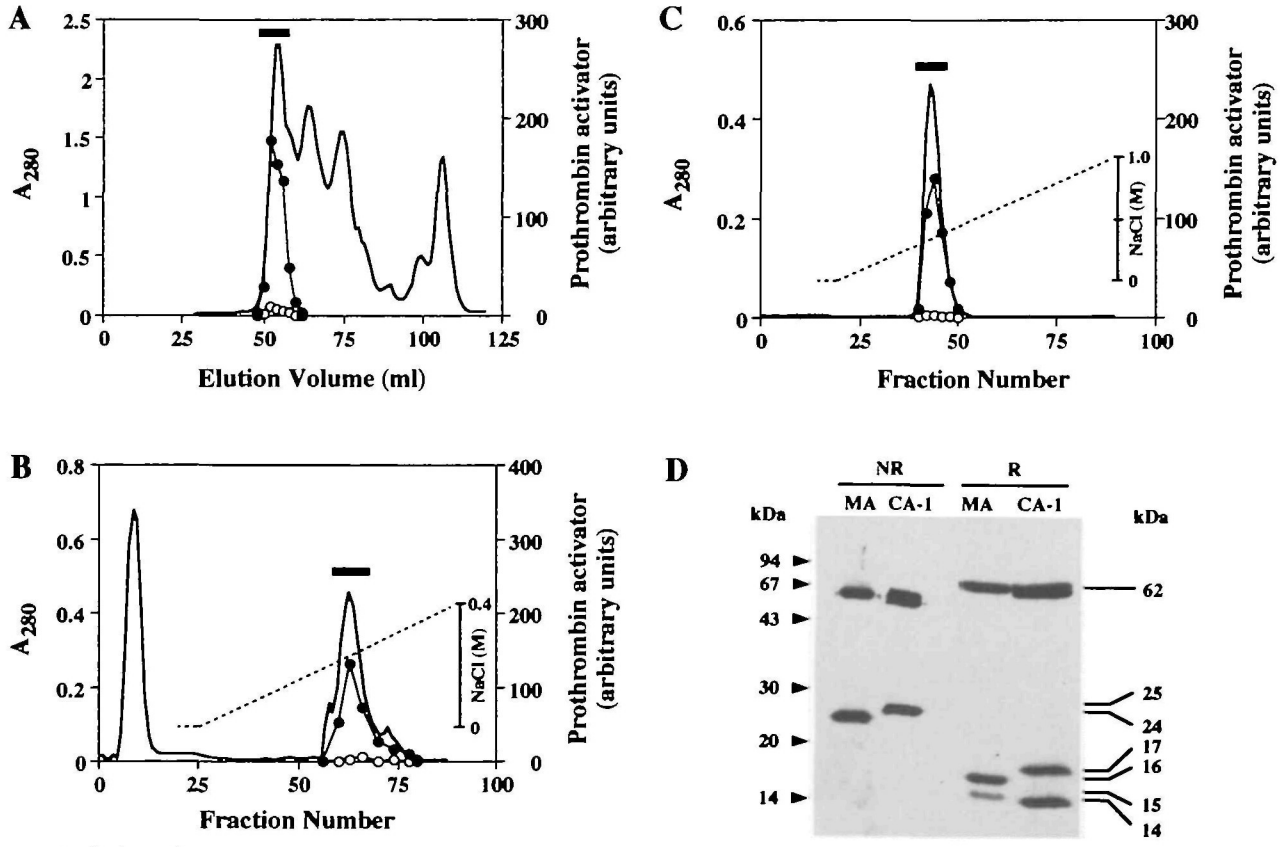

Fig. 1. **Isolation of multactivase.** A (step 1): The venom of *E. multisquamatus* (50 mg) was subjected to gel filtration on Superdex 200 pg. The fractions indicated by the bar were pooled. B (step 2): The active fractions from A were applied to a column of Q-Sepharose High Performance (1.6×20 cm) and then eluted with a linear gradient of NaCl (dotted line). Two-milliliter fractions were collected. The pooled fractions are indicated by the bar. C (step 3): The pooled fractions from B were applied to a Blue Sepharose CL-6B column (1.0×20 cm) and then eluted with a linear gradient of NaCl (dotted line). Two-milliliter fractions were collected. The pooled fractions (bar) contained apparently homogeneous multactivase. The prothrombin activator activity in each fraction was assayed as described under "MATERIALS AND METHODS," in either the presence (closed circles) or absence (open circles) of 3 mM Ca^{2+} ions. D: SDS-PAGE of multactivase. The isolated multactivase was compared with CA-1 on SDS-PAGE. MA, multactivase; NR, non-reducing conditions; R, reducing conditions (10% 2-mercaptoethanol).

glycoprotein Ib-binding protein alboaggregin-B (*17*), von Willebrand factor-dependent platelet coagglutinin botrocetin (*18*), and the hirudin-like anticoagulant protein, bothrojaracin (*19*).

Mechanism of Activation of Prothrombin by Multactivase—Multactivase activated prothrombin in a strictly Ca^{2+}-dependent manner. As shown in Fig. 3A, Ca^{2+} ions at millimolar concentrations were necessary for multactivase (half-maximal and maximal activation occurred at 0.2 and 1.0 mM, respectively), as was also the case for CA-1 (half-maximal and maximum activation occurred at 0.4 and 2 mM). Multactivase-induced prothrombin activation in the absence of Ca^{2+} ions was 2% of the maximum activity, whereas prothrombin activation by CA-1 without Ca^{2+} ions was less than 0.1% of the maximum activity (Fig. 3B).

Next, we examined the effects of phospholipids on multactivase-induced prothrombin activation. Phospholipids (especially phosphatidylserine) bind to a vitamin K-dependent coagulation factor *via* the Ca^{2+}-bound Gla domain and form the base for blood coagulation. When phospholipids (phosphatidylcholine/phosphatidylserine, 3 : 1, w/w) were applied to samples for prothrombin activation by multactivase, the phospholipids inhibited prothrombin activation in a dose-dependent manner (Fig. 4), suggesting that multactivase recognizes the Ca^{2+}-bound conformation of the Gla domain in prothrombin activation.

We also examined the inhibition of multactivase-induced prothrombin activation by prothrombin fragment 1 containing the Gla domain. If multactivase as well as CA-1 recognize the Gla domain in prothrombin activation, prothrombin activation by multactivase would be inhibited by fragment 1. As predicted, multactivase-induced prothrombin activation was effectively inhibited by fragment 1 in a concentration-dependent manner (data not shown). From these observations, we deduced that multactivase has an activation mechanism similar to that of CA-1 (*5*).

That the mechanism of activation of prothrombin by multactivase is identical to that of CA-1 was confirmed by the measurement of chromogenic activity in plasma derived from vitamin K antagonist-treated individuals (abnormal plasma). This abnormal plasma was rich in abnormal prothrombin with incompletely carboxylated Gla residues (PIVKA-II) and contained low level of normal prothrombin. The assessment of normal prothrombin levels in plasma with a defined ratio of normal and abnormal plasma was performed using multactivase. Multactivase-induced prothrombin activation decreased proportionately with an increase in the proportion of abnormal plasma (Fig. 5). It was apparent that multactivase selec-

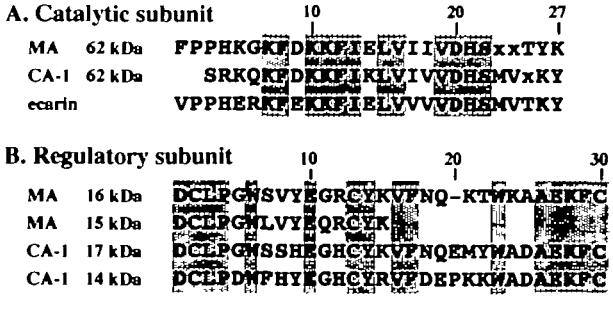

Fig. 2. **N-terminal amino acid sequences of multactivase.** The determined sequence of each polypeptide in multactivase is aligned with those of CA-1 (*5*) and ecarin (*12*). Identical residues are shaded. A: The catalytic subunits of multactivase, CA-1, and ecarin. B: The polypeptides comprising of the regulatory subunits of multactivase and CA-1.

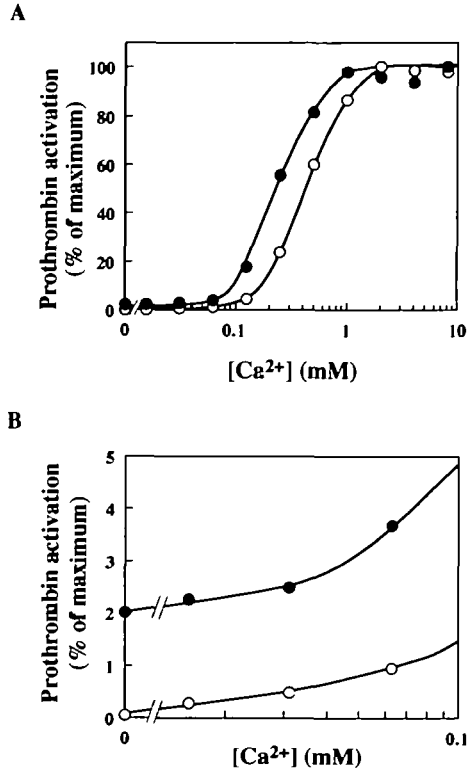

Fig. 3. **Ca^{2+} titration curve for prothrombin activation by multactivase.** A: The Ca^{2+} requirement of multactivase was measured using serial twofold dilutions from 8 mM Ca^{2+} ions. The Ca^{2+} concentration is presented on a logarithmic scale. B: The Ca^{2+} concentration between 0 and 0.1 mM. The Ca^{2+} concentration is presented on a logarithmic scales. Prothrombin at 1 μM mixed with TBS/BSA containing 4 pM multactivase (closed circles) or 4 pM CA-1 (open circles) and the indicated concentrations of Ca^{2+} ions was incubated for 20 min at 37°C. The thrombin generated was then quantified as described under "MATERIALS AND METHODS." The maximum activities were almost equivalent.

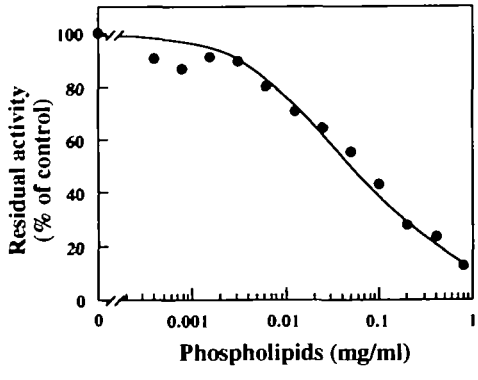

Fig. 4. **Effect of phospholipids on multactivase-induced prothrombin activation.** Prothrombin (50 nM) was incubated with 50 pM multactivase in the presence of 3 mM Ca^{2+} ions, and then the effect of phospholipids (phosphatidylcholine/phosphatidylserine, 3 : 1, w/w) was measured.

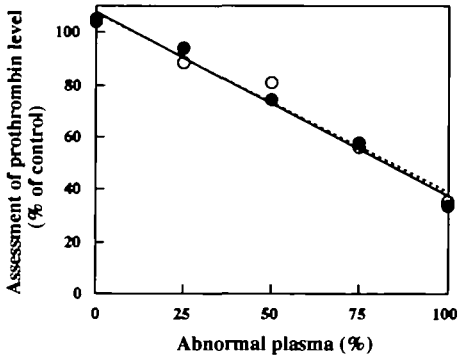

Fig. 5. **Multactivase-induced prothrombin activation requires the Gla domain in prothrombin.** Normal plasma and abnormal plasma from individuals who had been taking vitamin K antagonists were mixed at the indicated concentrations, and then normal prothrombin levels were assessed with multactivase (closed circles) and *E. multisquamatus* venom (open circles) as described under "MATERIALS AND METHODS."

tively recognized normal prothrombin with all the Gla residues intact, even in the presence of excess acarboxyprothrombin. These observations confirmed that its activation mechanism was the same as that of CA-1. Interestingly, the crude venom of *E. multisquamatus*, in place of purified multactivase, yielded the same results (Fig. 5), indicating that the crude venom contains no ecarin-like enzyme.

We next quantified normal prothrombin with the complete Gla domain in patients receiving warfarin treatment ($n=66$). The percentage of normal prothrombin activity measured with multactivase (final, 250 pM) was compared with the prothrombin time (%) by linear regression analysis (Fig. 6A). The correlation between the levels was significant ($y=1.01x+15.47$, $r=0.79$, $p<0.0001$). Interestingly, the same results were obtained with the crude venom of *E. multisquamatus* (final, 100 ng/ml) instead of the purified multactivase ($y=1.11x+12.45$, $r=0.80$, $p<0.0001$; Fig. 6B).

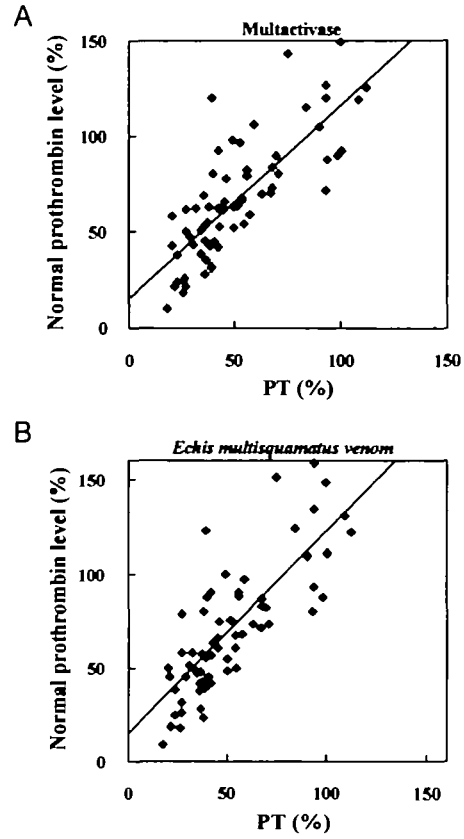

Fig. 6. **Assessment of normal prothrombin levels in abnormal plasma with multactivase and the venom of *E. multisquamatus*.** Normal prothrombin levels were assessed as described under "MATERIALS AND METHODS." The prothrombin time (PT) measured in each hospital was correlated with the normal prothrombin levels determined with multactivase (A: $y=1.01x+15.47$, $r=0.79$, $p<0.0001$) and with the venom of *E. multisquamatus* (B: $y=1.11x+12.45$, $r=0.80$, $p<0.0001$).

DISCUSSION

Viperidae snake venoms contain high levels of proteins that can act on the blood coagulation system (*2, 20*). Among them, a large number of metalloproteases and C-type lectin-like proteins have been documented. Both types of proteins have various actions; metalloproteases function as hemorrhagic factors, factor X activators, and prothrombin activators, while C-type lectin-like proteins act as factor IX/factor X-binding proteins (IX/X-bp), glycoprotein Ib-binding proteins, von Willebrand factor-dependent platelet coagglutinin, and α-thrombin inhibitors. However, hybrid proteins composed of metalloproteases and C-type lectin-like proteins have scarcely been reported. Only two such enzymes (RVV-X as a factor X activator and CA-1 as a prothrombin activator) have been confirmed (*5, 21-23*). The structures of factor X activators in the venoms of *Cerastes cerastes* (*24*) and *Bothrops atrox* (*25*) might also be homologous to those of these hybrid enzymes (*23*). The structure of multactivase was the same as that of CA-1. Therefore, multactivase was identified as one of these hybrid enzymes.

It is still unclear how such hybrid enzymes are synthesized and correctly folded. It has been suggested that these enzymes are each synthesized as a polypeptide composed of a metalloprotease domain, a disintegrin-like domain, a Cys-rich domain, and a C-type lectin-like domain, and become a mature protein on cleavage between the Cys-rich and C-type lectin-like domains (*26-28*). Kini (*27, 29*) also suggested the possibility that a C-type lectin-like domain is located on the amino terminal side of the metalloprotease domain. However, cDNA sequence analysis of the *T. flavoviridis* IX/X-bp A chain and B chain, which are homologous to the regulatory subunit of multactivase, revealed 5′-end noncoding bases, an open reading frame, 3′-end noncoding bases, a polyadenylation signal, and a poly(A)$^+$ region (*30*). Thus, both polypeptide chains of *T. flavoviridis* IX/X-bp are separately synthesized and then joined by an S-S bond. Furthermore, for the X-ray structure of *T. flavoviridis* IX/X-bp, it was recently reported that a central loop projects into the adjoining subunit and forms an intertwined dimer in a manner similar to domain swapping (*31*). Thus, each of the regulatory subunits of multactivase might be transcribed separately and then joined in a domain swapping manner through disulfide bridge(s) or non-covalent binding. Thus, multactivase could

be a useful tool for investigating the topology of the polypeptides in such hybrid enzymes because this is one of the most abundant enzymes of this class.

Solovjov *et al.* (*32*) quite recently isolated a prothrombin activator from the venom of *E. multisquamatus* and named it ecamulin. The structure of ecamulin, *i.e.* a 67-kDa single polypeptide subunit and a 27-kDa subunit comprising 14- and 13-kDa polypeptides linked through disulfide bridge(s), is almost identical to that of multactivase, suggesting that ecamulin and multactivase are homologous prothrombin activators. However, Solovjov *et al.* reported that the prothrombin activation by ecamulin does not require Ca^{2+} ions, similarly to that by ecarin, and that the biological activities of ecamulin and ecarin are very similar (*32*), although an experiment on the effect of Ca^{2+} ions on the prothrombin activation by ecamulin has not yet been performed. We detected no ecamulin-like Ca^{2+}-independent prothrombin activator activity in the venom of *E. multisquamatus*, and with the crude venom of *E. multisquamatus* in place of the purified multactivase the same results were obtained (Figs. 5 and 6B), indicating that the crude venom contains no ecarin-like enzyme. Therefore, ecamulin most likely has the same properties as those of a carinactivase-like enzyme, not an ecarin-like enzyme.

Warfarin is widely used as an oral anticoagulant for patients with thrombophilia. The dosage of warfarin is decided by monitoring of the prothrombin time (PT) in the optimal therapeutic range, because the suitable dose markedly differs between individuals. However, bleeding or thromboembolic complications sometimes occur during warfarin treatment in patients strictly dose-regulated by means of PT monitoring, and the use of monitoring of normal prothrombin levels decreases such complications (*33-35*). There have also been reports that a normal prothrombin level reflected most of the clotting activity of plasma from orally anticoagulated individuals (*36, 37*). Therefore, the determination of normal prothrombin levels in plasma is indispensable for the determination of warfarin dose, and more convenient methods should be developed. The results shown in Fig. 6A indicate that multactivase is an excellent candidate as a diagnostic reagent for monitoring normal prothrombin levels in plasma.

In conclusion, multactivase isolated from *E. multisquamatus* venom is a novel CA-1-like prothrombin activator. Multactivase belongs to the group 1B-prothrombin activators (*5, 6*). Multactivase is not only a useful probe for the investigation of how hybrid enzymes are biosynthesized and correctly folded, but also may be useful as a diagnostic reagent for determining normal prothrombin levels in plasma. This activator should be a good model for elucidating the details of the evolution of the metalloproteases and C-type lectin-like proteins in snake venoms. The assessment of normal prothrombin levels in plasma with multactivase has advantages over methods involving Ca^{2+}-dependent anti-prothrombin antibodies (*33-35*) because the former is easier and faster than enzyme-linked immunosorbent assaying and radioimmunoassaying with such antibodies.

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