The venom of the viper Echis carinatus contains a metalloprotease, ecarin, that is a potent prothrombin activator. We here show that the venom is also rich in another prothrombin activator, which does not belong to any known category of prothrombin activators. The novel enzyme, designated carinactivase-1 (CA-1), consists of two subunits held together non-covalently but very tightly. One subunit is a 62-kDa polypeptide that has metalloprotease activity and is homologous to the single-chain enzyme ecarin; the other subunit of 25 kDa consists of two disulfide-linked polypeptides of 17 and 14 kDa, and this subunit resembles the anticoagulant in the habu snake venom, IX/X-bp, that specifically binds the Gla domains of coagulation factors IX and X in a Ca\(^{2+}\)-dependent fashion. The activation of prothrombin by CA-1 requires Ca\(^{2+}\) ions at millimolar concentrations and in the absence of Ca\(^{2+}\) ions this enzyme is virtually inactive. By contrast, activation by ecarin is completely independent of Ca\(^{2+}\) ions. CA-1, unlike ecarin, does not activate prothrombin derivatives, in which binding of Ca\(^{2+}\) ions has been perturbed, namely prethrombin-1 and acarboxyprothrombin. Furthermore, the isolated catalytic subunit, although its activity is greatly reduced as compared to that of the holoenzyme, no longer requires Ca\(^{2+}\) ions for the activation of prothrombin. Reconstitution with the non-catalytic 25-kDa subunit restores high level activity and the dependence on Ca\(^{2+}\) ions. Finally, prothrombin activation by CA-1 is inhibited by prothrombin fragment 1, and the isolated non-catalytic subunit is capable of binding fragment 1 in the presence of Ca\(^{2+}\) ions. From these observations, we postulate the following unique mechanism for the activation of prothrombin by CA-1. The enzyme primarily recognizes the Ca\(^{2+}\)-bound conformation of the Gla domain in prothrombin via the 25-kDa regulatory subunit, and the subsequent conversion of prothrombin to active thrombin is catalyzed by the 62-kDa catalytic subunit.

Compounds that affect the mammalian blood coagulation system, in particular those that cause acute thrombosis, are often the major active principals of the lethal toxins in viper venoms (1, 2). Thrombogenic components in these venoms exhibit considerable heterogeneity in terms of function as well as of structure. Many types of protease that convert quiescent clotting proenzymes to their active forms (or inactive procofactors to active cofactors) are known, and various prothrombin activators have been reported (2). To date, three types of prothrombin activator have been identified in venoms (3): group 1 enzymes, which are metalloproteases whose actions on prothrombin are independent of any plasma or exogenous cofactors; group 2 enzymes, which are Gla-containing, factor Xa-like serine proteases that require factor Va, anionic phospholipids and Ca\(^{2+}\) ions, resembling in this respect the physiological activator factor Xa; and group 3 enzymes, which are hybrid proteins that consist of factor Xa-like catalytic subunits and factor Va-like regulatory subunits and require phospholipids and Ca\(^{2+}\) ions for their action. The group 1 enzymes are widely distributed in venoms of many kinds of vipers, e.g. genera Echis and Bothrops, and they are presumably the most toxic since they are resistant to the natural coagulation inhibitors (serpins) present in mammalian plasma, such as antithrombin-III. Another difference between metalloprotease-type prothrombin activators and the physiological activator factor Xa or the venom serine proteases involves the cleavage sites in the prothrombin molecule. The metalloenzymes cleave only the bond between the A chain and the B chain (Arg\(^{220}\)Ile\(^{221}\) in human prothrombin; Arg\(^{221}\)Ile\(^{222}\) in the bovine protein) with the resultant production of meizothrombin, which is ultimately converted to \(\alpha\)-thrombin by autolysis (4). The serine-type enzymes cleave one additional site (the junction between fragment 2 and the A chain; Arg\(^{271}\)Thr\(^{272}\) in the human protein) to produce \(\alpha\)-thrombin directly (5).

The venom of Echis carinatus contains a high level of a metallo-type prothrombin activator and has been widely used in laboratory studies as a convenient tool for the production of thrombin from prothrombin. The enzyme in E. carinatus venom, named ecarin, is a single-chain protein of 55 kDa that exhibits very strict substrate specificity. Prothrombin is the only protein that is cleaved by ecarin in plasma, and other structurally related coagulation factors, e.g. factors IX and X, are scarcely affected (6). The primary structure of ecarin was recently determined by molecular cloning (7). The mature protein consists of three independent motifs. From N to C terminus, there is a metalloprotease catalytic domain of approximately 200 amino acid residues, a disintegrin-like domain of approximately 90 residues, and a Cys-rich domain of approximately 120 residues. At present, however, the roles of non-catalytic domains remain unclear as to the factors that determine the strict specificity of this enzyme, and further information, in particular those about the three-dimensional structure, is necessary to clarify these issues. A significant number of proteins with the same domain organization and with unique respective functions has recently been identified in mammalian tissues (see Ref. 8, and references therein). Thus, this venom protein should serve as a good model in efforts aimed at an understanding of the biochemistry of these mammalian proteins as well as of details of the evolution of these proteins.
During the purification of ecarin, we found a novel prothrombin activator in the same venom preparation, which could not be assigned to any of the above mentioned categories. This enzyme, designated carinactivase-1 (CA-1), is strongly dependent on Ca\(^{2+}\) ions for the activation of prothrombin, in sharp contrast to ecarin, whose action is unaffected by exogenous Ca\(^{2+}\) ions. In the present report, we describe the purification of CA-1 and discuss the relationship between its structure and function and the unique mechanism by which it activates prothrombin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The venom of Malian E. carinatus leucogaster used for the isolation of CA-1 and ecarin was obtained from Latoxan (Resans, France). Gels for chromatography were from Pharmacia Biotech Inc. Bovine serum albumin (essentially fatty acid-free, ELISA grade) and phospholipids were from Sigma. Lyophilized pooled plasma from normal subjects was from the National Institutes of Health, Bethesda, MD. Bovine prethrombin-1 and fragment 1 and human factor Xa activities were assayed as described below. The active fractions were lyophilized and stored at \(-80^\circ\)C. A new lot of pooled plasma from normal subjects was used for assays on each occasion. Other methods—SDS-PAGE was performed by the method of Laemmli (19), with bovine serum albumin as the standard.

**RESULTS**

The venom of E. carinatus was fractionated by gel filtration and assayed for activation of prothrombin (Fig. 1A). Ecarin activity, which could be detected in the absence of Ca\(^{2+}\) ions, was found in the first protein peak. When the assay was conducted in the presence of a millimolar concentration of Ca\(^{2+}\) ions, the extent of activation of prothrombin was considerably enhanced. Since the activity of purified ecarin did not show any Ca\(^{2+}\) dependence (see below), it was clear that this fraction also contained another, hitherto unidentified prothrombin activator(s) whose activity was dependent on Ca\(^{2+}\) ions. We isolated this activity, as described below.

This fraction was applied to a column of Blue Sepharose. As shown in Fig. 1B, two peaks of the activity of a Ca\(^{2+}\)-dependent prothrombin activator were identified, and we designated these activities CA-1 and CA-2, respectively. Ecarin was eluted at higher concentrations of NaCl and was clearly separated from them.

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1 The name carinactivase follows the recommended principles for nomenclature of exogenous hemostatic factor of the International Society on Thrombosis and Haemostasis (9).
2 The abbreviations used are: CA-1 (\(-\)), carinactivase-1 (\(-\)); TBS, Tris-buffered saline; BS\(^3\), bis(sulfosuccinimidyl) suberate PAGE; polyacrylamide gel electrophoresis; CRD, carbohydrate-recognition domain.
from CA-1 and CA-2 at this step. Subsequent purification and characterization revealed that CA-2 was almost identical to CA-1 in terms of the molecular structure and enzymological features, and the main focus of this report is on CA-1. Isolation of CA-1 was accomplished by a third chromatography on Q-Sepharose (Fig. 1C). In a typical purification, we obtained 2 mg of CA-1 and 0.1 mg of ecarin from 100 mg of crude venom. The activity of CA-1 was irreversibly abolished by the incubation with EDTA or with heavy metals such as Co$^{2+}$ and Mn$^{2+}$, but it was resistant to inhibitors of serine, thiol, or carboxyl proteases. Thus, CA-1 appeared to be a metalloenzyme, as is ecarin.

SDS-PAGE of CA-1 is shown in Fig. 2A (lane 1). Two bands (60/62-kDa doublet plus 25 kDa) were obtained under non-reducing conditions, and three bands (62/64, 17, and 14 kDa) were obtained under reducing conditions. The doublet appearance of the larger polypeptide was probably due to microheterogeneity, as discussed below. We were unable to separate these polypeptides by any subsequent chromatographic procedures under non-denaturing conditions. The polypeptides also comigrated as a single band in native-PAGE. Dissociation of the polypeptides required rather extreme conditions. For example, guanidine hydrochloride (≥4 M) or SDS (≥0.1%) was effective, but urea was ineffective up to 8 M. Fig. 2B depicts the chromatograms after gel filtration in either the absence or the presence of 4 M guanidine HCl. Without the denaturant, a single, symmetrical peak that contained all three polypeptides was obtained. By contrast, two peaks were obtained in the presence of the denaturant; the first peak contained the 60/62-kDa polypeptide, and the second peak contained the 25-kDa component (see Fig. 2A). Thus, it appeared that CA-1 was a protein that consisted of two heterogeneous subunits held together non-covalently but very tightly, and that the 25-kDa subunit consisted of two different disulfide-linked polypeptides. The stoichiometry of the two subunits was 1 to 1. When the holoenzyme was subjected directly to protein sequence analysis, almost equimolar amounts (after correction for recovery of each amino acid) of three amino acids, corresponding to residues in the sequences of each of the three polypeptides (see below), were found after each sequencing cycle.
The N-terminal amino acid sequence of each polypeptide in CA-1 was analyzed (Fig. 3A). The 60/62-kDa chain had a sequence highly homologous to that of ecarin (7) and IX/X-bp (20). Identical residues are shaded. Two N-terminal residues of the 62-kDa polypeptide, indicated by dots, are absent from the 60-kDa polypeptide. B, schematic representation of the structures of CA-1, ecarin, and IX/X-bp. The apparent molecular mass of each polypeptide is shown.

The N-terminal amino acid sequence of each polypeptide in CA-1 was analyzed (Fig. 3A). The 60/62-kDa chain had a sequence highly homologous to that of ecarin (7), and this subunit did, indeed, have metalloprotease activity, as described below. The 60-kDa polypeptide had an identical sequence to that of the A chain and the B chain, as does ecarin. By contrast to the A chain and the B chain, the C chain of thrombin was extremely slow (less than 1/100 of that of ecarin). As shown in Fig. 4B, the 60-kDa polypeptide of CA-1 cross-reacted with antiserum against IX/X-bp. The determination sequence of each polypeptide in CA-1 is shown.

**Fig. 3. Structure of CA-1.** A, N-terminal amino acid sequence of CA-1. The determined sequence of each polypeptide in CA-1 is aligned with those of ecarin (7) and IX/X-bp (20). Identical residues are shaded. Two N-terminal residues of the 62-kDa polypeptide, indicated by dots, are absent from the 60-kDa polypeptide. B, schematic representation of the structures of CA-1, ecarin, and IX/X-bp. The apparent molecular mass of each polypeptide is shown.
reaction with the natural substrate, hydrolysis by CA-1 (and also by ecarin) of a peptidyl fluorogenic substrate, which was synthesized to resemble the scissile site in prothrombin, was unaffected by exogenous Ca\(^{2+}\) ions (Table I). Thus, it seemed likely that Ca\(^{2+}\) ions were required for the recognition of prothrombin by CA-1; prothrombin is capable of binding Ca\(^{2+}\) ions via the N-terminal Gla domain and undergoes a dramatic change in conformation at Ca\(^{2+}\)-concentrations around 1 mM. It appeared possible that CA-1 might recognize the Gla domain of prothrombin with bound Ca\(^{2+}\) ions, primarily via the IX/X-bp-like 25-kDa subunit, which is not present in ecarin.

To examine this possibility, we investigated the activation of prothrombin in detail using prothrombin derivatives and isolated CA-1 subunits. First, we tested prethrombin-1, which lacks prothrombin fragment 1, the N-terminal portion of prothrombin, while that of ecarin was influenced by neither of these factors.

Next, we evaluated the role of the subunits of CA-1 (Table II). When we examined the amidolytic activity of the fluorogenic substrate, which was synthesized to resemble the scissile site in bovine prothrombin, we found that the isolated 62-kDa catalytic subunit had enzymatic activity almost equivalent to that of the intact CA-1, but the rate of activation of prothrombin, the natural substrate, was much reduced. The 25-kDa subunit had no enzymatic activity (data not shown). Removal of the 25-kDa subunit also led to the loss of the dependence on Ca\(^{2+}\) ions. It appeared, therefore, that the 62-kDa component is the metalloprotease catalytic subunit, while the 25-kDa non-catalytic component is an accessory, regulatory subunit. Reconstitution with the 25-kDa subunit restored both the high potency and the dependence on Ca\(^{2+}\) ions. By contrast, the rate of activation of prethrombin-1 was unaffected by reconstitution, and the rate of the reaction was very close to that for prothrombin activation in the absence of the 25-kDa subunit and/or Ca\(^{2+}\) ions. These results strongly suggested that CA-1 recognized the conformation of the Gla domain of prothrombin with bound Ca\(^{2+}\) ions via its 25-kDa subunit.

In order to obtain further evidence, we conducted three additional experiments. In the first one, we examined the effect of fragment 1 on the activation of prothrombin. As is shown in Fig. 5, the activation by CA-1, but not by ecarin, was effectively inhibited by fragment 1. This result indicated that the low reactivity of prethrombin-1 with CA-1 was due to the absence of fragment 1 and not due to a secondary change in conformation of the protein upon liberation of the N-terminal portion.

In the second experiment, we used plasma from individuals who had taken a vitamin K antagonist, in which abnormal prothrombin with incompletely carboxylated Gla residues was present concomitant with the decreased level of normal prothrombin. A batch of plasma with a clotting activity of 20% of that of normal controls, which had been determined by a standard assay of prothrombin time, was utilized. The plasma was mixed with prothrombin-deficient plasma, the activator, and Ca\(^{2+}\) ions, and the time required for clot formation was measured. The prothrombin content of the tested plasma was estimated with serially diluted normal control plasma as the reference. When CA-1 was used as the activator, the results were similar to those obtained with the physiological activator factor Xa (Table III). By contrast, with ecarin, higher values were obtained, probably representing the sum of normal and abnormal prothrombins since it is known that ecarin can activate abnormal prothrombin as well as normal prothrombin (24). It was apparent that CA-1 selectively recognized normal pro-

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**TABLE I**

| Substrate       | CA-1 + Ca\(^{2+}\) | CA-1 - Ca\(^{2+}\) | Ecarin + Ca\(^{2+}\) | Ecarin - Ca\(^{2+}\) |
|-----------------|--------------------|--------------------|----------------------|----------------------|
| Prothrombin     | 8.4                | 0.030              | 5.7                  | 5.5                  |
| Prethrombin-1   | 0.015              | 0.014              | 5.1                  | 5.6                  |
| Fluorogenic     | 2.5                | 2.5                | 2.2                  | 2.1                  |

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**TABLE II**

| Substrate       | Intact   | 62-kDa subunit  | 62-kDa + 25-kDa subunits |
|-----------------|----------|-----------------|--------------------------|
|                  | + Ca\(^{2+}\) | + Ca\(^{2+}\) | + Ca\(^{2+}\) |
| Fluorogenic      | 1.7      | 1.6             | 1.5                       |
| Prothrombin      | 10.1     | 0.035           | 0.033                     |
| Prethrombin-1    | 0.014    | 0.039           | 0.013                     |

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**Fig. 5.** Inhibition of the CA-1-induced activation of prothrombin by fragment 1. Prothrombin (50 nM) was incubated with 10 pM CA-1 (closed circles) or 50 pM ecarin (closed triangles) in the presence of 10 mM Ca\(^{2+}\) ions, and the effect of fragment 1 was examined.
with fragment 1 was effectively blocked by the addition of an excess of the cold 25-kDa subunit (lane 5), indicating that this interaction was specific. These results together demonstrate that the unique structure of CA-1 explains the unique mechanism of its activation of prothrombin; the 25-kDa subunit first recognizes the N-terminal Gla domain of prothrombin in a Ca\(^{2+}\)-dependent fashion, and then the 62-kDa catalytic subunit cleaves the bond between the A and B chains, generating meizothrombin. Participation of Ca\(^{2+}\) ions in the exposure of the Gla domain recognition site on the regulatory subunit has not been proven and is hypothetical at present. For further details, see under "Discussion."

**DISCUSSION**

The results described herein clearly show that the venom of *E. carinatus* contains a hitherto novel type of prothrombin activator. This finding necessitates reconsideration of the classification of exogenous prothrombin activators. We propose that the previously defined group 1 enzymes (3) be divided into two subgroups, i.e. the ecarin-like (CA\(^{2+}\)-independent) metalloproteases (perhaps termed group 1A) and the carinactivase-like (CA\(^{2+}\)-dependent) enzymes (group 1B).

We screened the venoms of various Viperidae snakes for carinactivase-like activity (detailed screening data will be published elsewhere).\(^3\) All the venom preparations from *Echis* snakes contained both ecarin-like and carinactivase-like activators, although the total activities as well as the relative abundance of two enzymes varied depending upon the source. However, we failed to detect carinactivase-like activity in venoms of Viperidae snakes in genera other than *Echis*, although ecarin-like activities were found in some of them.

CA-1 appears to be a hybrid protein that is well adapted for the efficient and selective activation of prothrombin in the plasma of target animals after envenomation. The 25-kDa subunit of CA-1 exhibits striking structural similarity to the anticoagulant IX/X-bp in the *T. flavoviridis* venom. IX/X-bp is a heterodimeric protein that consists of two homologous polypeptide chains (20), and it recognizes CA\(^{2+}\)-bound conformations of the Gla domains in factors IX and X (22, 23). The function of the 25-kDa subunit is also similar to that of IX/X-bp, i.e. the CA\(^{2+}\)-dependent recognition of the Gla domain of prothrombin. Thus, the non-catalytic component is the regulatory subunit (Fig. 7). The specificity of IX/X-bp is very strict; it never binds other vitamin K-dependent coagulation factors such as prothrombin with the Gla residues intact, even in the presence of excess acarboxyprothrombin. Ecarin did not recognize such differences, and it seemed to recognize only the scissile site.

In the third experiment, we investigated the association between fragment 1 and the isolated 25-kDa subunit directly. We employed a cross-linking technique. The 125I-labeled 25-kDa subunit was incubated with fragment 1 (F1; 2 \(\mu\)l) in the presence and absence of 10 mM Ca\(^{2+}\) ions at 37°C for 60 min, and then it was cross-linked with BS\(^3\). The resultant SDS-stable complex was subjected to SDS-PAGE followed by analysis with a BAS-2000 Bioimaging Analyzer. In the lane 5, an excess of the cold 25-kDa subunit (6 \(\mu\)l) was included to confirm the specificity of the interaction. Positions of molecular mass markers are shown on the left. On the right, the arrowhead indicates the 25-kDa subunit, the double arrowhead shows the subunit dimer, and arrows show complexes of fragment 1 and fragment 1 polymers (and the subunit (and/or the subunit dimer). Detailed methods are given under "Experimental Procedures."
The Gla domain in prothrombin is a calcium-dependent anion-binding domain that is involved in thrombin activation (21). This domain is highly conserved among various prothrombin activators, and it is known that the Gla domain is essential for the binding of Ca$^{2+}$ ions, which facilitates the cleavage of prothrombin by the enzyme prothrombinase complex (22). The binding of Ca$^{2+}$ ions is a prerequisite for the subsequent binding to coagulation factors (23). It is possible that the regulatory subunit of CA-1 might also bind Ca$^{2+}$ ions and, upon binding of Ca$^{2+}$ ions, the recognition site for the Gla domain of prothrombin would be exposed (Fig. 7).

The results indicate that IX/X-bp and the regulatory subunit of CA-1 are highly analogous not only in terms of structure but also in terms of function, even though they have opposite toxicological effects. IX/X-bp is an anticoagulant, and it should thrombosis via the generation of thrombin. In addition to IX/X-bp from the habu snake venom, proteins structurally related to the regulatory subunit of CA-1 are widely distributed in viper venoms. We have identified homologues of IX/X-bp in venoms of Bothrops jararaca (27) and Deinagkistrodon acutus (28). Moreover, numerous proteins with structures very similar to IX/X-bp but with totally different pharmacological actions have been found in venoms of various Viperidae snakes, and appear to constitute a unique protein family. Ligands for these proteins are very heterogeneous. For example, botrocinin from B. jararaca binds von Willebrand factor (29), bothjaracrin from the same venom binds the anion-binding exosite in α-thrombin (30), and alboagregin from T. albolabris binds platelet glycoprotein Ib (31). Each of these venom proteins has two homologous polypeptide chains, and each chain constitutes the domain structure, known as CRD. The name CRD originally referred to the "carbohydrate-recognition domain" because this structure was first identified as the minimum functional motif of Ca$^{2+}$-dependent animal lectins such as asialoglycoprotein receptor. It is now known that the CRD is widely distributed in the animal kingdom, from invertebrates such as sea urchins to mammals, and it seems to be a fundamental motif that acts as an important domain in the construction of proteins (32, 33).

Another component in CA-1, the catalytic subunit, also has numerous relatives in the venoms of Viperidae. These relatives include ecarin in the same venom and hemorrhagic factors in T. flavoviridis and Crotaulus atrox venoms (26, 34). In addition, many proteins that resemble to these venom metalloproteases have recently been identified in mammalian tissue, in particular in reproductive organs, e.g. the sperm protein fertilin (PH-30) (35). Together, all these proteins constitute a superfamily for which the name ADAM has been proposed (8).

It is noteworthy that the factor X activator RVV-X in Vipera russelli venom has a structure very similar to that of CA-1. This protein also has three polyepitope chains, i.e. 57.6-, 19.4-, and 16.4-kDa chains, with a stoichiometry of 1:1:1, and these chains are held together by disulfide bonds, as recently proven unequivocally by Gowda et al. (36). In an earlier report, Takeya et al. (37) determined the complete amino acid sequences of the 57.6- and 16.4-kDa chains, and showed that the 57.6-kDa chain has a structure very similar to that of ecarin while the 16.4-kDa chain has a sequence homologous to that of IX/X-bp. Furthermore, the 19.4-kDa chain also has an N-terminal sequence homologous to IX/X-bp. The action of RVV-X is also dependent on both Ca$^{2+}$ ions and the Gla domain in factor X (38, 39). Therefore, it seems likely that the catalytic mechanism of RVV-X might be similar to that of CA-1. However, isolation of the intact catalytic chain of RVV-X appears to be impossible and thus unequivocal biochemical evidence cannot be obtained, because the chain is covalently linked to IX/X-bp-like chains. Both CA-1 and RVV-X are composed of two different components, which have a totally different genetic origin, and both proteins are likely to have originated from a single ancestral hybrid protein. It is still unclear how they are synthesized and correctly folded. The topology of the polypeptides in these enzymes is also unknown. These issues require further investigations.

In conclusion, we have shown that a newly isolated novel prothrombin activator, CA-1, has a unique machinery for the recognition and subsequent processing of its substrate. CA-1 should be useful as a convenient probe for biochemical studies in vitro of prothrombin and as a good diagnostic reagent for monitoring normal prothrombin levels in plasma (cf. Table III). Furthermore, this enzyme should be a good model for attempts to elucidate details of the evolution and the biosynthesis of multi-subunit proteins.

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Isolation and Characterization of Carinactivase, a Novel Prothrombin Activator in *Echis carinatus* Venom with a Unique Catalytic Mechanism

Daisuke Yamada, Fujio Sekiya and Takashi Morita

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