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Cation Binding Properties of the Multiple Subforms of RVV-X, the Coagulant Protein from *Vipera russelli*†

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ABSTRACT: The factor X activating enzyme from Russell's viper venom (RVV-X) has been shown to exist in multiple subforms, distinguished from each other by their isoelectric points. The differences in isoelectric points were due, at least in part, to dissimilarities in the respective sialic acid contents of the subforms. No functional difference was, however, discovered between any of the subforms. All of the subforms were found, by equilibrium ultrafiltration, to bind Ca^{2+} reversibly. At least two equivalent Ca^{2+} binding sites were observed on each protein molecule (M_r 79 000), with a K_D of 50 ± 15 μM at pH 7.4 and 25 °C. A new substrate for RVV-X, which does not bind Ca^{2+}, apoprotein AI from human high-density lipoprotein, was used to show that this reversibly bound Ca^{2+} was not essential for enzymic activity. All subforms have also been shown, by atomic absorption analysis, to contain nonexchangeable metal ions, to the extent of 1 mol of Ca^{2+} and 0.7 mol of Zn^{2+} per mol of protein. No Mn^{2+} or Mg^{2+} was detected. This nonexchangeable Ca^{2+} and Zn^{2+} could only be removed from the protein by incubation at pH 3.0 or by treatment with 6 M guanidine hydrochloride, conditions under which the protein lost activity irreversibly.

The venom from Russell's viper (RVV)[1] has long been known to possess a potent stimulatory effect toward blood coagulation (MacFarlane & Barnett, 1934). Williams & Esnouf (1962) purified a coagulant protein from RVV to apparent homogeneity, which they subsequently showed to be a potent activator of factor X (Esnouf & Williams, 1962): the protein with this activity is now designated RVV-X. Schiffman et al. (1969) showed that another coagulant protein could be separated from RVV, which functioned by stoichiometric acti-

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[1] Abbreviations: RVV, Russell's viper venom; RVV-X, the factor X activating enzyme from RVV; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; L-TAME, α-*N*-tosyl-L-arginine methyl ester; DEAE-cellulose, diethylaminoethylcellulose; QAE-Sephadex, quaternary aminoethyl-Sephadex; iPr_2PF, diisopropyl fluorophosphate.

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vation of factor V: this protein is now designated RVV-V.

Several different purification procedures have subsequently been published for RVV-X with, in several cases, conflicting data as to the physical properties of the purified protein. Although Williams & Esnouf (1962) reported significant L-TAME esterase activity in their preparations of RVV-X, this activity has subsequently been shown to be due to a contaminating protein, with the coagulant protein having no significant activity toward synthetic substrates (Jackson et al., 1971; Furukawa et al., 1976; Kisiel et al., 1976b). The subunit composition of the protein is still controversial with Furie & Furie (1975) observing only a single subunit of molecular weight 60 000, whereas Furukawa et al. (1976) and Kisiel et al. (1976b) propose a heavy chain of molecular weight 60 000, disulfide linked to one or more light chains of molecular weight 10 000–20 000.

There is also controversy regarding the susceptibility of RVV-X to iPr_2PF and, hence, whether the enzyme can be classified as a serine protease. Furukawa et al. (1976) assert that RVV-X is inhibited by iPr_2PF, whereas the experiments of Jackson et al. (1971) and Kisiel et al. (1976b) suggest no such inhibition.

During purification of RVV-X in our own laboratory, we had routinely observed multiple peaks of activity on ion-exchange chromatography, suggesting subforms of the enzyme. We felt that the properties of these subforms should be investigated in order to determine whether past controversies resulted from studies on different subforms possessing disparate properties. A further reason for our interest in RVV-X is that it has recently been shown to activate two vitamin K dependent plasma proteins in addition to factor X: factor IX (Lindquist et al., 1978) and protein C (Kisiel et al., 1976a). Since we are interested in the role of metal ions in the activation of these two plasma proteins by physiological activators and also by RVV-X (Amphlett et al., 1978; Byrne & Castellino, 1978; Amphlett et al., 1981a,b), it was essential to study the effects of metal ions on RVV-X.

Materials and Methods

Materials. Crude lyophilized RVV was obtained from Calbiochem, Sigma Chemical Co., and the Miami Serpentarium. $^{45}CaCl_2$ and $Na^{125}I$ were purchased from New England Nuclear. Sodium boro[^{3}H]hydride and ethylenediaminetetra[2-^{14}C]acetic acid were obtained from Amersham. Guanidine hydrochloride (extreme purity) was purchased from Heico.

Proteins. Bovine factor X was purified from fresh, citrated bovine plasma by the procedure of Bajaj et al. (1977) and tritiated in its sialic acid residues by the procedure of van Lenten & Ashwell (1971). Apo-AI, from human serum high-density lipoprotein, was prepared as described by Scanu et al. (1969). Purified apo-AI was radioiodinated by using the solid-state lactoperoxidase method of David (1972).

Protein Concentrations and Molecular Weight. Protein concentrations were determined from the absorption at 280 nm by using the following $\epsilon_{1cm}^{1\%}$ values: RVV-X, 11.8 [determined in this laboratory by the procedure of Babul & Stellwagen (1969)]: bovine factor X, 12.4 (Jackson & Hanahan, 1968); apo-AI, 11.9 (Edelstein & Scanu, 1980). The molecular weight of RVV-X was taken to be 79 000 (Kisiel et al., 1976b).

RVV-X Assays. The coagulant activity of samples of interest was assayed by the one-stage procedure of Williams & Esnouf (1962), using citrated normal bovine plasma. The proteolytic activity of RVV-X toward bovine factor X was determined by the peptide release assay of Silverberg et al. (1977). In a particular example of the assay, 0.02 mL of AcNeu^7-^{3}H-labeled factor X (0.3 mg/mL, 40 000 dpm/μg) was incubated at 37 °C with 0.001 mL of RVV-X (0.6 μg/mL) in 0.1 M NaCl/2.5 mM $CaCl_2$/50 mM Tris-HCl, pH 7.4. At appropriate time intervals, 0.003-mL aliquots were withdrawn and added to 0.04 mL of a solution containing 3 mg/mL bovine serum albumin/30 mM Tris–EDTA, pH 7.4. Subsequently, 0.04 mL of 15% trichloroacetic acid was added, and the amount of tritiated peptide released was quantitated after centrifugation and liquid scintillation counting of the supernatant. An analogous procedure was used to monitor the cleavage of radioiodinated apo-AI by RVV-X. Typically, 0.02 mL of [^{125}I]apo-AI (1 mg/mL, 2000 dpm/μg) was incubated at 37 °C with 0.001 mL of RVV-X (2 mg/mL). At intervals, 0.005-mL aliquots were treated as described above, and the amount of radioiodinated peptide released was determined by using a γ counter.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. In all cases, the procedure was similar to that described by Weber & Osborn (1969). Tritium-labeled protein was eluted from polyacrylamide gel slices with Soluene 350 (Packard) as recommended by Moore (1980). Slices were incubated overnight at 37 °C with 0.5 mL of Soluene 350 and 10 mL of scintillation cocktail [prepared by dissolving 46 g of 2,5-diphenyloxazole and 0.6 g of 1,4-bis(5-phenlyoxazol-2-yl)benzene in 3 L of toluene]. The activity of the eluted protein was then determined by liquid scintillation counting.

Isoelectric focusing was performed on an LKB 110-mL column with Pharmacia carrier ampholytes (pH range 3–10). Our exact procedures have been described (Sodetz et al., 1972).

Sialic Acid Determination and Removal. Sialic acid was determined by the thiobarbituric acid method of Warren (1959). Sialic acid was quantitatively removed by incubation with *Vibro cholerae* neuraminidase. In a typical incubation, 0.7 mL of RVV-X (2.5 mg/mL) in 0.15 M NaCl/10 mM $CaCl_2$/50 mM sodium acetate, pH 5.5, was incubated with 0.05 mL of neuraminidase (Gibco, 500 units/mL) at 37 °C for 12 h.

Sedimentation Velocity. Sedimentation coefficients of RVV-X samples were determined in the usual manner (Schachman, 1952), using a Beckman Model E analytical ultracentrifuge. For protein concentrations below 1.0 mg/mL, the photoelectric scanning optical system was used, and for concentrations above 1.0 mg/mL, the Schlieren optical system was employed.

Ligand Binding Studies. Binding of $^{45}Ca^{2+}$ and ethylene diaminetetra[2-^{14}C]acetic acid to RVV-X and apo-AI was quantitated by the Paulus ultrafiltration method (Paulus, 1969). Our exact methodology has been described previously (Amphlett et al., 1978).

Atomic Absorption Analysis. The Ca^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} contents of solutions of interest were determined by use of a Varian AA 775 atomic absorption spectrophotometer with a CRA 90 carbon rod atomizer. All relevant solutions were prepared by using distilled, deionized water in plastic tubes or beakers which had been washed with 20% HNO_3. In addition, all disposable pipet tips used for transferring and diluting solutions were cleaned with 20% HNO_3 and rinsed extensively with distilled, deionized water to minimize the possibility of metal ion contamination.

Results

Purification of RVV-X from Different Sources. When crude RVV (from Calbiochem) was subjected to chromatography on DEAE-cellulose, essentially by the procedure of Furie

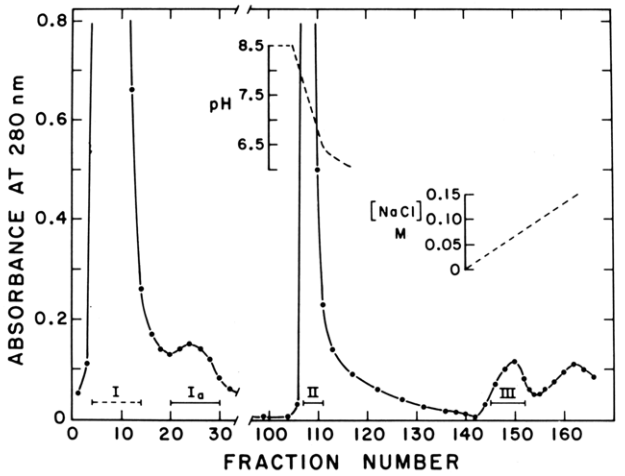

FIGURE 1: Fractionation of crude RVV (Calbiochem) on DEAE-cellulose. A quantity of 2 g of lyophilized crude venom was dissolved in 100 mL of 10 mM Tris–PO_4/1 mM benzamidine, pH 8.4, and dialyzed against this buffer at 4 °C overnight. After clarification by centrifugation, the protein was applied to a column (2.5 × 30 cm) of DEAE-cellulose equilibrated in this same buffer. Fractions (20 mL) were collected at a flow rate of 60 mL/h. After the column was washed extensively with equilibration buffer, a solvent containing 10 mM Tris–PO_4/1 mM benzamidine, pH 6.0, was applied at fraction 85, above. The pH of fractions eluted from the column with this buffer is shown by the dashed line. When the absorbance at 280 nm of the column eluate was less than 0.01, a linear gradient, consisting of 1 L of 0–0.25 M NaCl in 10 mM Tris–PO_4/1 mM benzamidine, pH 6.0, was applied. The progress of this gradient is indicated by a dashed line. Strong coagulant activity was detected in pools II and III, with a small amount of activity in pool Ia and trace amounts in pool I.

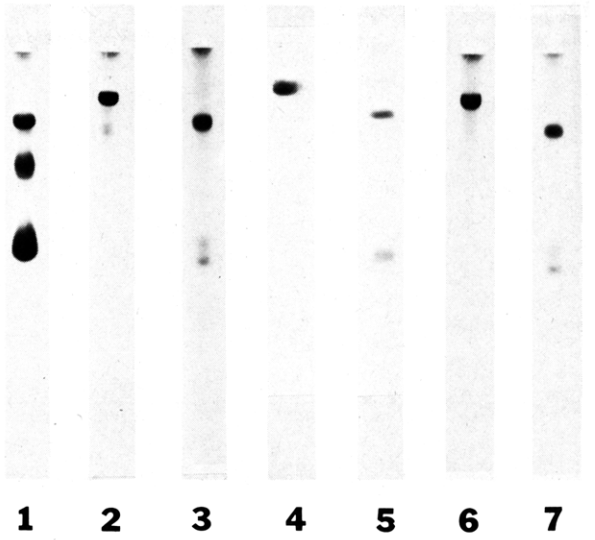

FIGURE 2: Reduced and nonreduced $NaDodSO_4$ electrophoretograms of purified fractions of Calbiochem RVV-X. The gel composition was 7.5% acrylamide/0.28% bis(acrylamide). Gel 1, a mixture of bovine serum albumin, egg ovalbumin, and ribonuclease A, reduced; gel 2, pool III from the DEAE-cellulose column (Figure 1), nonreduced; gel 3, same as gel 2 but reduced; gel 4, pool Ia from the DEAE-cellulose column after further purification by gel filtration on Sephacryl S-200 and isoelectric focusing, nonreduced; gel 5, same as gel 4 but reduced; gel 6, pool II from the DEAE-cellulose column after further purification by gel filtration on Sephacryl S-200, nonreduced; gel 7, same as gel 6 but reduced. The anode was at the bottom in all cases. The bands were visualized by staining with Coomassie Blue.

et al. (1974), at least three of the protein fractions were routinely found to contain coagulant activity (Figure 1). The protein eluted after application of 10 mM Tris–PO_4/1 mM benzamidine, pH 6.0, and the protein in the first peak eluted by the NaCl gradient at pH 6.0 (designated pools II and III, respectively) invariably contained the majority of the coagulant activity. The relative amounts of coagulant activity in pools II and III varied, depending upon the particular lot number of crude RVV, although there was always more total activity in pool II. There was also a trace of activity in the large pool of protein that was not bound by the column (pool I). In addition, a small protein peak, with high specific coagulant activity (pool Ia), was found to be retarded by the ion-exchange column and was eluted by exhaustive washing with equilibration buffer, 10 mM Tris–PO_4/1 mM benzamidine, pH 8.4.

$NaDodSO_4$ gel electrophoretic analysis showed that pool III from the DEAE-cellulose column was homogeneous on nonreduced gels, with an apparent molecular weight of approximately 80 000 (Figure 2). Under reducing conditions, a protein band was observed with an apparent molecular weight of 60 000, with a diffuse doublet in the molecular weight range of 10 000–20 000 also visible. The subunit composition of RVV-X indicated by these experiments is in complete agreement with that previously proposed by Kisiel et al. (1976b).

Pools Ia and II from the DEAE-cellulose column were heterogeneous when subjected to nonreduced $NaDodSO_4$ gel electrophoresis. The protein in both pools could be further purified by gel filtration using a column of Sephacryl S-200. When pool Ia was applied to this column, a single peak containing coagulant activity was eluted (Figure 3A). The pool containing coagulant activity is indicated by the bar under the peak. The protein in this pool was still heterogeneous on nonreduced $NaDodSO_4$ electrophoresis. This fraction was, therefore, subjected to preparative isoelectric focusing, yielding

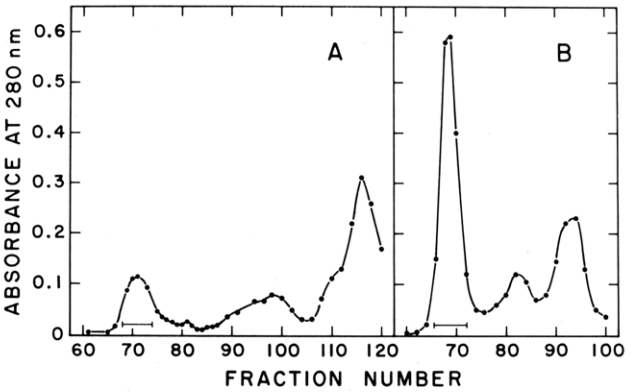

FIGURE 3: Gel filtration of crude Calbiochem RVV-X fractions on Sephacryl S-200. A total of 4 mL of concentrated crude RVV-X was applied to a column of Sephacryl S-200 (2.5 × 110 cm) equilibrated in 0.1 M NaCl/1 mM benzamidine/50 mM Tris-HCl, pH 7.4. Fractions (5 mL) were collected at a flow rate of 15 mL/h. The peaks containing coagulant activity were pooled as shown by the bars under the appropriate peaks. The RVV-X fractions applied to the column were the following: (A) pool Ia from the DEAE-cellulose column (Figure 1); (B) pool II from the DEAE-cellulose column (Figure 1).

a single band of coagulant activity at pH 7.85. $NaDodSO_4$ gel electrophoretic analysis showed that this fraction was now homogeneous and had a similar subunit composition to RVV-X from pool III of the DEAE-cellulose column. When pool II from the DEAE-cellulose column was applied to the Sephacryl S-200 column, a single peak of coagulant activity was again eluted (Figure 3B). The pool containing coagulant activity is indicated by the bar under the peak. The protein in this pool was homogeneous on nonreduced $NaDodSO_4$ gel electrophoresis and showed a similar subunit composition to RVV-X from pool III of the DEAE-cellulose column was examined by reduced $NaDodSO_4$ gel electrophoresis (Figure 2).

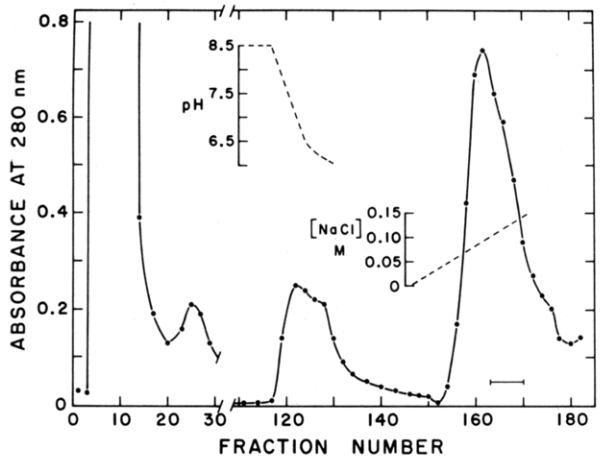

FIGURE 4: Fractionation of crude RVV (from the Miami Serpentarium) on DEAE-cellulose. The conditions used were as described in the legend to Figure 1. As in Figure 1, changes in the pH and NaCl concentration of eluted fractions are shown by dashed lines. Essentially all of the coagulant activity was found in the first peak eluted by the NaCl gradient (the fractions pooled are indicated by the bar under this peak).

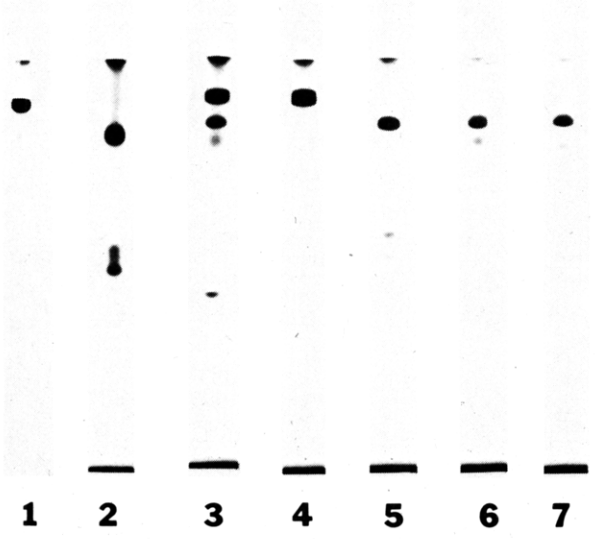

FIGURE 5: Reduced and nonreduced gel electrophoretograms showing stages in the purification of RVV-X from the Miami Serpentarium. Gel 1, Calbiochem RVV-X, nonreduced; gel 2, Calbiochem RVV-X, reduced; gel 3, pool of coagulant activity from DEAE-cellulose fractionation of Miami Serpentarium RVV (Figure 4), nonreduced; gel 4, pool of high coagulant activity obtained by QAE–Sephadex fractionation (Figure 6) of the crude pool from the DEAE-cellulose column (Figure 4), nonreduced; gel 5, same as gel 4 but reduced; gel 6, contaminating protein from fraction 42 of the QAE–Sephadex column (Figure 6), nonreduced; gel 7, same as gel 6 but reduced. The anode was at the bottom in all cases. The bands were visualized by staining with Coomassie Blue.

When crude RVV from either Sigma or the Miami Serpentarium was subjected to DEAE-cellulose chromatography (Figure 4), a significantly different elution profile was obtained from that observed with Calbiochem RVV. Essentially all of the coagulant activity (indicated by the bar under the peak) was obtained in the latter half of the first protein pool eluted by NaCl, pH 6.0. In contrast to the corresponding peak from the Calbiochem purification, the protein in this pool was still heterogeneous when subjected to nonreduced $NaDodSO_4$ gel electrophoresis (Figure 5). No further purification of the coagulant protein was effected by gel filtration on Sephacryl S-200. However, further purification was possible by subjecting the partially pure coagulant protein to chromatography on QAE–Sephadex (Figure 6). By judicious pooling of

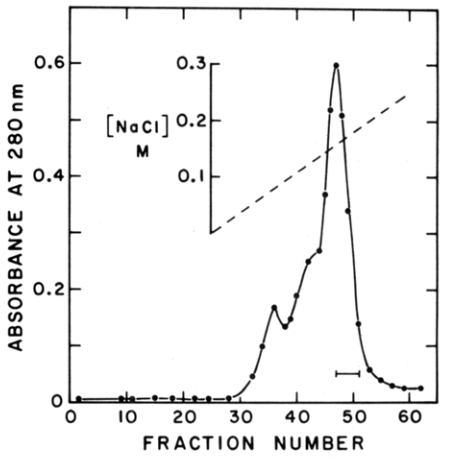

FIGURE 6: Fractionation of partially purified Miami RVV-X on QAE–Sephadex. The pool of coagulant activity from DEAE-cellulose fractionation of crude Miami RVV (Figure 4) was dialyzed against 50 mM Tris–PO_4/1 mM benzamidine, pH 7.5, and applied to a column of QAE–Sephadex (2.5 × 30 cm) equilibrated in this buffer. Fractions (15 mL) were collected at a flow rate of 45 mL/h. After the fractions were washed with equilibration buffer, a linear gradient consisting of 1 L (total volume) of 0–0.5 M NaCl in equilibration buffer was applied at fraction 22 in the above separation. The progress of this gradient is indicated by the dashed line. The pool of highest specific coagulant activity is indicated by the bar under the last peak.

Table I: Isoelectric Points of Different Subforms of RVV-X before and after Neuraminidase Treatment

subform[a]	p*I* of native protein	p*I* of asialo protein
Ia	7.85	nd[b]
II	7.65	7.80
III	6.50	7.20

[a] The proteins used in this study were all purified from Calbiochem RVV. The nomenclature of the subforms is based on the order of their elution from DEAE-cellulose (Figure 1). [b] Not determined.

fractions (as indicated by the bar under the peak in the elution profile), it was possible to isolate protein with high coagulant activity which was homogeneous on nonreduced $NaDodSO_4$ gel electrophoresis (Figure 5). The subunit composition of this protein appeared identical with that of the various subforms, described earlier, of RVV-X purified from Calbiochem RVV. The major contaminating protein in partially pure Miami or Sigma RVV-X, which was removed during chromatography on QAE–Sephadex, migrated as a single band on both nonreduced and reduced $NaDodSO_4$ gel electrophoresis, with an apparent molecular weight of 60000. The specific coagulant activity of this fraction was negligible compared to that of the subsequently eluted fraction.

Characterization of Different Forms of RVV-X. In view of the apparently identical subunit composition of the different forms of RVV-X described above, it was of interest to investigate whether any structural or functional differences might exist, in addition to those necessary to account for different elution behavior on anion-exchange chromatography. The isoelectric points of the various forms of Calbiochem RVV-X were found to be different (Table I) and to be consistent with the order of their elution during chromatography on DEAE-cellulose. Since RVV-X is known to contain sialic acid residues (Kisiel et al., 1976b), and varying contents of sialic acid are a common cause of glycoprotein heterogeneity, the sialic acid content of the two major forms of Calbiochem RVV-X was determined (Table II). The form with the lower isoelectric point was found to have a higher sialic acid content. In order

Table II: Sialic Acid Content of the Major Subforms of Calbiochem RVV-X before and after Treatment with *Vibrio cholerae* Neuraminidase

subform[a]	sialic acid content of native protein (mol/mol)	sialic acid content of neuraminidase-treated protein (mol/mol)
II	1.3 ± 0.2	<0.1
III	2.6 ± 0.3	<0.1

[a] Defined as in the legend to Table I.

to determine whether differences in sialic acid content alone were responsible for differences in the isoelectric points, sialic acid was completely removed from the two major forms of Calbiochem RVV-X by incubation with *Vibrio cholerae* neuraminidase (see Materials and Methods for experimental details). Removal of sialic acid was complete (Table II), and no degradation of the proteins occurred, as demonstrated by $NaDodSO_4$ gel electrophoresis. After removal of sialic acid, the isoelectric points of the two major forms of Calbiochem RVV-X increased and converged, although they still were not identical (Table I). Thus, although differences in sialic acid content are at least partially responsible for the different elution patterns on DEAE-cellulose, other charge differences in these proteins also exist.

The proteolytic activities of the different purified forms of RVV-X were next compared, using three different assays: a clotting assay and a peptide release assay, the latter utilizing tritiated bovine factor X (see Materials and Methods for details of both of these procedures); and cleavage of radioiodinated human high-density liporprotein apo-AI. This latter assay has not been used previously. RVV-X catalyzed hydrolysis of a trichloroacetic acid soluble peptide from apo-AI by specific bond cleavage(s). This peptide contains tyrosine, and, if the apo-AI is radioiodinated in its tyrosine residues, the rate of appearance of trichloroacetic acid soluble radioactivity can be used to readily monitor the rate of liberation of the soluble peptide. Regardless of the assay used, all forms of RVV-X tested (Miami RVV-X and the three forms purified from Calbiochem RVV) possessed the same specific activity. In the clotting assay, 5 ng of RVV-X catalyzed clotting of standard bovine plasma in 13.5 s. In the tritiated factor X cleavage assay, a concentration of 30 ng/mL RVV-X catalyzed an initial rate of cleavage of 0.03 mg of factor X mL^{-1} min^{-1} at an initial factor X concentration of 0.3 mg/mL. Apo-AI, at an initial concentration of 1.0 mg/mL, was cleaved at an initial rate of 1.0 mg mL^{-1} h^{-1}, at a RVV-X concentration of 0.1 mg/mL. In addition, the activities of the asialo RVV-X forms described above were determined. There was no significant difference between their activities (in either a clotting assay or a tritiated factor X peptide release assay) and the activities of RVV-X with a full complement of sialic acid. Hence, the differences in isoelectric point and sialic acid content are apparently not reflected in differences in proteolytic activity.

Ca^{2+} Binding to RVV-X. Binding of $^{45}Ca^{2+}$ to the various forms of RVV-X, described above, was measured by using the equilibrium ultrafiltration methodology of Paulus (1969). A Scatchard analysis of the binding data (Figure 7) showed that the protein does indeed bind Ca^{2+}, with essentially identical Ca^{2+} binding isotherms being obtained with both major forms of Calbiochem RVV-X and the single form of Miami RVV-X. In all cases, a single class of Ca^{2+} binding sites was observed, with a total of 2.0 ± 0.2 sites of K_D 50 ± 15 μM. Since the Paulus ultrafiltration procedure necessarily results in a high

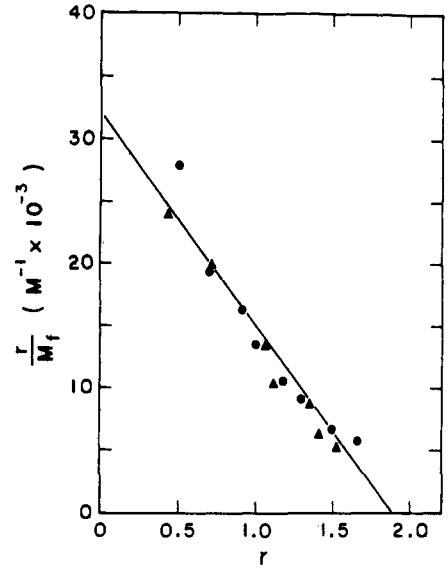

FIGURE 7: Scatchard plot representing the binding of $^{45}Ca^{2+}$ to RVV-X at 25 °C. r represents the moles of $^{45}Ca^{2+}$ bound per mole of protein, and M_f represents the free ligand concentration. The buffer employed was 50 mM Tris-HCl/0.1 M NaCl, pH 7.4. The form of RVV-X used to generate these data was from pool III of the DEAE-cellulose purification of Calbiochem RVV (Figure 1), but essentially similar results were obtained with all other forms. The initial protein concentrations in the binding experiments were the following: (●) 0.4 mg/mL; (▲) 0.24 mg/mL.

Table III: $s_{20,w}$ Values of RVV-X at Various Concentrations

RVV-X concn (mg/mL)	Ca^{2+} [a] (5 mM)	$s_{20,w}$ (S)
0.45	−	6.00
0.45	+	6.05
4.5	−	6.03
4.5	+	6.13

[a] The buffer employed was either 0.1 M NaCl/0.05 M Tris-HCl, pH 7.4 (−), or 0.1 M NaCl/0.0025 M $CaCl_2$/0.05 M Tris-HCl, pH 7.4 (+).

final concentration of protein relative to the initial concentration, binding experiments were performed at several different initial protein concentrations (0.24–0.40 mg/mL) in order to determine whether the high protein concentration might affect the results. Essentially identical binding isotherms were obtained at all initial protein concentrations used, suggesting that such an artifact did not occur. In addition, the effect of protein concentration on the $s_{20,w}$ of RVV-X was studied, in both the absence and the presence of sufficient Ca^{2+} to saturate its binding sites on the protein. As can be observed from the data of Table III, there is only a slight change in the $s_{20,w}$ over a protein concentration range of 0.45–4.5 mg/mL. This latter concentration approximates the final concentration of protein obtained in the ultrafiltration binding experiments, suggesting that aggregation of protein as a result of excessive protein concentration is unlikely to be occurring and influencing the results.

The effect of the Ca^{2+} binding sites on the proteolytic activity of RVV-X is difficult to assess by using the conventional substrates of the enzyme (factor X, factor IX, and protein C) since these substrates also bind Ca^{2+}. However, the novel protein substrate described above, apo-AI, does not bind significant Ca^{2+} (in binding experiments using equilibrium ultrafiltration, less than 0.1 mol of Ca^{2+} was bound per mol of apo-AI at a free Ca^{2+} concentration of 1.0 mM). Thus, any effect of Ca^{2+} on the rate of apo-AI cleavage, by RVV-X, should reflect the influence of the Ca^{2+} binding sites on the

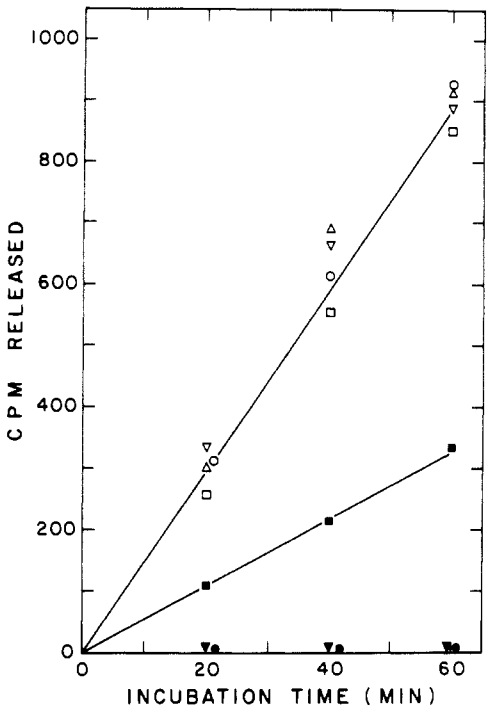

FIGURE 8: Cleavage of radioiodinated apo-AI by RVV-X in the presence of various chelating agents. Incubations and assays were performed as described under Materials and Methods with the following additions: (△) none; (○) $CaCl_2$ up to 5 mM; (□) 100 M EDTA; (■) 1 mM EGTA; (▽) 10 M EDTA; (▼) 100 M EDTA; (●) 1 mM *o*-phenanthroline.

latter protein. As shown in Figure 8, the rate of cleavage of apo-AI proceeded equally well in the absence of Ca^{2+} as in the presence of up to 5 mM Ca^{2+}. Atomic absorption analysis showed that, although all of the components of the activation mixture had been subjected to Chelex-100 treatment, there was still significant Ca^{2+} contamination (approximately 1 μM) in the activation mixture. To ensure that this low level of Ca^{2+} was not affecting the activity of the RVV-X, we carried out activations in the presence of the specific Ca^{2+} chelator ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Figure 8). At a concentration of 100 μM EGTA, sufficient to lower the free Ca^{2+} concentration to less than 10^{-9} M, the rate of apo-AI cleavage remained the same as in the absence of any additions. It is apparent, therefore, that the exchangeable Ca^{2+} binding sites described above have no significant effect on the proteolytic activity of RVV-X.

Although 100 μM EGTA had no inhibitory effect on apo-AI cleavage by RVV-X, higher concentrations were inhibitory (Figure 8). Other chelating agents, *o*-phenanthroline and ethylenediaminetetraacetic acid (EDTA), also were inhibitory, in the case of EDTA at substantially lower concentrations (100% inhibition was obtained at 100 μM EDTA, although no inhibition was observed at 10 μM EDTA). It was unlikely that these chelating agents were acting by sequestering some contaminating metal ion other than Ca^{2+} that was essential for activity, since the concentration of chelator required for inhibition was so high. In addition, extensive dialysis of both apo-AI and RVV-X against 0.1 mM EDTA/0.1 M NaCl/10 mM Tris-HCl, pH 7.4, followed by dialysis against 0.1 M NaCl/10 mM Tris-HCl, pH 7.4, to remove the EDTA, led to no change in the rate of cleavage of apo-AI by RVV-X. Another possible explanation for the inhibitory effect of these chelators was that they were binding directly to either the apo-AI or the RVV-X. Ultrafiltration binding experiments showed, however, that less than 0.1 mol of [^{14}C]EDTA was bound per mol of either apo-AI or RVV-X at free EDTA concentrations up to 0.5 mM. At this free EDTA concentration, complete inhibition of RVV-X-catalyzed cleavage of apo-AI was nonetheless observed.

Table IV: Effect of Various Treatments on the Nonexchangeable Metal Ion Content of RVV-X[a]

treatment	Ca^{2+} concn (mol/mol of RVV-X)	Zn^{2+} concn (mol/mol of RVV-X)
passage over Chelex-100[b]	1.2 ± 0.3	0.7 ± 0.1
extensive dialysis[b] against 2.5 or 1 mM EDTA or EGTA, or 0.5 mM *o*-phenanthroline	1.0 ± 0.2	0.7 ± 0.1
dialysis against 1 mM HCl	0.05 ± 0.02	0.03 ± 0.01
dialysis against 6 M Gdn·HCl	0.04 ± 0.02	0.02 ± 0.01

[a] Similar results were obtained on all subforms of RVV-X.
[b] The buffer employed was 0.05 M Tris-HCl/0.1 M NaCl, pH 7.4.

Nonexchangeable Metal Ion Content of RVV-X. Before the above Ca^{2+} binding experiments were carried out, all protein and buffer solutions used were passed over a small (0.5 × 5 cm) column of Chelex-100 resin in order to remove contaminating metal ions. The effectiveness of this procedure was assessed by atomic absorption spectroscopy. All solutions thus treated possessed only low levels of Ca^{2+} contamination (1 μM or less), with the exception of RVV-X solutions. Whichever subform of the protein was used, the Ca^{2+} content of Chelex-treated RVV-X solutions was consistently found to be approximately 1 mol of Ca^{2+} per mol of RVV-X. This Ca^{2+} could not be removed by dialysis for 5 days against 1 mM EDTA/0.1 M NaCl/10 mM Tris-HCl, pH 7.4, or 0.5 mM *o*-phenanthroline, in this same buffer, indicating that the Ca^{2+} was either very tightly bound or, more likely, nonexchangeable. These results are summarized in Table IV.

Since other proteins known to contain tightly bound or nonexchangeable Ca^{2+} often contain other divalent metal ions e.g., concanavalin A (Kalb & Levitzki, 1968) and thermolysin (Feder et al., 1971), the possible presence of Zn^{2+}, and Mg^{2+} in RVV-X was investigated by atomic absorption spectroscopy. The levels of Mn^{2+} and Mg^{2+} in RVV-X were shown to be minimal (less than 0.01 mol/mol) in all major subforms. The Zn^{2+} content of all subforms, however, consistently approximated 0.7 mol/mol and was not removed by dialysis for 5 days against 1 mM EDTA in 0.1 M NaCl/10 mM Tris-HCl, pH 7.4, as seen from the results of Table IV, indicating that it also was nonexchangeable.

Table IV shows that it was found possible to remove both nonexchangeable Zn^{2+} and Ca^{2+} from RVV-X by the use of denaturing conditions. After 5 h of dialysis against either 1 mM HCl or 6 M guanidine hydrochloride (Gdn·HCl), the contents of both Zn^{2+} and Ca^{2+} were less than 0.05 mol of metal ion/mol of protein. At this point, the coagulant activity of the dialyzed protein was approximately 5% of that of the native protein. It is not clear whether this residual activity resides only in the small fraction of protein molecules that retained their metal ions (it is difficult to effect 100% removal of metal). Alternatively, it may reflect a much reduced, but still significant, level of activity of the metal-free protein. In order to determine whether metal-free protein could recover activity upon incubation with Zn^{2+} and Ca^{2+} under nondenaturing conditions, metal-free protein, prepared by HCl or Gdn·HCl dialysis, as described above, was dialyzed against 2.5 mM $ZnCl_2$/2.5 mM $CaCl_2$/0.1 M NaCl/10 mM Tris-HCl, pH 7.4, at 4 °C for 48 h. After this treatment, the activity of the RVV-X was nonetheless very low (less than 5% of the starting activity), indicating that the loss of activity was likely irreversible.

Discussion

The results presented here have shown that RVV-X, isolated from crude RVV from three different suppliers, can be obtained in at least three subforms with different isoelectric points. The significance of this variation is unclear, but it may reflect differences between individual snakes from which the venom was obtained. The differences in isoelectric points between the subforms have been found to be due largely, though not entirely, to differences in their content of sialic acid. No other physical or functional differences have, however, been observed between the subforms. Thus, they all possess identical subunit compositions, $s_{20,w}$ values, activities toward protein substrates, and metal binding capacities. It would appear, therefore, that the functional differences between the subforms are minimal.

The subunit composition of RVV-X is controversial, with Kisiel et al. (1976b) proposing a heavy chain (M_r 60 000) linked by disulfide bonds to one or two light chains (M_r 10 000–20 000). On the other hand, Furie & Furie (1975) have suggested, on the basis of $NaDodSO_4$ gel electrophoretograms, a single subunit of molecular weight 60 000. However, only reduced gels were presented by these latter authors, and the light chain may not have been visible due to its poor staining properties. In our experiments, every purified coagulant fraction had a subunit composition consistent with the former model. Although a contaminating protein was found in certain preparations of RVV-X with a subunit composition similar to that proposed by Furie & Furie (1975), once purified this protein had negligible coagulant activity.

The reversible binding of Ca^{2+} by RVV-X was of interest in relation to our earlier studies on the Ca^{2+}-requiring activation of vitamin K dependent plasma proteins by RVV-X (Byrne & Castellino, 1978; Amphlett et al., 1981a,b). A protein substrate, apo-AI, for RVV-X was found which was unique in its inability to bind Ca^{2+}. It was therefore possible to study the effects of Ca^{2+} on the enzyme alone, without the complicating consideration that Ca^{2+} might also affect the substrate. The Ca^{2+} concentration had no effect on the rate of cleavage of apo-AI by RVV-X, indicating that the reversible Ca^{2+} binding sites on the latter protein need not be occupied for expression of enzymic activity. This observation lends further support to our earlier arguments that the effects of Ca^{2+} on the activation of vitamin K dependent proteins are mediated by interaction of the cation with the substrate, rather than with the enzyme.

The inhibition of the RVV-X-catalyzed cleavage of apo-AI by relatively high concentrations of chelating agents is difficult to explain. EDTA was a very effective inhibitor at a concentration of 100 μM; yet at this concentration, it was not bound to either RVV-X or apo-AI. At an EDTA concentration of 10 μM, there was, however, no inhibition. Since precautions had been taken to exclude gross metal ion contamination (the use of deionized, distilled H_2O and treatment of all buffer and protein solutions with Chelex resin prior to use), any remaining metal ion should have been sequestered by EDTA at a concentration of 10 μM. Thus, the inhibition of EDTA is unlikely to occur by chelation of an essential trace metal ion. It is possible that EDTA binds to the apo-AI·RVV-X complex much more tightly than to either component individually and that it can inhibit cleavage by this binding. This hypothesis is, however, difficult to test experimentally.

RVV-X is by no means unique in regard to its tight binding of Ca^{2+}, in addition to the exchangeable Ca^{2+}. The α-amylases (Stein et al., 1964), concanavalin A (Kalb & Levitzki, 1968), and thermolysin (Feder et al., 1971) all have tightly bound Ca^{2+}, which can be removed only with difficulty. In the latter two cases, transition-metal ions were found to be bound tightly in addition to Ca^{2+}. This has been found to be the case with RVV-X, with each protein molecule containing approximately 1 mol of tightly bound Ca^{2+} and 0.7 mol of tightly bound Zn^{2+}. The functional significance of the tightly bound metal ions is difficult to evaluate since they could only be removed from RVV-X by using denaturing conditions that irreversibly destroyed the activity of the enzyme. Several possibilities can be offered, using examples in the literature. For instance, the Ca^{2+} in thermolysin is apparently required for thermostability, whereas Zn^{2+} is essential for enzymic activity (Feder et al., 1971). In other cases, the function of Zn^{2+} is principally to facilitate interaction between subunits of a protein, as is the case with bovine insulin.

In conclusion, we have shown that RVV-X has multiple subforms but that the differences between these subforms are minimally significant. Exchangeable Ca^{2+} binding by RVV-X has been observed although no clear evidence for their functional importance has been obtained. This extremely interesting observation is under further study in our laboratory.

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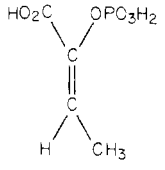
Stereospecificity of (*E*)- and (*Z*)-Phosphoenol-α-ketobutyrate with Chicken Liver Phosphoenolpyruvate Carboxykinase and Related Phosphoenolpyruvate-Utilizing Enzymes†

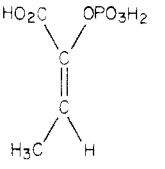
Thomas H. Duffy, Howard J. Saz, and Thomas Nowak*,‡

ABSTRACT: The diastereomers (*E*)- and (*Z*)-phosphoenol-α-ketobutyrate (PEB) were synthesized and purified as analogues of the substrate phosphoenolpyruvate. The two isomers are distinguishable by their differences in ^1H chemical shifts. These analogues have been shown to have no substrate activity with phosphoenolpyruvate carboxykinase, enolase, or pyruvate phosphate dikinase. These isomers do exhibit substrate activity with pyruvate kinase, however. The Mn^{2+}-activated pyruvate kinase shows no significant differences in K_m or V_{max} values between *E*- and *Z*-PEB. The Mg^{2+} enzyme also shows no substantial differences in V_{max} for the diastereomers but shows a dramatic difference between the K_m of *Z*-PEB (K_m = 5 μM) and of *E*-PEB (K_m = 500 μM). The cation thus appears to affect the specificity of the enzyme for these ligands. These analogues showed specificity of inhibition of Mn^{2+}-activated pyruvate kinase. The *Z* isomer (K_I = 0.45 μM) is more effective than the *E* isomer (K_I = 15 μM) in causing inhibition. PRR titrations demonstrate that the dissociation constants, K_3, of these ligands from the enzyme-Mn complex agree with the K_I values and that the *Z* isomer is more like phosphoenolpyruvate than is *E*-PEB with respect to ligand binding to the enzyme. The enzyme phosphoenolpyruvate carboxykinase demonstrates the opposite stereospecificity of inhibition. The *E* isomer (K_I = 1.5 μM) is a more potent inhibitor than is the *Z* isomer (K_I = 32 μM). PRR titration studies demonstrate agreement between K_3 and K_I values. The titration of the enzyme-Mn with the *Z* isomer resembles the titration of the enzyme-Mn complex with phosphoenolpyruvate. The Mn^{2+}-activated enolase exhibits absolute stereospecificity of inhibition with K_I (*Z*-PEB) = 11 μM and K_I (*E*-PEB) > 3 mM. The Mg^{2+}-activated enzyme shows no change in inhibition by *Z*-PEB (K_I = 8 μM) but is susceptible to inhibition by *E*-PEB (K_I = 80 μM). The cation appears to affect the type of interaction which occurs between *E*-PEB and enolase. PRR studies of enolase-Mn, however, demonstrate that not only does *Z*-PEB show a strong interaction with the enzyme (K_3 = 4 μM) but *E*-PEB does also (K_3 = 8 μM). The nature of this interaction and failure to elicit inhibition is still unclear.

The synthesis of both the *E* and the *Z* isomers of phosphoenolbutyrate (PEB)[1] has been previously reported by Sprinson and his colleagues (Adlersberg et al., 1977).



Z-PEB



E-PEB

Initial reports, using a *Z*,*E* (80:20) mixture, showed competitive inhibition vs. P-enolpyruvate with rabbit muscle pyruvate kinase (Woods et al., 1970). Subsequent studies have demonstrated that the *E*,*Z* mixture (Bondinell & Sprinson, 1970) and the pure *Z* isomer (Stubbe & Kenyon, 1971) also serve as substrates for pyruvate kinase. These preliminary studies demonstrated a stereospecific protonation of PEB by pyruvate kinase (Bondinell & Sprinson, 1970; Stubbe & Kenyon, 1971). The stereospecificity was identical with that demonstrated by Rose (1970), who used the isotopically asymmetric [3-^2H, ^3H]P-enolpyruvate. These experiments showed that the enolate of pyruvate, formed during the catalytic reactions, is specifically protonated by the enzyme on the *si* face of C-3 to yield pyruvate. Subsequent studies with pure *Z* and *E* isomers of PEB demonstrated that although they served as substrates for pyruvate kinase, absolute stereospecific protonation was not observed (Adlersberg et al., 1977). These results suggest that some population of the isomeric enolates of α-ketobutyrate dissociates from the enzyme prior to protonation.

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[1] Abbreviations: PEB, phosphoenol-α-ketobutyrate; P-enolpyruvate, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; 2-PGA, D(+)-2-phosphoglycerate; OAA, oxalacetate; PRR, solvent proton longitudinal relaxation rate.

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