The Activities of Recombinant γ-Carboxyglutamic-Acid-Deficient Mutants of Activated Human Protein C toward Human Coagulation Factor Va and Factor VIII in Purified Systems and in Plasma†

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Received September 23, 1993; Revised Manuscript Received December 7, 1993®

ABSTRACT: The dependence of the activity of recombinant activated human protein C (r-APC) on each of its nine γ-carboxyglutamic (Gla) residues (sequence positions 6, 7, 14, 16, 19, 20, 25, 26, and 29) has been assessed in purified systems and in plasma using r-mutants in which each Gla residue of r-APC was individually altered to an Asp (D) residue. The assays employed included a factor Va inactivation assay in the prothrombinase system with purified components and in plasma. In addition, a factor VIII inactivation assay in the tenase system, also with purified components, was utilized. Compared to wild-type protein (wtr-APC), the r-mutants that possessed nearly full activity in all assays were the $Gla^6 \rightarrow D$ variant ([Gla^6D]r-APC]) as well as [Gla^{14}D]r-APC and [Gla^{19}D]r-APC. In addition, another mutant ($Q^{32} \rightarrow Gla$) in which a Gla was substituted for Gln (Q) at position 32, a situation that exists with other vitamin-K-dependent clotting proteins (e.g., factor IX and prothrombin), displayed full activity in all assays. Those mutants that possessed very-low-to-no activity in all assays included [Gla^{16}D]r-APC and [Gla^{26}D]r-APC. The other mutants showed partial and, in some cases, differential activity in these assay systems, with [Gla^{25}D]r-APC being the most remarkable example. In this case, the factor V/Va plasma assay and the plasma-based activated partial thromboplastin time assay yielded <25% activity, whereas nearly full activity was observed for this variant in the prothrombinase and tenase assays with purified components. Although not as pronounced as with [Gla^{25}D]r-APC, similar differences existed for several other mutants in the plasma-based and purified system assays. This suggests that a factor(s) in plasma may diminish the activity of these undercarboxylated forms of r-APC. Finally, none of the r-APC mutants showed significant differential activity toward fVa and fVIII, demonstrating that determinants on APC for each of these substrates are likely the same.

Protein C (PC)[1] is the zymogen precursor of the serine protease APC, an enzyme which functions both as an anticoagulant and a profibrinolytic factor. The anticoagulant properties of APC are derived from the ability of this enzyme to catalyze, by limited proteolysis, the inactivation of coagulation cofactors fV/fVa (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980). Ca^{2+}, phospholipid, and a plasma cofactor, PS (Walker, 1981), enhance the ability of APC to inactivate these plasma cofactors. The profibrinolytic role of APC is grounded in the ability of this enzyme to neutralize plasminogen activator inhibitor-1, thus allowing plasminogen activators to function in plasminogen activation and consequently in clot lysis (Griffin et al., 1981; Sakata et al., 1986). Both of these roles of APC serve to maintain the fluidity of blood.

PC is synthesized as a single-chain protein containing 419 amino acids plus a 42-residue signal polypeptide (Foster & Davie, 1984; Foster et al., 1985). In addition to cleavage of the signal sequence (Foster et al., 1985), a dipeptide, K^{156}–R^{157}, is processed out of the protein prior to secretion (Beckmann et al., 1985). This results in plasma PC, which exists as a light chain of 155 residues disulfide-linked to a heavy chain of 262 amino acids. Other processing events also occur prior to secretion, including glycosylation of four N-residues (Kisiel, 1979), β-hydroxylation at D^{71} (Drakenberg et al., 1983), and γ-carboxylation at nine precursor E residues, the latter of which exist at sequence positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 of the light chain (DiScipio & Davie, 1979). APC arises from PC as a result of activation by thrombin or, on cell surfaces, by the thrombin/thrombomodulin complex (Esmon et al., 1982). Activation is accompanied by release of a dodecapeptide from the amino terminus of the heavy chain. Ca^{2+} inhibits activation by thrombin (Amphlett et al., 1981) and stimulates this same activation by the thrombin/thrombomodulin complex (Esmon et al., 1982). The catalytic triad of amino acids responsible for the serine proteolytic activity of APC is present in the heavy chain of the enzyme.

As a result of γ-carboxylation of PC, this protein gains the ability to interact with Ca^{2+} and PL. Such binding events are necessary for the proper functioning of PC and its activated product, APC. That the Gla domain is essential for elucidation of the anticoagulant properties of PC/APC has been demonstrated by the diminution of this activity after blockage of

† Supported by Grant HL-19982 from the National Institutes of Health and the Kleiderer/Pezold Family Endowed Professorship (to F.J.C.).

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® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: PC, protein C; APC, activated protein C; PS, protein S; fV, coagulation factor V; fVa, activated coagulation factor V; ifVa, fVa inactivated as a result of proteolysis with APC; fVIII, coagulation factor VIII; fVIIIa, activated coagulation factor VIII; ifVIII, fVIII inactivated as a result of proteolysis with APC; fIX, coagulation factor IX; fIXaβ, activated coagulation factor IX; fX, coagulation factor X; fXa, activated coagulation factor X; r, recombinant; TBS, 50 mM Tris–HCl/100 mM NaCl, pH 7.4; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine/bovine brain phosphatidylserine; S2222, benzoyl-L-Ile-L-Glu(OR)-Gly-L-Arg-*p*-nitroanilide; S2366, L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/94/0433-1869$04.50/0

γ-carboxylation by administration of warfarin (Sugo et al., 1985) and by mutagenesis of individual Gla residues in PC/APC (Zhang et al., 1992). From these latter studies, those individual Gla residues that play a role in sustaining the overall anticoagulant activity of APC have been identified. Further investigations have shown that anticoagulant losses accompanying mutation of individual Gla residues of APC have been linked to defective functional binding of the PC/APC Gla mutants to Ca^{2+} (Zhang & Castellino, 1992) and to PL (Zhang & Castellino, 1993).

To assess the functional roles of individual Gla residues of APC, only APTT assays have been performed to date on these Gla deficient r-APC variants (Zhang et al., 1992). This assay, while clinically valuable, suffers from an important defect for studies such as these, viz., that Ca^{2+} concentrations are necessarily very low at times in the assay when APC is inactivating the plasma cofactors. Therefore, in order to determine whether poorly functional Ca^{2+}-binding-deficient r-APC mutants could be corrected at higher Ca^{2+} concentrations, assays are required wherein Ca^{2+} levels could be more widely varied. To solve this problem, we have performed assays in prothrombinase and tenase systems using purified components and synthetic substrates. These assays also allowed us to assess whether fVa and fVIII were equally responsive to defects in APC.

MATERIALS AND METHODS

Proteins. r-PC was expressed in human 293 cells as described previously (Zhang & Castellino, 1990). r-APC was obtained by activation of r-PC with thrombin (Enzyme Research Laboratories, South Bend, IN). Here, a solution of r-PC (5 μg) was incubated with thrombin (1.5 μg) in a solution containing 20 mM Tris–HCl/100 mM NaCl, pH 7.4 (final volume, 100 μL). The activation was carried out for 45 min at 37 °C. The activation mixture was then loaded onto a column (1 mL) of SP-Sephadex, equilibrated with a buffer containing 20 mM Tris–HCl/100 mM NaCl, pH 7.4. The r-APC was eluted from the column with a solution of 20 mM Tris–HCl/75 mM NaCl, pH 7.4. The concentrations of the various r-APCs were adjusted in each of the assays, to be described below, to the same amidolytic activities, using the chromogenic substrate S2366 (Helena Laboratories, Beaumont, TX).

Human plasma fV was provided by Dr. Paula Tracy (Burlington, VT). It was converted to fVa as a result of incubation in borosilicate glass tubes of fV (1 μM) with thrombin (final concentration, 3 NIH units/mL) for 15 min at 37 °C. The final volume was 13 μL. The thrombin in the solution was inhibited by addition of DAPA (final concentration, 10 μM) (Katzman et al., 1981). The final volume after activation was adjusted to 50 μL with a buffer of 20 mM Hepes–NaOH/150 mM NaCl/0.1% bovine serum albumin, pH 7.4.

Human r-fVIII was provided by Dr. Randall Kaufman (Cambridge, MA). Bovine fX (Bajaj & Mann, 1973), bovine prothrombin (Bajaj & Mann, 1973), and bovine fIX (Amphlett et al., 1978) were purified as described. Bovine fIX was converted to fIXaβ as published earlier (Amphlett et al., 1979).

Determination of Ca^{2+} Concentrations by the Ca^{2+}-Selective Electrode. The concentrations of free Ca^{2+} in plasma were determined potentiometrically using a Ca^{2+}-selective electrode (Orion Research, Inc., Boston, MA) with a Ag/AgCl reference electrode. The procedure employed has been described in detail (Colpitts & Castellino, 1993).

Factor Va Inactivation Assays. A two-stage assay was employed for measurements of the initial rates of activation of prothrombin in the complete prothrombinase system with purified components and the effect of r-APCs on these rates. These experiments were carried out at 37 °C in borosilicate glass tubes by incubation of (final concentrations) fVa (0.1 μM) with the particular r-APC (0.5 nM) for different time periods (usually 0–12 min) in a buffer consisting of 20 mM Hepes–NaOH/150 mM NaCl/2 mM $CaCl_2$ or 15 mM $CaCl_2$/20 μM PL, pH 7.4. The amount of fVa remaining was determined by fluorimetric assay with purified components in the complete prothrombinase system (Solymoss et al., 1988). Here, 9 μL of the above fVa/ifVa mixture was added to the reaction mixture (final volume, 1.0 mL) containing 1.39 μM prothrombin/2.5 mM $CaCl_2$/20 μM PL/3 μM DAPA. The activation reaction was accelerated by addition of 8 nM (final concentration) fXa. The fluorescence resulting from uptake of DAPA by the generated thrombin was monitored continuously with use of a SLM-Aminco (Urbana, IL) 8000C spectrofluorometer. The excitation and emission wavelengths were 335 and 565 nm, respectively.

Factor VIII Inactivation Assays. Once again, a two-stage assay was used. In the first stage, the abilities of wtr-APC (wild type) and the r-APC variants to inactivate r-fVIII were examined. Here, an amount of fVIII (5.76 nM, final concentration) was incubated with PL (40 μM in phosphate, final concentration), the desired r-APC (ca. 0.5 nM, final concentration, small adjustments were made such that equal amidolytic activities of all r-APCs were present at this stage), and $CaCl_2$ (2.5 mM, final concentration). The final volume was 150 μL in a buffer of 25 mM Hepes–NaOH/150 mM NaCl, pH 7.4. The times of these incubations were varied from 0–10 min at 37 °C. For activation of the remaining fVIII in the fVIII/ifVIII mixture prior to assay in the fX system, the complete volume of 150 μL of the above fVIII inactivation mixture was incubated with fIXaβ (6.7 nM, final concentration) for protection of fVIIIa activity (Lollar et al., 1984) and thrombin (0.12 units/mL, final concentration) at 37 °C for 1.5 min. The amount of fVIIIa present was then assayed by its ability to stimulate the initial activation rate of fX. For this stage, 165 μL of the above solution was added to another solution containing, in a spectrophotometer cuvette at 37 °C, final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM of the chromogenic substrate S2222 (Helena Laboratories, Beaumont, TX) in 25 mM Hepes–NaOH/150 mM NaCl, pH 7.4. The final volume was 790 μL. After the base line was recorded for 1 min, substrate hydrolysis was accelerated by addition of 10 μL of a solution of 32 μM fX. The rate of amidolysis of S2222 by the fXa generated in the activation was recorded continually for 2–5 min at 405 nm.

Data Analysis for the Effects of APC on the Prothrombinase and Tenase Assays. The data obtained in both of these assays were treated the same, with the exception being that rate assays were employed in the case of fVIII inactivations to obtain the concentrations of fXa generated in the tenase assay, whereas in the fVa assays, the amount of thrombin generated in the prothrombinase assay was determined directly by titration with DAPA.

Under the assay conditions described above, the initial rates of thrombin (fXa) production were linear and proportional to the concentration of fVa (fVIIIa) in the reaction mixture. The initial rates of thrombin (fXa) generation in the assay were calculated for different incubation times of fVa (fVIIIa) with the different r-APC mutants. From these rates, the

percentage of fVa (fVIIIa) remaining was determined. The plot of fVa (fVIIIa) remaining versus the incubation time of fVa (fVIIIa) with the particular r-APC was fit to a first-order exponential decay curve, from which the pseudo-first-order rate constant for the inactivation of fVa (fVIII) by the r-APC mutant was calculated. The ratios of these rate constants for the mutant r-APC to wtr-APC were used to determine the percent activity of the mutant r-APC in each of these assays.

Prothrombin Time Assays. To determine the activity of the r-APC mutants toward inactivation of fV/fVa in plasma, a modified prothrombin time assay was used at 37 °C. Since several sets of conditions were employed in these assays, only the general features of the protocol are outlined here. The exact conditions of each assay are detailed in the appropriate figure legends.

A quantity of 100 μL of PC-deficient plasma (Sigma Diagnostics, St. Louis, MO) containing 40 μM (in phosphate) PL was incubated for 2 min at 37 °C. The inactivation of fV/fVa and fVIII/fVIIIa was accelerated by addition of the desired level of 100 μL of $CaCl_2$ immediately followed by addition of the desired amount of the particular r-APC under investigation. After an incubation time of 1 min, clotting was accelerated by inclusion of a solution of 100 μL of fXa. After we compared the mutant r-APCs with the wtr-APC, the logarithms of the clotting times were then plotted against the logarithms of the concentrations of the r-APC used. Parallel lines were obtained for all r-APC mutants used, and the activity of the r-APC mutants relative to that of wtr-APC was obtained as the concentration ratio of the mutant r-APC to wtr-APC at the same clotting time.

RESULTS

A series of r-APC mutants with each Gla residue conservatively replaced with a D residue has been generated in earlier work and assayed for their overall anticoagulant activities in the clinically relevant APTT assay system (Zhang et al., 1992). This allowed an assessment of the role of each of these Gla residues as to their importance in the Ca^{2+}-dependent properties of r-APC that are required for activity to be expressed. However, since many of these same mutants were defective in their Ca^{2+}-binding properties (Zhang & Castellino, 1992), it was of interest to determine whether full activity could be reached at levels of Ca^{2+} that were higher than those present in the APTT assay. Simple elevation of Ca^{2+} in this assay was not possible at times during which APC was incubating with the PC-deficient plasma, since clotting would rapidly occur upon addition of Ca^{2+}. Determination of the free concentration of Ca^{2+} with use of the Ca^{2+}-selective electrode in the first stage of the APTT assay (when the r-APC is incubated with the citrated plasma) gave a value of approximately 80 μM, a concentration far less than optimal for inactivation of fVa and fVIII by APC. Because of this, two nonclotting-based assays were employed that allowed determination of the activity of r-APC and its r-variants toward fVa and fVIII.

In the first assay, the rate of inactivation of purified fVa by wtr-APC or the desired r-APC mutant was determined with aid of the prothrombinase assay. Here, a concentration of fVa/PL was preincubated with the desired concentration of Ca^{2+} and the r-APC of choice, and the relative amount of active fVa remaining was determined by its ability to serve as a cofactor in the activation of prothrombin by a complex of fXa/Ca^{2+}/PL. First-order plots of the time courses of inactivation of fVa by wtr-APC, as well as by one of the mutants which showed nearly full activity ([Gla^{14}D]r-APC) and another which displayed virtually no activity ([Gla^{26}D]r-APC) in this assay, are illustrated in Figure 1. From these plots, the relative activities of each of these r-APCs have been calculated. These activities, as well as those obtained by similar assays of all of the other r-APC mutants, are summarized in Table 1.

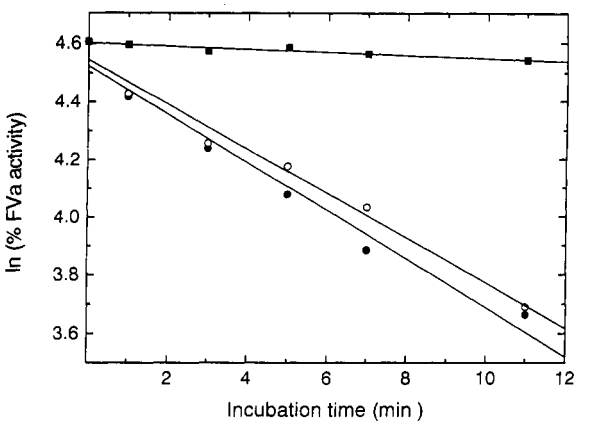

FIGURE 1: Time course of inactivation of human fVa by wtr-APC and r-APC mutants at 25 °C, pH 7.4. After various times of incubation of fVa (0.1 μM, final concentration) with the particular r-APC (0.5 nM, final concentration) in the presence of PL (20 μM, final concentration) and Ca^{2+} (2 or 15 mM, final concentration), an aliquot of the mixture was added to a cuvette containing (final concentrations) prothrombin (1.39 μM), $CaCl_2$ (2.5 mM), PL (20 μM), and DAPA (3 μM). The activation reaction was accelerated by addition of fXa (8 nM, final concentration). The fluorescence increase resulting from uptake of DAPA by the generated thrombin was monitored continuously by spectrofluorimetry. The initial rates of thrombin production were calculated from these experiments at each incubation time of APC/fVa (relative to that of wtr-APC which was assumed to be 100% active), and first-order kinetic replots are illustrated. The ratio of the rate constants for the mutants to that of wtr-APC was employed to calculate the percent activity of each of the mutants. The concentrations of each APC solution were adjusted so that each provided the same amidolytic activities. The symbols are as follows: (●) wtr-APC, (○) [Gla^{14}D]r-APC, and (■) [Gla^{26}D]r-APC. A control in the absence of APC provided a result similar to (■).

Table 1: Activities of r-APC Mutants in Various Assay Systems

	percent activity				
		prothrombin	fVa		
variant	APTT[a]	time[b]	2 mM Ca^{2+}	15 mM Ca^{2+}	fVIII[c]
wtr-APC[d]	100	100	100	100	100
Gla^{6}D[e]	86	118	93	nd[f]	100
Gla^{7}D[e]	6	6	25	30	34
Gla^{14}D[e]	92	114	93	nd[f]	89
Gla^{16}D[e]	<2	<1	15	15	10
Gla^{19}D[e]	80	74	96	nd[f]	99
Gla^{20}D[e]	<2	<1	22	20	25
Gla^{25}D[e]	24	15	92	94	85
Gla^{26}D[e]	<2	<1	7	7	7
Gla^{29}D[e]	9	3	20	51	34
$Q^{32}Gla$[e]	98	133	94	nd[f]	72

[a] The Ca^{2+} concentration determined by Ca^{2+}-specific electrode analysis was approximately 80 μM. These data were taken from Zhang et al. (1992). [b] The free Ca^{2+} concentration was 2.5 mM. [c] The Ca^{2+} concentration was 2.5 mM. [d] Refers to the wild-type recombinant molecule. [e] The location and nature of the mutation in r-APC. [f] Not determined.

Similar assays were conducted for the inactivation of fVIII by the r-APC mutants, employing components of the tenase complex for assay. In choosing the protocols for these experiments, we fully realize that fVIIIa is a better substrate than fVIII for APC. However, due to severe problems with stability of fVIIIa in controls wherein fVIIIa was incubated in the absence of r-APC, we employed fVIII as the substrate

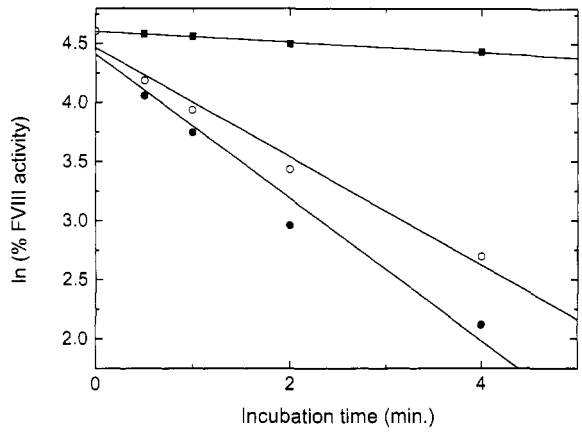

FIGURE 2: Time course of inactivation of human fVIII by wtr-APC and r-APC mutants at 37 °C, pH 7.4. A quantity of fVIII (5.8 nM, final concentration) was incubated with the particular r-APC (0.5 nM, final concentration) in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). After this, fIXaβ (6.7 nM, final concentration) and thrombin (0.12 units/mL) were added in order to activate the remaining fVIII. The amount of fVIIIa present was then assayed by a fX activation assay. For this stage, the fVIIIa solution was added to another solution containing, in a spectrometer cuvette, final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM of the chromogenic substrate S2222. The final volume was 790 μL. After the base line was recorded for 1 min, substrate hydrolysis was accelerated by addition of 10 μL of a solution of 32 μM fX. The rate of amidolysis of S2222 by the generated fXa was determined spectrophotometrically. The initial rates of fXa production were calculated from these experiments at each incubation time of APC with fVIII (relative to that of wtr-APC which was assumed to be 100% active), and first-order kinetic replots are illustrated. The ratio of the rate constants for the mutants to that of wtr-APC was employed to calculate the percent activity of each of the mutants. The concentrations of each r-APC solution used were adjusted so that each provided the same amidolytic activities. The symbols are as follows: (●) wtr-APC, (○) [Gla^{14}D]r-APC, and (■) [Gla^{16}D]r-APC. A control in the absence of APC provided a result similar to (■).

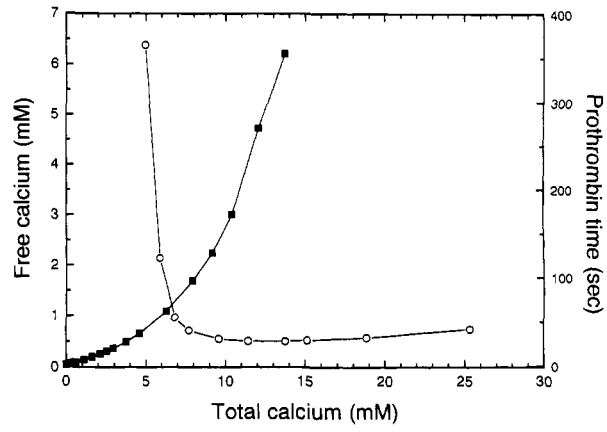

FIGURE 3: Effect of the concentration of Ca^{2+} on the modified prothrombin time at 37 °C. A quantity of 100 μL of the indicated levels of Ca^{2+} (dissolved in TBS) was incubated for 2 min at 37 °C with 100 μL of citrated normal plasma containing 40 μM (in phosphate) PL, and the free Ca^{2+} concentrations (■) in this mixture were determined with use of the Ca^{2+}-selective electrode. Clotting times (○) were then determined at each of these Ca^{2+} concentrations after addition of 100 μL of a 0.3 μg/mL solution of fXa (dissolved in a TBS solution also containing 0.5% bovine serum albumin).

for APC since far greater stability of this protein was observed in control experiments. Thus, our strategy was to examine the temporal dependence of the inactivation of fVIII by the r-APC mutants. Assays of the relative amount of fVIII present in the inactivation mixtures were conducted by rapidly activating the remaining fVIII in the fVIII/ifVIII mixture with thrombin and adding this solution to the otherwise complete tenase components, viz., fX/fIXaβ/Ca^{2+}/PL. The initial rates of formation of fXa were then monitored as a measure of the amounts of fVIIIa present. Typical inactivation rate data of fVIII by wtr-APC and the same two mutants selected for display in Figure 1 are shown in Figure 2. From the relative first-order rate constants calculated from these data, the percent activity (relative to that of wtr-APC) for these mutants, as well as all others examined in this study, is summarized in Table 1.

Finally, we designed a clotting assay that allowed variation of the Ca^{2+} concentration at the stage in which fV/fVa inactivation occurred in the presence of the r-APC mutants. In this plasma-based fV/fVa assay, r-APC (or the desired mutant) was incubated with PC-deficient plasma in the presence of PL and a desired level of Ca^{2+}. The Ca^{2+} concentration can be significantly increased in this step, as compared to in the APTT assay, since clotting cannot occur due to the absence of activated clotting factors (thromboplastin reagent was not added). At a suitable time of incubation of the plasma/PL/Ca^{2+}/r-APC mixture (1 min), clotting was initiated by addition of fXa. Clotting times were then measured. This particular procedure for determination of APC activity bypasses fVIII/fVIIIa inactivation and only measures the remaining levels of functional fV/fVa in plasma.

We first evaluated the levels of Ca^{2+} that could be tolerated in this modified prothrombin time assay. These data are presented in Figure 3. Here, quantities of Ca^{2+} were added to citrated plasma, and the amount of free Ca^{2+} present in the plasma was determined by Ca^{2+}-selective electrode analysis. The modified prothrombin time assay described above was then performed at each addition of Ca^{2+}. The data show that total Ca^{2+} levels of 15 mM (approximately 7 mM free Ca^{2+}) can be accommodated in this assay. It is noteworthy to observe that under conditions of the APTT assay, where no exogenous Ca^{2+} is present in the assay at the times in which APC is incubating with fV/fVa and fVIII/fVIIIa, the free (available) Ca^{2+} for these Ca^{2+}-dependent reactions is only approximately 80 μM in this citrated plasma. Prior to using this modified prothrombin time assay for the purpose intended, it remained to evaluate the effects in plasma of Ca^{2+} on the APC-catalyzed inactivation of fV/fVa. Titrations with Ca^{2+} (Figure 4) demonstrated that optimal levels of free Ca^{2+} for this stage of the assay were reached at approximately 1.5–2.0 mM (6–8 mM exogenous Ca^{2+}) for wtr-APC, after which a degree of inhibition occurred. This confirms an earlier observation on this same point (Bakker et al., 1992). This inhibition of wtr-APC inactivation of fV/fVa in plasma at high Ca^{2+} concentrations was also observed in the prothrombinase and tenase assays with purified components. In these cases, approximately 15%–20% inhibition was seen when the Ca^{2+} concentration was increased from 2 to 15 mM. Since the activities of each r-APC mutant are relative to that of wtr-APC at the designated Ca^{2+} concentration, this small degree of inhibition by high levels of Ca^{2+} most likely does not affect the conclusions reached.

Given the suitability of this modified prothrombin time assay as a means of examining in plasma the inactivation of fV/fVa by APC at higher concentrations of Ca^{2+} than were possible in the APTT assay, we performed this assay with all r-APC mutants described herein. The free Ca^{2+} concentration in these experiments was 2.5 mM. An example of the data obtained is shown in Figure 5 for wtr-APC and [Gla^{19}D]r-APC. The relative activities of all the r-APC mutants examined by this assay are listed in Table 1.

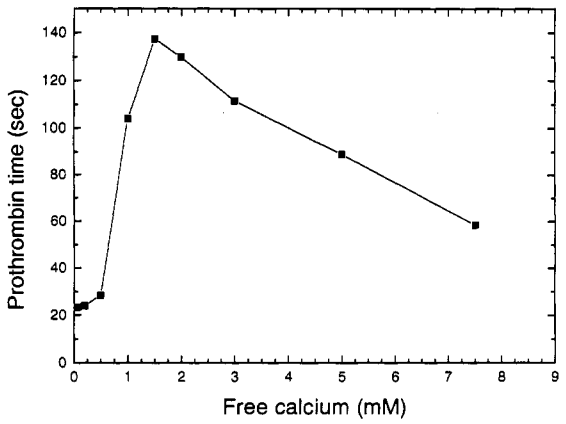

FIGURE 4: Effect of Ca^{2+} on the inactivation of fVa by wtr-APC in plasma at 37 °C. To 100 μL of citrated PC-deficient plasma containing 40 μM (in phosphate) PL was added 100 μL of the desired levels of $CaCl_2$ dissolved in TBS (the concentrations of free Ca^{2+} in the resulting solution were determined with use of the Ca^{2+}-selective electrode) immediately followed by addition of 10 μL (containing 1 μg) of r-APC dissolved in TBS. After incubation for 1 min, clotting was then accelerated by addition of 100 μL of a 1.0 μL/mL solution of fXa (dissolved in a TBS solution also containing 0.5% bovine serum albumin and $CaCl_2$, at concentrations such that the final total concentration of Ca^{2+} was 15 mM). The clotting times (prothrombin times) were then measured.

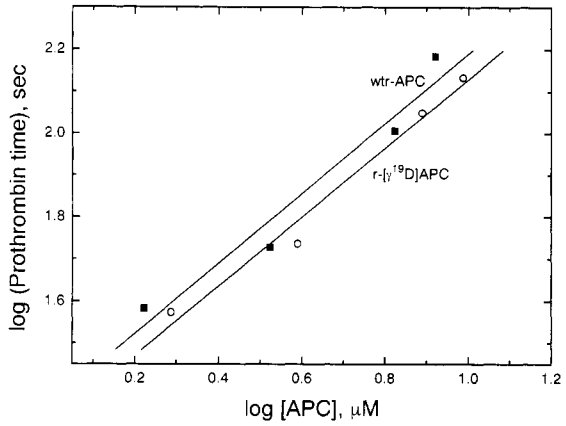

FIGURE 5: Activity of r-APC toward fV/fVa in plasma. The modified prothrombin time assay of Figure 4 was employed. To 100 μL of PC-deficient plasma containing 40 μM (in phosphate) PL was added 100 μL of 21 mM $CaCl_2$ dissolved in TBS (final concentration of free $CaCl_2$ in the assay mixture was 2.5 mM) immediately followed by addition of 10 μL of a range of levels (0.1–1.0 μg) of the r-APC or the desired r-APC mutant dissolved in TBS. Clotting was accelerated by inclusion of 100 μL of a 1.0 μg/mL solution of fXa (dissolved in a TBS solution also containing 25.5 mM $CaCl_2$ and 0.5% bovine serum albumin). The logarithm of the clotting times was then plotted against the logarithm of the r-APC concentrations, and the activities of the r-APC mutants relative to that of wtr-APC were obtained from the concentration ratio of the mutant r-APC to wtr-APC at the same clotting time. All stock r-APC solutions were adjusted to equal amidolytic activities. Shown in this figure are the effects of APC concentrations on clotting (prothrombin) times of (■) wtr-APC and (○) [Gla^{19}D]r-APC (r-[γ^{19}D]APC).

DISCUSSION

In this communication, we have examined the activities in a variety of assays of all r-APC preparations which contained conservative mutations at each of the Gla residues of this protein. The chemical and physical properties of all of these r-mutants have been described in previous reports (Zhang et al., 1992; Zhang & Castellino, 1992, 1993). Considering this, the most important considerations for the purposes of this report are that each mutant was fully γ-carboxylated at all of the available precursor E residues (Zhang et al., 1992) and that all of the relative activities of the mutants have been normalized to equal amidolytic activities of the respective r-APC mutants. These latter specific activities were not greatly different for any of these mutants. However, we nonetheless believed that such a normalization was the most productive and effective manner of proceeding with this work.

While we had performed APTT assays on these mutant proteins in a past publication (Zhang et al., 1992), experiments that allowed us to assess the importance of each of the Gla residues for expression of the overall anticoagulant activity of r-APC and subsequent studies on the Ca^{2+} binding properties of these proteins (Zhang & Castellino, 1992) suggested that other types of assays might be additionally revealing. More specifically, in the APTT assay, exogenous Ca^{2+} is not present during times in which the particular APC is added to the PC-deficient plasma to inactivate fV/fVa and fVIII/fVIIIa, despite the fact that these are Ca^{2+}-requiring reactions. Most of the endogenous Ca^{2+} present in the plasma has been complexed by citrate. This situation is not readily correctable in this assay since additions of Ca^{2+} would lead to rapid clotting, thus confounding the assay. There is some endogenous free Ca^{2+} present in the assay components (estimate, 80 μM) which allows the reaction to proceed, but at far less than optimal rates. This is not a particular problem clinically since all results are obtained under identical conditions. However, for our purposes, we needed to evaluate whether those r-APC mutants which showed low activity in the APTT assay and abnormal Ca^{2+}-binding properties would produce higher activities at elevated levels of Ca^{2+}. In addition, the APTT assay does not discriminate as to whether each defective r-APC possesses similar activities toward both fV/fVa and fVIII/fVIIIa, since selective inactivation of either of the cofactors would produce the same overall result. The question as to whether the same determinants on APC are required for inactivation of the fV/fVa cofactor system, as well as for that of fVIII/fVIIIa, is one of great importance and had not been addressed to this time. With the mutant proteins that we possessed, we were in an excellent position to do so.

Given the above considerations, we employed assays of the r-APC-catalyzed inactivation of fVa and fVIII in systems using purified components, in the former case by examining the rate of thrombin formation from prothrombin and in the latter case by measuring the rate of formation of fXa from fX, in otherwise complete prothrombinase and tenase systems, respectively. Synthetic substrates were employed in each case for measurement of thrombin and fXa production, thereby circumventing the need for clotting-based assays. Because of this, we were able to vary the Ca^{2+} concentrations to considerably higher levels in these assays than were possible in the APTT assays.

Relative activities toward fVa and fVIII of each of the r-APC mutants are summarized in Table 1 and compared to the anticoagulant activities for these same mutants obtained previously in the APTT assay system. The mutants that possessed full activity in the APTT assay, viz., [Gla^{6}D]r-APC, [Gla^{14}D]r-APC, and [Gla^{19}D]r-APC, also possessed activities toward each of these substrates to an extent that was nearly the same as that of wtr-APC. Three mutants that displayed virtually no activity in the APTT assay, viz., [Gla^{16}D]r-APC, [Gla^{20}D]r-APC, and [Gla^{26}D]r-APC, also possessed very low activities in the fVa and fVIIIa assays. In the case of [Gla^{20}D]r-APC, activity was enhanced toward both substrates at higher levels of Ca^{2+}, but the maximal activity attainable was <30%. This demonstrates that partial correction with Ca^{2+} is possible in assays using purified

components and argues that the higher Ca^{2+} concentrations required to place this mutant into its proper Ca^{2+}-dependent conformation (Zhang & Castellino, 1992), through binding to the first set of internal Ca^{2+}-binding sites (Nelsestuen, 1976; Prendergast & Mann, 1977; Strickland & Castellino, 1980; Keyt et al., 1982), play a role in the loss of activity of this mutant. That only partial correction is obtained at elevated levels of Ca^{2+} is consistent with the finding that altered Ca^{2+}-binding properties observed with this mutant probably contributed to its loss of activity. The fact that activity could not be fully restored with Ca^{2+} is consistent with the finding that the Ca^{2+}-dependent association of the Ca^{2+}-dependent form of APC with PL, which occurs through the second set of surface Ca^{2+}-binding sites (Nelsestuen, 1976), is greatly deficient in the case of [Gla^{20}D]r-APC (Zhang & Castellino, 1993). Similar situations are also believed to be the case with the [Gla^{7}D]r-APC and [Gla^{29}D]r-APC mutants.

The mutants [Gla^{16}D]r-APC and [Gla^{26}D]r-APC possess virtually no activity in these two cofactor inactivation assays (a slight level of activity is found for the $Gla^{16} \rightarrow$ D mutant in the fVa and fVIII cofactor assays), as well as in the APTT assay, and this large diminishment of activity accompanying mutations at these sites cannot be significantly reversed by increases in the Ca^{2+} concentrations. Even the most conservative of mutations, i.e., to a D residue, at Gla^{16} and Gla^{26} seems to destroy both the adoption of the Ca^{2+}-dependent conformation of r-PC and r-APC (Zhang & Castellino, 1992) as well as the ability of these mutants to interact with PL (Zhang & Castellino, 1993). In all structural and functional properties of r-PC and r-APC that we have examined, these residues appear to be the most essential for the integrity of functionally relevant Ca^{2+} binding. As is the case with both Gla^{16} and Gla^{26} substitutions, these residues appear to adversely affect every functional Ca^{2+}-dependent property of r-PC and r-APC that has been evaluated to date. Assuming that a parallel exists for the Gla domain of PC with the crystal structure of the prothrombin fragment-1/Ca^{2+} complex, we conclude that Gla^{16} and Gla^{26} mainly coordinate internal core Ca^{2+} ions (Soriano-Garcia et al., 1992). Thus, substitutions at these locations should not be well tolerated in the Ca^{2+}-dependent functions of this protein.

The results most difficult to fully explain are with the mutant [Gla^{25}D]r-APC. This protein adopts a normal Ca^{2+}-dependent conformation as recognized by the Ca^{2+} dependency of its intrinsic fluorescence perturbations by Ca^{2+} and by a Ca^{2+} dependency of binding of a Ca^{2+}-dependent monoclonal antibody (Zhang & Castellino, 1992). On the other hand, the Ca^{2+} dependency of its interaction with PL is altered in a small way from wild-type counterpart (Zhang & Castellino, 1992). This suggests that a slightly different protein/Ca^{2+}/PL complex may exist that could result in inhibition of its activity in the APTT assay. This activity loss is not observed in assays against fVa and fVIII (Table 1), perhaps also suggesting that components in plasma may interact with this altered protein and result in its inhibition. This component is not protein S, as demonstrated in separate assays.[2] Some confirmation of this point is present in the considerations that follow.

Since we ultimately desired to examine these mutant enzymes in a coagulation assay, we developed a workable plasma-based fV/fVa assay. Here, the desired r-APC was incubated with PC-deficient plasma at various levels of Ca^{2+}. Since thromboplastin is not present, clotting factors are not activated and a clot does not form. The r-APC then inactivates plasma fV/fVa and fVIII/fVIIIa. Upon addition of fXa at the desired time, thrombin is formed and clotting occurs. This latter clotting assay is then specific for evaluation of components of the prothrombinase complex—in this case, the amount of fV/fVa present. The results of this assay for all mutants (Table 1) are in good agreement with the APTT assays and show that in virtually all cases of the anticoagulation-defective mutants the plasma-based coagulation assays yield lower activities than those calculated from the cofactor assays with purified components. This further suggests that a component in plasma may exist that interferes with the ability of the modified r-APC mutants to inactivate fV/fVa and fVIII/fVIIIa.

Finally, we constructed and expressed a mutant, [Q^{32}Gla]r-APC, which contains an additional Gla residue at a location wherein Gla exists on other vitamin-K-dependent clotting proteins, such as factor IX and prothrombin. This altered protein contained full r-APC activity, suggesting that functional Ca^{2+}-binding properties of r-APC were not substantially altered by the presence of this Gla residue. Interestingly, electron density at this residue was not observed in the crystal structure of the prothrombin fragment-1/Ca^{2+} complex (Soriano-Garcia et al., 1992), suggesting that it is a disorganized side chain without significant impact in coordination of Ca^{2+}. Our results with r-APC are consistent with a similar conclusion.

In summary, we have examined the activities of the complete set of Gla mutants in plasma-based fV/fVa and fVIII/fVIIIa coagulation assays and in assays of fVa and fVIII using purified components in nonclotting assays. In general, those mutants which showed defective Ca^{2+}-binding properties in the first set of internal sites responsible for placing r-APC in its Ca^{2+}-dependent conformation, viz., [Gla^{16}D]r-APC and [Gla^{26}D]r-APC, also showed no substantial activity in these assay systems. Partial activity was observed with the mutants [Gla^{7}D]r-APC, [Gla^{20}D]r-APC, [Gla^{25}D]r-APC, and [Gla^{29}D]r-APC that were defective in their second set of surface Ca^{2+} sites, which are mainly responsible for r-APC/PL interactions. Higher levels of Ca^{2+} did not fully restore activities in these latter cases, suggesting that these Gla residues were responsible for stabilizing properly oriented r-APC/PL complexes that are suitable for effective substrate hydrolysis. Full activity was found with three mutants, [Gla^{6}D]r-APC, [Gla^{14}D]r-APC, and [Gla^{19}D]r-APC, the results of which demonstrate that not all Gla residues, and/or Ca^{2+} coordinatioin sites, are essential for activity of r-APC. No substantial differences were seen in the relative activities of r-APC for fVa and fVIII, suggesting that the determinants for such activity on r-APC are very similar for these two substrates.

[2] Jhingan, A. Unpublished experiments.

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