Roles of Two Conserved Cysteine Residues in the Activation of Human Adenovirus Proteinase†

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ABSTRACT: The roles of two conserved cysteine residues involved in the activation of the adenovirus proteinase (AVP) were investigated. AVP requires two cofactors for maximal activity, the 11-amino acid peptide pVIc (GVQSLKRRRCF) and the viral DNA. In the AVP−pVIc crystal structure, conserved Cys104 of AVP has formed a disulfide bond with conserved Cys10 of pVIc. In this work, pVIc formed a homodimer via disulfide bond formation with a second-order rate constant of 0.12 M^{-1} s^{-1}, and half of the homodimer could covalently bind to AVP via thiol−disulfide exchange. Alternatively, monomeric pVIc could form a disulfide bond with AVP via oxidation. Regardless of the mechanism by which AVP becomes covalently bound to pVIc, the kinetic constants for substrate hydrolysis were the same. The equilibrium dissociation constant, K_d, for the reversible binding of pVIc to AVP was 4.4 μM. The K_d for the binding of the mutant C10A-pVIc was at least 100-fold higher. Surprisingly, the K_d for the binding of the C10A-pVIc mutant to AVP decreased at least 60-fold, to 6.93 μM, in the presence of 12mer ssDNA. Furthermore, once the mutant C10A-pVIc was bound to an AVP−DNA complex, the macroscopic kinetic constants for substrate hydrolysis were the same as those exhibited by wild-type pVIc. Although the cysteine in pVIc is important in the binding of pVIc to AVP, formation of a disulfide bond between pVIc and AVP was not required for maximal stimulation of enzyme activity by pVIc.

Human adenovirus contains a gene for a proteinase whose activity is essential for the synthesis of infectious virus (*1*). Late in an adenovirus infection, the virus-encoded proteinase becomes activated inside young virions. Approximately 70 proteinase molecules (*2*) cleave multiple copies of six virion precursor proteins more than 3200 times to render a virus particle infectious. The adenovirus proteinase (AVP)[1] is encoded by the L3 23K gene whose protein product has a molecular mass of 23 kDa (*3*).

AVP is unusual in that it requires cofactors for maximal activity. One cofactor, pVIc, is the 11-amino acid peptide (GVQSLKRRRCF) derived from the C-terminus of virion precursor protein pVI (*4*, *5*). Preceding the sequence for pVIc is the AVP consensus cleavage sequence, IVGL-G. Thus, AVP can cleave pVI to liberate its own cofactor. The other cofactor is the viral DNA to which AVP binds nonspecifically, i.e., independent of any specific nucleic acid sequence, i.e., to a polyanion with a high negative charge density (*4*). Binding of the cofactors to AVP increases the specificity constant, k_{cat}/K_m, for substrate hydrolysis (*6*). Compared to that for AVP alone, the k_{cat}/K_m increases 1130-fold with an AVP−pVIc complex, 110-fold with an AVP−viral DNA complex, and 34100-fold in the presence of both pVIc and viral DNA.

The crystal structure of the AVP−pVIc complex is known at a resolution of 2.6 Å (*7*) and revealed that AVP is not structurally homologous to any protein structure in the databases. However, AVP shares some common secondary structural elements with papain. When the common secondary structure elements are aligned and the amino acids of the active-site region of papain (*8*) and AVP are compared, it is clear that AVP is a new type of cysteine proteinase. In positions identical to Cys25, His159, and Asn175 of papain are Cys122, His54, and Glu71 of AVP, respectively. Even Gln19 of papain, presumed to participate in the formation of an oxyanion hole (*9*), aligns with Gln115 of AVP. The main chain nitrogen atoms of the two active-site Cys residues also match; in papain, this atom is proposed to join with Gln19 to form the oxyanion hole (*10*). This remarkable juxtaposition of catalytic elements strongly suggested that AVP employs the same catalytic mechanism as papain (*11*) and that AVP was an example of convergent evolution (*12*).

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[1] Abbreviations: Ad2, human adenovirus serotype 2; AVP, human adenovirus proteinase; AVP−pVIc, noncovalent or covalently linked heterodimer of AVP and pVIc; DTNB, 5,5′-dithiobis(2-nitrobenzoate); K_d, equilibrium dissociation constant; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; NP-40, Nonidet P-40; pVIc, 11-amino acid cofactor (GVQSLKRRRCF) originating from the C-terminus of virion precursor protein pVI; C10A-pVIc, mutant pVIc with alanine at position 10; C10S-pVIc, mutant pVIc with serine at position 10; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]-3-propanesulfonate.

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The pVIc Cys10 residue forms a disulfide bond with Cys104 of AVP. In this work, the roles of these two conserved cysteine residues involved in the activation of AVP were investigated. Although AVP contains eight cysteine residues, only Cys122 and Cys104 are conserved throughout adenovirus serotypes (*13*). The penultimate amino acid residue in pVIc, Cys10, is also conserved. The ability of pVIc to form a disulfide-linked homodimer was quantitatively characterized, and activation of AVP by dimeric and monomeric pVIc was characterized. The level of binding of pVIc cysteine mutants, C10A-pVIc and C10S-pVIc, to AVP in the presence and absence of DNA was measured as were the effects of these mutations on the enzyme activity of AVP−mutant pVIc complexes. Finally, the question of whether the disulfide bond between Cys104 of AVP and Cys10 of pVIc is required for stimulation of enzyme activity was addressed.

MATERIALS AND METHODS

Materials. Synthetic peptides with the sequences GVQSLKRRRCF (pVIc), GVQSLKRRRAF (C10A-pVIc), GVQSLKRRRSF (C10S-pVIc), and GVQSLK*RRRAF (fluorescein-labeled C10A-pVIc), where K* is a lysine residue with a fluorescein molecule attached to its ϵ-amino group, were obtained from Research Genetics (Huntsville, AL). The 12mer single-stranded (ss) DNA (GACGACTAGGAT) was purchased from Life Technologies (Rockville, MD).

Protein Concentration. The concentration of AVP was determined using a molar absorbance coefficient at 280 nm of 26 510 M^{-1} cm^{-1}, calculated according to the method of Gill and von Hippel (*14*). The concentration of pVIc was determined by titration of its cysteine residue with Ellman's reagent and confirmed by quantitative amino acid analysis.

Concentration of pVIc. The concentration of thiolate anion was determined with an Ellman's assay. Assays were performed in a volume of 0.516 mL in 50 mM Tris (pH 8.0) containing 350 μM DTNB and pVIc. Reaction mixtures were incubated at 25 °C for 10 min before the absorbance at 412 nm was measured. The concentration of thiolate anion was calculated by subtracting the absorbance in an assay lacking pVIc from that measured in its presence, using a molar extinction coefficient for thionitrobenzoate at 412 nm of 14 150 M^{-1} cm^{-1} (*15*).

pVIc Dimerization Reactions. pVIc was dissolved in nitrogen-saturated 50 mM Tris-HCl (pH 8.0) to a concentration of 0.1, 0.3, 0.7, or 1.0 mM, and incubated at 37 °C. At various times, aliquots were removed and the amount of thiolate anion present was titrated by an Ellman's assay. The presence of the pVIc dimer was verified by size exclusion chromatography.

AVP−pVIc Complex Formation. AVP−pVIc complexes for activity assays were formed by incubation of either 25 μM AVP with 65 μM dimeric pVIc or 100 μM AVP with 150 μM monomeric pVIc, at 4 °C for 0.5−16 h. All buffers were saturated with nitrogen.

Proteinase Activity Assays. The fluorogenic substrate (Leu-Arg-Gly-Gly-$NH)_2$-rhodamine was synthesized as described previously (*4*). Assays were carried out using either an ISS (Urbana, IL) PC-1 spectrofluorometer equipped with a thermostated sample chamber or a Photon Technologies International (PTI) (Monmouth Junction, NJ) QuantaMaster spectrofluorometer equipped with a thermostated sample chamber. For both instruments, the excitation wavelength was 492 nm and the emission wavelength was 523 nm. For the ISS spectrofluorometer, the band-pass was 8 nm, and for the PTI spectrofluorometer, the band-pass was 4 nm. For each peptide, the substrate concentrations used to measure K_m and k_{cat} varied between 0.2 and 5 times the K_m.

Assay for the Formation of a Disulfide Bond between AVP and pVIc. Reaction mixtures containing 12.5 μM AVP and 20 μM monomeric or dimeric pVIc were incubated at either 4 or 37 °C in 5 mM sodium phosphate, 20 mM NaCl, and 0.1 mM EDTA. After various time intervals, aliquots were removed and diluted 1:500 into cuvettes containing 10 mM Tris (pH 8.0), 0.005% NP-40, 15 μM (Leu-Arg-Gly-Gly-$NH)_2$-rhodamine, and either 20 μM pVIc or no additional pVIc. The increase in fluorescence was monitored as a function of time. Complexes were formed at 5 times the K_d for the reversible binding of pVIc to AVP; then they were diluted to 0.01 times the K_d, and the amount of enzyme activity was measured. The substrate concentration was 5 times the K_m. The assays in which aliquots were diluted into cuvettes containing no additional pVIc measured the activity of AVP molecules that had formed a covalent complex with pVIc, whereas those diluted into cuvettes containing additional pVIc measured the activity of AVP to which pVIc was reversibly or covalently bound.

The mixtures for reactions being used to assess the effect of DNA on the formation of the disulfide bond between AVP and pVIc contained 1 μM 12mer ssDNA, 0.5 μM AVP, 1 μM monomeric pVIc in 5 mM sodium phosphate, 20 mM NaCl, and 0.1 mM EDTA. After incubation at 4 or 37 °C for various time intervals, aliquots were removed and diluted 1:200 into cuvettes containing 10 mM Tris (pH 8.0), 0.005% NP-40, 1 μM 12mer ssDNA, 15 μM (Leu-Arg-Gly-Gly-$NH)_2$-rhodamine, and either 1 μM pVIc or no additional pVIc. The increase in fluorescence was monitored as a function of time. For some assays, Ad2 DNA, at a concentration of 280 ng/mL, was used in place of 12mer ssDNA; the concentration of (Leu-Arg-Gly-Gly-$NH)_2$-rhodamine was 4 μM. These assays measured only the activity of the AVP−DNA complex that had formed a covalent bond with pVIc. Complexes were formed at 5 times the K_d for the reversible binding of pVIc to AVP in the presence of DNA; then they were diluted to 0.05 times the K_d, and the amount of enzyme activity was measured. The substrate concentration was 5 times the K_m.

Equilibrium Dissociation Constants. The equilibrium dissociation constant, K_d, for the binding of the wild-type and three mutant pVIcs to AVP was determined as follows. Increasing concentrations of pVIc, $(pVIc)_i$, were added to a constant amount of AVP, $(AVP)_o$. The concentration of the ligand was varied between 0.2 and 5 times the K_d. After 5 min at 37 °C, substrate was added and the rate of increase in fluorescence with time, F_i, measured. A plot of F_i versus $(pVIc)_i$ yielded a rectangular hyperbola. From the data in this graph, the concentration of pVIc bound, $(pVIc)_b$, was obtained:

$$(pVIc)_b = (AVP)_o[F_i/F_{max}]$$

where F_{max} is the maximal rate of substrate hydrolysis, i.e., the rate when AVP is saturated with pVIc. The concentration

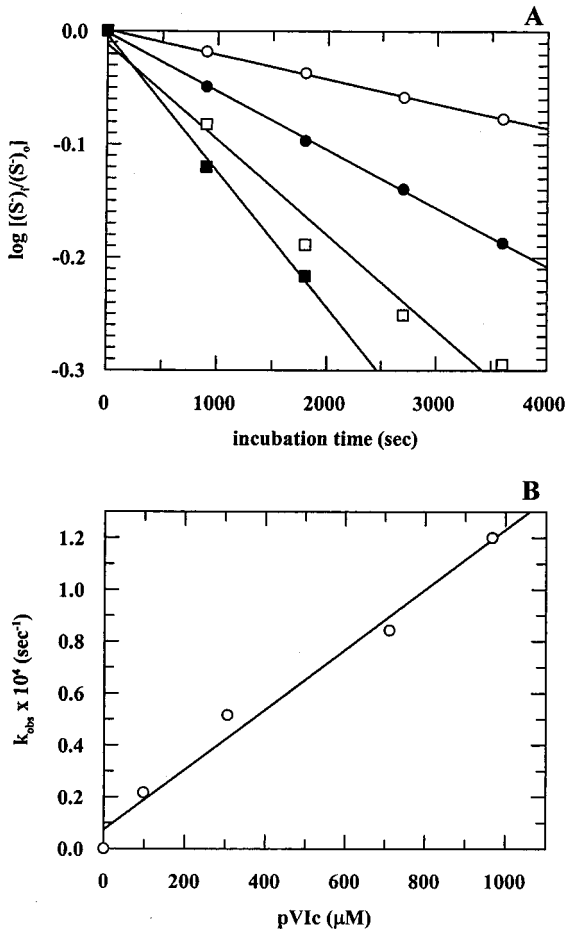

FIGURE 1: Kinetics of formation of homodimers of pVIc. pVIc was dissolved in 50 mM Tris (pH 8.0) to concentrations of 0.1 (○), 0.3 (●), 0.7 (□), and 1.0 mM (■) and incubated at 37 °C. At various times, aliquots were removed and the amount of thiolate anion present was titrated by an Ellman's assay. (A) Pseudo-first-order reaction kinetics. The log of the concentration of thiol, $(S^-)_i$, in pVIc at time t divided by $(S^-)_o$, the initial pVIc concentration, is plotted as a function of time. (B) Second-order rate constant. The slopes of the lines in Figure 1A are plotted vs pVIc concentration.

of pVIc free, $(pVIc)_f$, was calculated by:

$$(pVIc)_f = (pVIc)_i - (pVIc)_b$$

And, from a plot of $(pVIc)_f$ versus $(pVIc)_b$, the K_d can be calculated as the value of $(pVIc)_f$ that yields one half-maximal $(pVIc)_b$.

RESULTS

Kinetics of Formation of pVIc Homodimers. pVIc has been shown to be able to form a homodimer (*5*). The kinetics of homodimer formation were studied by incubating 0.1, 0.3, 0.7, or 1.0 mM pVIc in 50 mM Tris (pH 8.0) at 37 °C and monitoring as a function of time the concentration of monomeric pVIc remaining by an Ellman's assay. When the log of the percent change in the monomeric pVIc concentration was plotted versus time, straight lines were observed with half-times of 232 min for 0.1 mM pVIc, 97 min for 0.3 mM pVIc, 57 min for 0.7 mM pVIc, and 41 min for 1 mM pVIc (Figure 1A). Thus, the reaction was pseudo-first-order. A plot of the slopes of the lines in Figure 1A versus the pVIc concentrations gave a straight line, indicating that the second-order rate constant for the formation of homodimers was 0.12 M^{-1} s^{-1} (Figure 1B). The presence of homodimers was confirmed by size exclusion chromatography (data not shown), which indicated a molecular mass of 2700 Da.

Equilibrium Dissociation Constants for the Binding of Monomers and Homodimers of pVIc to AVP and k_{cat} and K_m for Substrate Hydrolysis, in the Absence and Presence of DNA. The K_d for the binding of monomeric pVIc to AVP was 4.4 μM, and 0.09 μM in the presence of 12mer ssDNA (*24*) (Table 1). The K_d for the binding of the homodimer of pVIc to AVP was 0.48 μM, and 0.02 μM in the presence of 12mer ssDNA. With both forms, the k_{cat} values for the resultant AVP−pVIc complexes were ~0.1 s^{-1}, and the K_m values were ~1.2 μM (Table 1). In the presence of 12mer ssDNA, the k_{cat} values increased ~6-fold and the K_m values increased ~3-fold.

Equilibrium Dissociation Constants for the Binding of AVP to Mutants of pVIc with an Altered Cysteine Residue. The function of the cysteine residue, Cys10, in pVIc is unknown. To assess its role in the binding of pVIc to AVP, the cysteine in pVIc was mutated to an alanine (C10A-pVIc) or to a serine (C10S-pVIc), or the cysteine was alkylated. The K_d for the binding of pVIc to AVP is 4.4 μM (*24*). In similar binding experiments, the K_d values for these three variants were much higher. For example, a binding isotherm with a maximal concentration of 80 μM for the mutant C10A-pVIc gave an estimated K_d of 440 μM with a standard error of 700 μM. These experiments indicated the K_d values of the three mutants may be at least 100-fold greater than that of wild-type pVIc. These data also indicated Cys10 in pVIc is important in the binding of pVIc to AVP.

Equilibrium Dissociation Constants for the Binding of AVP to Mutants of pVIc with an Altered Cysteine Residue, in the Presence of DNA. To determine whether the presence of DNA can affect the binding of the three variants of pVIc to AVP, the binding experiments were repeated in the presence of 1 μM 12mer ssDNA (Table 1). Surprisingly, in the presence of DNA, the variant pVIc molecules bound to AVP much more tightly. The K_d for the binding of C10A-pVIc was 6.93 μM. For C10S-pVIc and alkylated pVIc, the K_d values were 7.60 and 0.14 μM, respectively.

A competition assay using fluorescence anisotropy was used to confirm that the activity assays described above were measuring the level of binding of AVP to C10A-pVIc. AVP was incubated with 12mer ssDNA and C10A-pVIc that contained a fluorescein molecule attached to the ϵ-amino group of the lysine residue. The concentration of 12mer ssDNA was 5-fold higher than its K_d for binding to AVP. After binding equilibrium had been reached, increasing amounts of wild-type pVIc were added, and the change in anisotropy was measured (data not shown). There was a decrease in the anisotropy as the wild-type pVIc concentration was increased. This indicated a complex of AVP with fluorescein-labeled C10A-pVIc had formed and that wild-type pVIc competed with the fluorescein-labeled C10A-pVIc for the same binding site on AVP. In conclusion, the binding of AVP to DNA dramatically reduced the K_d values for the binding of the mutants of pVIc to AVP.

Kinetic Constants for Substrate Hydrolysis by AVP in a Complex with Mutants of pVIc with Altered Cysteine Residues, in the Presence of DNA. Since AVP−DNA

Table 1: Equilibrium Dissociation Constants for the Binding of pVIc and of Mutants of pVIc to AVP in the Absence or Presence of 12mer ssDNA and Macroscopic Kinetic Constants for Substrate Hydrolysis

	in the absence of DNA			in the presence of DNA		
peptide	K_d (μM)	K_m (μM)	k_{cat} (s^{-1})	K_d (μM)	K_m (μM)	k_{cat} (s^{-1})
monomeric pVIc	4.43 ± 0.45	0.84 ± 0.30	0.129	0.09 ± 0.01	3.68 ± 0.11	0.59
dimeric pVIc	0.48 ± 0.06	1.48 ± 0.52	0.065	0.02 ± 0.005	2.70 ± 0.24	0.60
C10A-pVIc	>440			6.93 ± 1.1	8.06 ± 2.14	0.43
C10S-pVIc	>440			7.60 ± 0.67	10.62 ± 1.39	0.39
alkylated pVIc	>440			0.14 ± 0.02	3.28 ± 1.17	0.22

Table 2: Macroscopic Kinetic Constants for Substrate Hydrolysis by AVP in the Presence of pVIc or Mutants of pVIc, and in the Presence of Ad2 DNA

form of pVIc	K_m (μM)	k_{cat} (s^{-1})	form of pVIc	K_m (μM)	k_{cat} (s^{-1})
monomeric pVIc	0.41 ± 0.12	1.23	C10S-pVIc	1.28 ± 0.77	1.22
dimeric pVIc	0.40 ± 0.27	1.18	alkylated pVIc	0.59 ± 0.09	1.09
C10A-pVIc	0.28 ± 0.09	1.34			

complexes can readily be saturated by the three mutants of pVIc with altered cysteine residues whereas AVP cannot, the effects of these mutations on the kinetics of substrate hydrolysis by AVP in the presence of 12mer ssDNA could be assessed. For comparison, for the wild-type pVIc monomer, the K_m was 3.68 μM and the k_{cat} was 0.59 s^{-1}. With C10A-pVIc, C10S-pVIc, and alkylated pVIc, the K_m values were 8.06, 10.62, and 3.28 μM, respectively, and the k_{cat} values were 0.43, 0.39, and 0.22 s^{-1}, respectively (Table 1). When these experiments were repeated but with Ad2 DNA substituted for 12mer ssDNA, the K_m and k_{cat} values for the three mutant pVIcs became identical to those exhibited by wild-type pVIc (Table 2).

Kinetics of Formation of a Disulfide Bond between AVP and pVIc. The cysteine residue in pVIc forms a disulfide bond with Cys104 of AVP (*7*). An assay was developed to measure the kinetics of disulfide bond formation between AVP and pVIc. In the assay, AVP was incubated with a concentration of either monomeric or dimeric pVIc that was 5 times greater than the K_d. After certain time intervals, the reaction mixtures were diluted into solutions of substrate and the rate of substrate hydrolysis was measured. The dilution reduced the pVIc concentration 100-fold lower than its K_d for AVP. Thus, during the activity assay, reversibly bound pVIc will have dissociated, and the only active enzyme will be that which had formed a disulfide bond with pVIc.

The pVIc dimer formed a disulfide bond to AVP with a half-time of 25 s (Figure 2). The half-time for formation of a disulfide bond with monomeric pVIc was much longer, 1740 s. These results are not unexpected as the only mechanism for disulfide bond formation by dimeric pVIc is thiol−disulfide exchange. Thiol−disulfide exchange is much faster than oxidation which must occur when monomeric pVIc forms a disulfide bond between its thiol and Cys104 of AVP.

Assays were also performed to see if the presence of DNA affected the rate of formation of a disulfide bond between AVP and pVIc. AVP was incubated with monomeric pVIc at a pVIc concentration of 5 times the K_d in the presence of 12mer ssDNA such that all the AVP was bound to the DNA. After certain time intervals, the reaction mixtures were diluted into solutions of substrate and DNA and the rate of substrate hydrolysis was measured. The dilution reduced the pVIc concentration 20-fold lower than its K_d for AVP, whereas the DNA concentration remained the same. The presence of 12mer ssDNA had a slight effect on the rate of formation of a disulfide bond between monomeric pVIc and AVP. The half-time decreased 26%, to 1288 s.

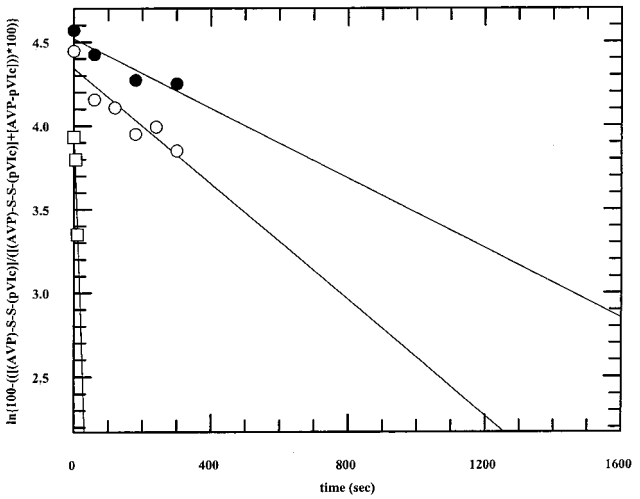

FIGURE 2: Kinetics of formation of a disulfide bond between AVP and pVIc and the effect of 12mer ssDNA on the formation of that bond. AVP (12.5 μM) was preincubated at 37 °C for the indicated times in the presence of 20 μM monomeric pVIc (●), 0.5 μM monomeric pVIc and 1 μM 12mer ssDNA (○), or 20 μM dimeric pVIc (□). Aliquots were removed, diluted 500-fold to a concentration 100-fold lower than the K_d for the reversible binding of monomeric pVIc to AVP, and assayed for enzyme activity. As a control, aliquots were removed, diluted 500-fold but into the same concentration of pVIc, pVIc and DNA, or dimeric pVIc, and assayed for enzyme activity. The rates of substrate hydrolysis were divided by the rates of substrate hydrolysis from the control reactions. This ratio was multiplied by 100; the resultant number was subtracted from 100, and the natural logarithm of that number was plotted vs time. The slopes of the lines were 0.0017 (●), 0.001 (○), and 0.058 (□) s^{-1}.

That this assay was indeed monitoring formation of a disulfide bond between pVIc and AVP was confirmed by immunoblot analysis. Immunoblots of AVP−pVIc complex formation, after SDS−PAGE under reducing and nonreducing conditions (W. J. McGrath and W. F. Mangel, unpublished observations), were probed with an anti-pVIc antibody. In the reduced gels, only a band that comigrated with pVIc (MW = 1350) was observed, and in the nonreduced gels, a band that comigrated with AVP (MW = 23 000) was observed.

Kinetics of Activation of AVP by pVIc. Although both dimeric and monomeric pVIc can form a disulfide bond with AVP, is formation of a disulfide bond necessary for activation of the enzyme or is just binding of pVIc to AVP sufficient to obtain maximal enzyme activity? This question can be answered by comparing the time required for

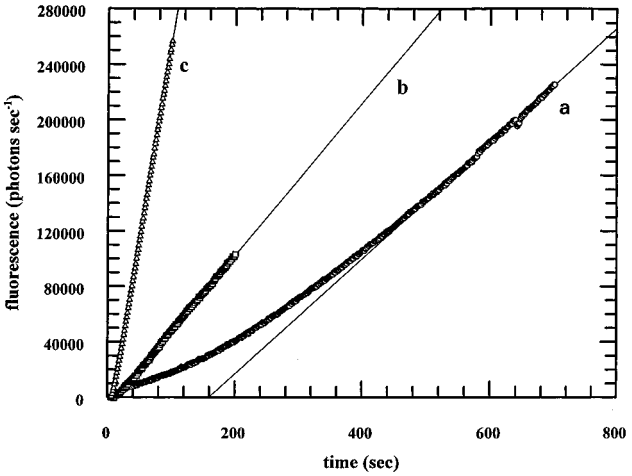

FIGURE 3: Time to attain the maximal rate of substrate hydrolysis upon addition of pVIc to AVP, in the presence or absence of DNA. Monomeric pVIc (a), dimeric pVIc (b), or monomeric pVIc in assays containing 1 μM 12mer ssDNA (c) was added to cuvettes containing 25 nM AVP and saturating concentrations of (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine, and the increase in fluorescence was monitored as a function of time.

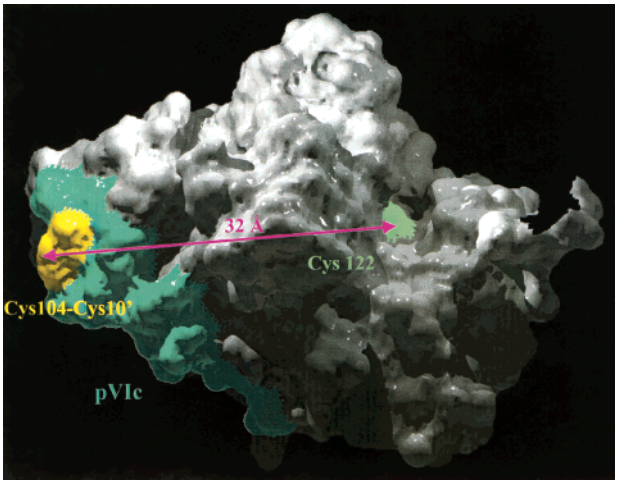

FIGURE 4: Distance on the surface between the disulfide bond between Cys10 of pVIc and Cys104 of AVP and the active-site nucleophile Cys122. The solvent accessible surface of the AVP−pVIc complex is shown in gray. The pVIc peptide is colored cyan; free cysteines are colored green, and the disulfide bond between pVIc Cys10 and AVP Cys104 is colored yellow.

formation of the disulfide bond to the time required to obtain maximal enzyme activity. The time required to obtain maximal enzyme activity upon addition of AVP to dimeric or monomeric pVIc was measured as follows. AVP was incubated with either dimeric or monomeric pVIc at a concentration 5-fold higher than its K_d value, in the presence of substrate, and enzyme activity was monitored as a function of time. Upon addition of dimeric pVIc, the extent of substrate hydrolysis was linear with time. A plot of the concentration of the cleaved substrate versus time yielded a straight line that went through the origin; i.e., it exhibited no lag (Figure 3). Upon addition of monomeric pVIc, there was a lag of ~3 min before the maximal rate of substrate hydrolysis was achieved. In the presence of 12mer ssDNA, the lag was much shorter with the maximal rate occurring within 0.11 min of mixing. Since formation of the disulfide bond takes longer than achievement of the maximal rate of substrate hydrolysis, formation of a disulfide bond is therefore not necessary for maximal activation of the enzyme by pVIc.

DISCUSSION

Conserved Cysteines. The objectives of this study were to investigate the roles of two conserved cysteine residues in the activation of AVP by pVIc. The crystal structure of the AVP−pVIc complex provided some insight into the functions of the conserved cysteines and raised several important questions. Cys104 is involved in a disulfide bond with the conserved Cys10 of pVIc. The active-site nucleophile, the Cys122−His54 ion pair, is located in a groove on the surface of the enzyme (*7*). Surprisingly, pVIc, which greatly increases the k_{cat}/K_m for substrate hydrolysis by AVP, binds quite far from the active-site nucleophile Cys122; the pVIc Cys10 residue is 32 Å away from Cys122 (Figure 4). Site-directed mutagenesis studies have demonstrated the importance of these cysteine residues (*16*−*19*).

Activation of AVP by Thiol−Disulfide Exchange. How is AVP activated by pVIc? Webster et al. (*5*) proposed a model for the activation of AVP by pVIc in which the active-site nucleophile of AVP forms a disulfide bond with another cysteine residue, and as a result, the enzyme is inactive. To activate the enzyme, pVIc must first form a homodimer, and then AVP is activated by a thiol−disulfide interchange reaction.

pVIc Homodimer Formation. pVIc can certainly form a homodimer, but both the homodimer and the monomer of pVIc can bind and activate AVP. The kinetics of homodimer formation were pseudo-first-order with a second-order rate constant of 0.12 M^{-1} s^{-1}. At a pVIc concentration of 0.1 mM, the half-time for homodimer formation was 3.9 h. The K_d values for the binding of the homodimer and the monomer of pVIc to AVP were 0.48 and 4.4 μM, respectively. The 10-fold lower K_d of the homodimer compared to the monomer is probably the reason the dimer was thought to be the active, physiologically relevant form of pVIc (*5*). Once pVIc was bound to AVP, the kinetic constants for substrate hydrolysis were the same, whether binding occurred via the dimer or monomer. Thus, a homodimer is not required for the activation of AVP by pVIc.

Under the assay conditions for stimulation of AVP by pVIc described in Materials and Methods, do homodimers form and then activate the enzyme by thiol−disulfide exchange? The time it took to obtain maximal enzyme activity upon addition of pVIc to AVP was ~160 s. The half-time for formation of a pVIc homodimer was 3.9 h. Thus, it is unlikely under these assay conditions that momomeric pVIc formed a dimer which then activated AVP.

Can homodimers form in vivo in the virus particle? There are at most only 340 molecules of pVIc per virion (*20*). And, pVIc is a very basic protein (pI 11.31) that binds to 12mer dsDNA with an apparent K_d of 693 μM (*6*). The viral precursor to pVIc, pVI, is also a DNA binding protein (*21*). Thus, if pVIc is in contact with the viral DNA, its rate of diffusion may be severely limited which would make homodimers more difficult to form. A more plausible model for activation of AVP by pVIc would be one in which both AVP and pVI bind to DNA. AVP cleaves pVI at the proteinase consensus cleavage site near the C-terminus of

pVI, liberating pVIc which then binds to the enzyme that released it.

The Cysteine in pVIc Is Involved in Binding of pVIc to AVP. The cysteine residue in pVIc is clearly involved in the binding of pVIc to AVP, but it is not absolutely required for pVIc to bind to AVP. The K_d values of C10A-pVIc, C10S-pVIc, or alkylated pVIc were more than 100-fold greater than the K_d of 4.4 μM for wild-type pVIc binding to AVP. Surprisingly, the presence of DNA suppressed the effects on binding to AVP of alterations in the cysteine residue of pVIc. Equilibrium binding constants were greatly lowered. In the presence of 12mer ssDNA, the K_d for the binding of C10A-pVIc or C10S-pVIc to AVP was ~7 μM, ~80-fold higher than the K_d for wild-type pVIc. Alkylated pVIc bound even more tightly. Its K_d was 0.14 μM. Furthermore, once these mutant pVIc molecules were bound to AVP in the presence of 12mer ssDNA, the macroscopic kinetic constants for substrate hydrolysis were similar to those of wild-type pVIc bound to AVP in the presence of DNA. For example, the k_{cat} with C10A-pVIc was 0.43 s^{-1}, whereas that with wild-type pVIc was 0.6 s^{-1}. When Ad2 DNA was used instead of 12mer ssDNA, the k_{cat} with C10A-pVIc was 1.34 s^{-1} whereas that with wild-type pVIc was 1.20 s^{-1}. Thus, the binding of the mutant pVIcs to AVP bound to DNA resulted in macroscopic kinetic constants for substrate hydrolysis similar to those from wild-type pVIc bound to AVP bound to DNA.

The Disulfide Bond between pVIc and AVP Is Not Required for Stimulation of AVP by pVIc. Formation of a disulfide bond between Cys104 of AVP and Cys10 of pVIc is not required for stimulation of enzyme activity by pVIc. At a concentration of the homodimer 5-fold greater than its K_d for AVP, the half-time for formation of a disulfide bond with AVP was 25 s. Yet when the same concentration of homodimer was added to a solution of AVP and substrate, the rate of substrate hydrolysis was constant, with no lag before reaching the maximal rate. More convincingly, at a concentration of monomeric pVIc 5-fold greater than its K_d, the half-time for formation of a disulfide bond was 1740 s. Yet when the same concentration of the monomer was added to a solution of AVP and substrate, there was a lag of 3 min before the maximal rate of substrate hydrolysis was reached. Because upon addition of pVIc to AVP and substrate, the maximal rate of substrate hydrolysis is reached before a disulfide bond can form, formation of a disulfide bond between Cys104 of AVP and Cys10 of pVIc is therefore not necessary for stimulation of AVP by pVIc.

How can DNA suppress the effects of mutations at position 10 of pVIc? pVIcs without Cys10 have difficulty in binding to AVP, except in the presence of DNA. One possibility is that when AVP binds to DNA, its pVIc binding site adopts a more favorable conformation for the binding of pVIc. This seems to be the case in the binding of wild-type pVIc to AVP in the absence and presence of DNA. With monomeric, wild-type pVIc, the K_d for AVP drops from 4.4 μM in the absence of DNA to 0.09 μM in the presence of 12mer ssDNA (*24*). Certainly, formation of a disulfide bond between AVP and pVIc is not necessary for activation of AVP. Once the mutant pVIcs are bound to AVP in the presence of DNA, the kinetic constants for substrate hydrolysis are the same as those exhibited by wild-type pVIc under similar conditions.

Previous work (*5*, *18*, *19*) indicated activation of AVP is dependent on the cysteine residue in pVIc, suggesting that the mechanism may involve the formation of a disulfide bond. The pVIc mutant C10A does not stimulate AVP at concentrations up to 74 μM (*5*). No increase in tryptophan fluorescence is observed when pVIc is treated with iodoacetamide, or when Cys10 in pVIc is replaced with a Ser or an Asp residue (*22*); 150 μM was the highest mutant peptide concentration that was tested. We show here the cysteine residue in pVIc is not required for activation and that AVP can be activated by pVIc with, for example, an alanine in place of the cysteine. The major effect of replacing the cysteine in pVIc with an alanine is that the K_d for AVP is increased enormously, to much greater than 400 μM. Others showed that the C10A-pVIc mutant binds to AVP almost as well as does wild-type pVIc (*23*). However, analysis of the titration curve implies the K_d for the binding of the mutant pVIc to AVP is less than 2 μM, a value we obtained with the C10A-pVIc mutant but only in the presence of DNA (polyanion).

Although formation of a disulfide bond between Cys104 of AVP and Cys10 of pVIc was not required for stimulation of AVP activity by pVIc, it may be required to keep AVP permanently activated in the virion. Perhaps the role of pVIc in the activation of AVP is to form a strap between the two domains of AVP that optimally aligns the Cys−His dyad to a geometry which favors the maintenance of the ion pair. Given the relatively low concentration of pVIc in the virion, reversible binding of pVIc to AVP might not allow the enzyme to be sufficiently activated so that it can cleave all the virion precursor proteins. One way to ensure sufficient activation is the formation of a disulfide bond. In the virus particle, AVP has formed a disulfide bond with pVIc (W. J. McGrath and W. F. Mangel, unpublished observation).

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