Effect of Residue 65 Substitutions on Thermal Stability of Tissue Plasminogen Activator Kringle-2 Domain

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ABSTRACT: We have used differential scanning calorimetry to measure the effect of replacements of valine 65 on thermal stability of the isolated kringle-2 domain of tissue plasminogen activator (t-PA). The role of this site in stability was examined because a human t-PA variant having this valine (residue 245 in t-PA numbering) replaced with a methionine has been described [Johnston, M. D., & Berger, H. (1987) U.K. Patent Application GB 2176702A]. Mutants of kringle-2 having valine 65 replaced with Met, Leu, Ile, Thr, Ala, or Ser were constructed by using site-directed mutagenesis in conjunction with a restricted site selection strategy. Isolated kringle-2 domains were expressed in *Escherichia coli* and purified as previously described for the wild-type domain [Cleary, S., Mulkerrin, M. G., & Kelley, R. F. (1989) *Biochemistry 28*, 1884–1891]. None of these substitutions results in a significant perturbation of the native conformation of kringle-2 as judged by far-UV circular dichroism and equilibrium dialysis measurements of L-lysine affinity. A two-state analysis of the heat capacity profile observed for heating a solution of wild-type (w-t) kringle-2 containing 100 mM citrate, pH 4.5, provides values of 64.3 ± 0.8 °C for T_g (melting temperature), 81 ± 5 kcal/mol for ΔH_g, and 1.2 ± 0.9 kcal/(mol·deg) for ΔC_p. Thermal denaturation of w-t kringle-2 is reversible in the pH range 3–6 as indicated by the observation of similar heat capacity profiles for consecutive heating cycles and also recovery of spectroscopic and lysine binding properties upon cooling the heat-denatured protein. The melting temperature increases with pH from a value of 43 °C at pH 3 to a limiting value of 75 °C at pH 6. ΔH_g is linearly dependent on T_g, yielding a ΔC_p of 0.9 kcal/(mol·deg). A stability profile calculated for w-t kringle-2 at pH 4.5 suggests that this domain has maximum stability at −8 °C with a corresponding ΔG of 9 kcal/mol. A two-state analysis of the pH 4.5 melting curve observed for the kringle-2 mutant having valine 65 replaced with methionine (V65M) indicates a T_g of 54.4 °C and ΔH_g of 67.8 kcal/mol. Thermal denaturation of this mutant domain is also reversible and displays a variation of melting temperature with pH similar to that observed for w-t kringle-2. ΔH_g is also linearly dependent on T_g, providing a ΔC_p value of 1.1 kcal/mol. By use of a mean value of 1.0 kcal/(mol·deg) for ΔC_p, the V65M substitution is calculated to lower the ΔG for folding of kringle-2 by 2.2 kcal/mol. Replacement of valine 65 with Leu, Ala, or Ser has a similar effect on melting temperature at pH 4.5 as the V65M mutation. In contrast, replacement with Ile has no effect on T_g, and substitution with Thr results in a 4.3 °C decrease in T_g. These results suggest that a β-branched residue at position 65 is required for stability of the kringle fold. The β-branched residue may contribute to stability either through hydrophobic interactions with the Cγ methyl or by restricting the backbone conformation in either the native or denatured state.

Kringles are small (ca. 80 residues) protein domains which have a characteristic three-disulfide bond pattern. Kringle domains were first observed in prothrombin, and on the basis of sequence homology they were observed to occur singly or multiply in a variety of other proteins including urokinase (Steffens et al., 1982), factor XII (McMullen & Fujikawa, 1985), apolipoprotein *a* (Eaton et al., 1987), plasminogen (Sottrup-Jensen et al., 1978), and tissue plasminogen activator (t-PA)[1] (Pennica et al., 1983). Kringles appear to function in protein–protein interactions as exemplified by the role of some of the t-PA and plasminogen kringles in binding of these proteins to fibrin (Thorsen, 1975; Thorsen et al., 1981; van Zonneveld et al., 1986; Verheihen et al., 1986).

Thermal denaturation experiments with plasminogen and proteolytic fragments of plasminogen have suggested that kringle domains are independent folding units (Castellino et al., 1981; Novokhathy et al., 1984). Trexler and Patthy (1983) have shown that the reduced form of the isolated kringle-4 domain of plasminogen can be oxidatively refolded in vitro. We have previously shown that the 174–263 portion of t-PA, a fragment consisting of the kringle-2 domain and a few flanking residues, can be expressed in functional form in *Escherichia coli* by secretion into the periplasmic space (Cleary et al., 1989). In this report, we describe results of experiments on the thermal stability of the t-PA kringle-2 domain produced in *E. coli*. We have also examined the effect of single amino acid substitutions of valine 65[2] on the thermal stability of kringle-2. We became interested in the role of residue 65 in the folding of kringles because a variant human t-PA having a methionine in this position has been described (Johnston & Berger, 1987). This observation is surprising since all other kringle sequences have either valine or threonine in this position [cf. Tulinsky et al. (1988)]. Residue 65 is positioned at the end of a peptide segment that is strongly conserved between kringles and forms one strand of a central β-sheet in the prothrombin fragment 1 kringle (Park & Tulinsky, 1986). These features suggested that residue 65 has an important role

[1] Abbreviations: t-PA, tissue plasminogen activator; DSC, differential scanning calorimetry. Mutants are designated in shorthand notation where the wild-type residue is specified in one-letter code followed by the sequence position and the one-letter code for the residue in the mutant. For example, V65I is a mutant with the wild-type residue at 65, a valine, replaced with isoleucine.

[2] We use a numbering system based on alignment of kringle sequences to plasminogen kringle-5 as suggested by Tulinsky et al. (1988). Residue 65 in kringle-2 is valine 245 in the t-PA numbering system.

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in the folding of t-PA kringle-2, and we expected the methionine substitution to result in decreased thermal stability. Differential scanning calorimetry experiments indicate that the methionine substitution results in a 2 kcal/mol decrease in the free energy (ΔG) for folding of kringle-2. In addition, results of thermal denaturation experiments with kringle-2 mutants having valine 65 replaced with either Ala, Leu, Ser, Ile, or Thr suggest that β-branching of residue 65 is required for optimal stability of the t-PA kringle-2 domain.

Materials and Methods

DNA Manipulations. Plasmid DNA was isolated according to the procedure of Birnboim and Doly (1979). DNA fragments produced by restriction enzyme cleavage were separated by electrophoresis in 1.0% low melting point agarose gels and recovered as described by Weislander (1979). Olgionucleotide-directed mutagenesis was performed as described by Smith and Gillam (1981). DNA sequences were determined by using the dideoxynucleotide chain termination method (Sanger et al., 1977).

Kringle-2 Mutagenesis. Plasmid pSB-5 (Cleary et al., 1989) designed for secretion of wild-type kringle-2 in *E. coli* by using the alkaline phosphatase promoter and stII enterotoxin signal sequence was used for mutagenesis. This plasmid was replicated in single-stranded form for use as the template in the first mutagenesis step. A restriction site selection strategy was used to produce a set of mutants involving replacement of valine 65. First, valine 65 was replaced with threonine by using the oligonucleotide $^{5'}$ATGCCAAGCCATGGTGCCAC-ACGCTGAAGA$^{3'}$, which also creates a unique *Nco*I restriction enzyme cleavage site. (The codon for residue 65 is shown in boldface type, and the *Nco*I recognition sequence is underlined). *E. coli* JM101 was transformed to ampicillin resistance with the mutagenesis reaction mixture, and double-stranded DNA was isolated. The mutant population was enriched by cleaving the DNA with *Nco*I, and singly cut plasmid was isolated by agarose gel electrophoresis, religated, and used to transform JM101. Single-stranded DNA was prepared from a few colonies and a mutant having the *Nco*I site, and the Thr 65 codon was identified by sequencing the kringle-2 coding segment of the plasmid. This template (designated V65T-1) was then used for a second round of mutagenesis in which the threonine codon was changed to a codon for either methionine, leucine, isoleucine, serine, or alanine with selection for loss of the *Nco*I restriction site. The following oligonucleotides were used to direct these changes:

Met 65	$^{5'}$GGATGCCAAGCCCTGGTGCCAC**ATG**CTGAAGAAC$^{3'}$
Ser 65	$^{5'}$GGATGCCAAGCCCTGGTGCCAC**TCG**CTGAAGAAC$^{3'}$
Ile 65	$^{5'}$GGATGCCAAGCCCTGGTGCCAC**ATC**CTGAAGAAC$^{3'}$
Leu 65	$^{5'}$GGATGCCAAGCCCTGGTGCCAC**CTG**CTGAAGAAC$^{3'}$
Ala 65	$^{5'}$GGATGCCAAGCCCTGGTGCCAC**GCC**CTGAAGAAC$^{3'}$

The products of in vitro replication were digested with *Nco*I prior to transformation of JM101. Plasmid DNA was isolated and cleaved with *Nco*I, and JM101 was transformed and plated on agar plates containing ampicillin. Single-stranded DNA was prepared from individual colonies, and positive mutants having no base changes other than those targeted by the oligonucleotide were identified by DNA sequencing of the kringle-2 coding segment.

Purification of Kringle-2 Mutants. For expression of kringle-2, the mutant plasmids were transformed into *E. coli* strain 16C9. ELISA assay (Cleary et al., 1989) of crude extracts of the transformed cells indicated that all of the mutant proteins except for V65T were expressed in *E. coli* at levels comparable to that observed for the wild-type protein. *E. coli* transformed with the V65T-1 plasmid did not express kringle-2. However, 16C9 transformed with a plasmid having the *Nco*I site removed but retaining the Thr 65 codon (V65T-2) did express high levels of kringle-2. The mutant proteins were purified from *E. coli* cell paste as described previously for the wild-type domain (Cleary et al., 1989). All of the purified mutant proteins appeared homogeneous by SDS–PAGE and had amino acid compositions identical with that of the wild-type protein except for the changes due to the amino acid replacement (data not shown). Protein concentrations were determined by absorbance measurements using the extinction coefficient (ϵ_{280} = 28 mM^{-1} cm^{-1}) determined for the wild-type protein (Cleary et al., 1989).

Circular Dichroic Spectroscopy. Circular dichroic spectra were recorded on an Aviv/Cary spectropolarimeter using quartz cylindrical cells of 1-cm path length. The temperature of the solution inside the cuvette was maintained at 25 °C by a circulating water bath. A time constant of 1.0 s, spectral bandwidth of 1.0 nm, and wavelength interval of 0.5 nm were used.

Equilibrium Dialysis Measurements of L-Lysine Binding. Equilibrium dialysis was performed as described previously (Cleary et al., 1989) except that only a single concentration of L-lysine was used in determining the binding constant rather than determining a complete binding curve. A stock solution of 200 μM L-lysine containing 0.2 μCi/mL [^{14}C]-L-lysine, 50 mM Tris–HOAc, pH 8, and 50 mM sodium acetate was prepared and added to one side of the dialysis cell (cells having equal volumes for both compartments were used). To the other side of the cell was added a solution of mutant or wild-type protein (ca. 80–100 μM) which had been dialyzed against the same buffer. After overnight equilibration at ambient temperature, the free (S_f) and bound (S_b) L-lysine concentration was determined by scintillation counting and the total protein concentration (P_t) was determined by absorbance measurements. The dissociation constant was then calculated by using the following equation, which assumes one binding site for ligand:

$$K_D = [(P_t - S_b)S_f]/S_b$$

Differential Scanning Calorimetry. Protein solutions were prepared for thermal denaturation experiments by extensive dialysis at ambient temperature. For pH values in the range of 3–6, a buffer prepared by mixing varied ratios of 0.1 M citric acid and 0.1 M trisodium citrate was used. A buffer of 100 mM sodium phosphate was used for pH values of 7.2 and 7.4. All thermal denaturation experiments were performed on a Microcal (Northhampton, MA) MC-2 scanning calorimeter. About 2 mL of protein solution was loaded into the sample cell of the calorimeter, and an equal volume of the dialysis solution was loaded into the reference cell. The cell compartment was sealed and pressurized to 25 psi with nitrogen. At least 1 h was allowed for temperature equilibration prior to initiating a heating cycle. Unless noted otherwise, all thermal denaturation experiments were performed by using a heating rate of 1 °C/min. Heat capacity profiles were analyzed as a two-state process by using a computer program supplied by Microcal based on a nonlinear least-squares algorithm.

Results

Thermal Denaturation of Wild-Type t-PA Kringle-2. A typical excess heat capacity profile observed upon heating a

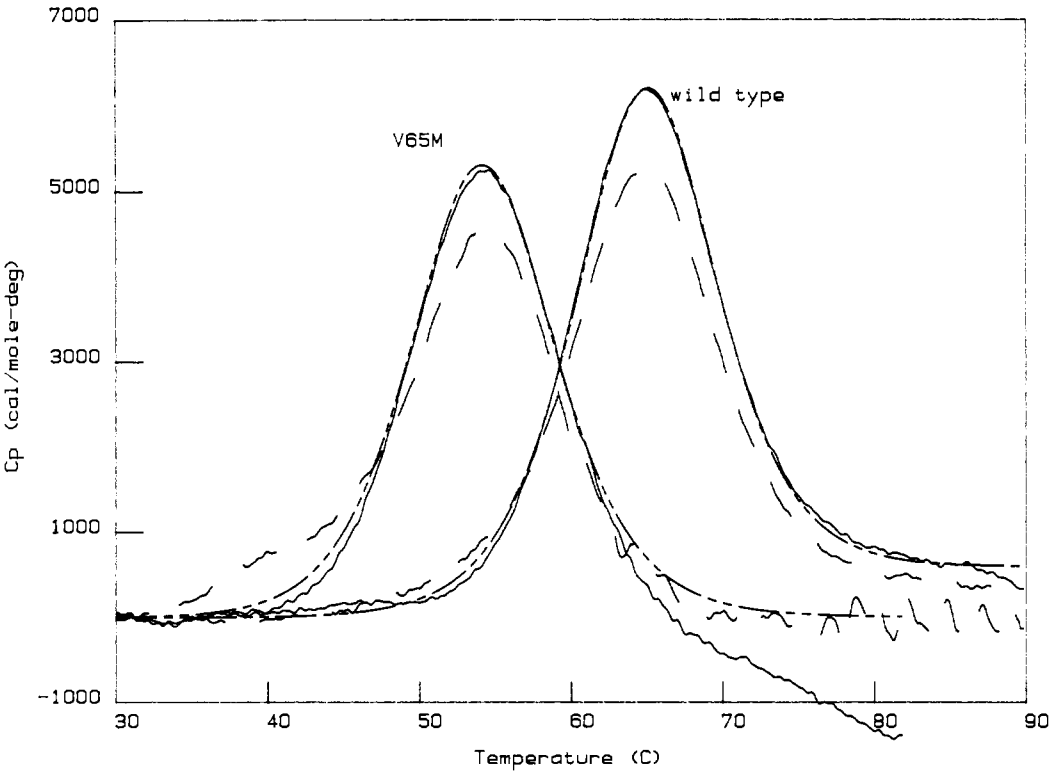

FIGURE 1: Differential scanning calorimetry of t-PA kringle-2 domain. Heat capacity profiles observed for heating solutions of wild-type or V65M kringle-2 containing 100 mM citrate, pH 4.5, are shown as the solid lines. (The protein concentration for these experiments was about 1.5 mg/mL.) Second melts of the same sample are represented by the dashed lines. These curves were generated from the raw data by first subtracting the heat capacity of the native protein and then normalizing for the moles of protein in the calorimeter cell. The native protein heat capacity was determined by least-squares analysis of the data collected below the transition zone. Curves calculated from a two-state analysis of the first melting profile observed for each protein are shown as the double-dashed lines.

solution of native wild-type t-PA kringle-2 domain containing 100 mM citrate, pH 4.5, is shown in Figure 1. An endothermic transition having a maximum at about 65 °C is observed for thermal denaturation of kringle-2. This transition was analyzed as a two-state unfolding process

$$N \rightleftharpoons D$$

by using a nonlinear least squares algorithm in which T_g, ΔC_p, and ΔH_g are variable parameters.[3] In this procedure ΔC_p, the change in heat capacity accompanying denaturation, is determined as the difference in the base-line heat capacities of native and denatured protein extrapolated to T_g (see Figure 1). ΔH_g was determined from the area of the heat capacity transition and was constrained to be equal to the van't Hoff enthalpy during the fitting process. This analysis yields values of 65.1 °C for T_g, 0.6 kcal/(mol·deg) for ΔC_p, and 75.0 kcal/mol for ΔH_g. As shown by the good overlap between the observed and calculated profiles, this transition is precisely described by a two-state process. Thermal denaturation of kringle-2 at pH 4.5 is reversible as indicated by the observation of a similar excess heat capacity profile upon cooling and reheating the same protein solution. Deconvolution of the second melting curve shown in Figure 1 provides values of 64.9 °C for T_g 68.9 kcal/mol for ΔH_g, and 0.3 kcal/(mol·deg) for ΔC_p. A comparison of the ΔH_g values obtained for the two melting curves indicates that thermal denaturation at pH 4.5 is >90% reversible. The small decrease in ΔH_g for the second melt likely results from a small amount of aggregation of the heat-denatured protein. Indeed, the protein solution appeared slightly turbid upon removal from the calorimeter cell after the second heating cycle.

The error in measurement of the thermodynamic quantities was determined by repeating the thermal denaturation experiments using fresh solutions of native protein. Mean values of 64.3 ± 0.8 °C for T_g, 81 ± 5 kcal/mol for ΔH_g, and 1.2 ± 0.9 kcal/(mol·deg) for ΔC_p were determined from the first heating cycle on solutions of kringle-2 containing 100 mM citrate, pH 4.5. These mean values were calculated from experiments using protein concentrations between 0.5 and 3 mg/mL and heating rates in the range of 0.5–1.5 °C/min and also include measurements made on different lots of kringle-2. These results show that both T_g and ΔH_g for denaturation of kringle-2 can be precisely measured by using DSC and are relatively independent of both protein concentration and heating rate. There is considerable variation in the value determined for ΔC_p that we believe is the result of a slight tendency of the heat-denatured protein to aggregate. Aggregation of the denatured protein gives rise to an abnormal high temperature base line and thus error in calculation of ΔC_p since this parameter is determined from a single melting curve by extrapolation of this base line. This problem in determination of ΔC_p from a single melting profile has been observed for a variety of proteins (Privalov & Khechinashvili, 1974).

Reversibility of Thermal Denaturation. Reversibility of thermal denaturation was further examined by measuring the recovery of spectroscopic and lysine binding properties. These experiments were performed on a sample of wild-type protein that had been subjected to four consecutive heating–cooling cycles. This sample was centrifuged to remove some aggregated material and is referred to as "renatured" kringle-2.

[3] We use the convention of Becktel and Schellman (1987), where the subscript "g" denotes the melting temperature; at this temperature ΔG for folding is zero. The subscript "s" is used to denote the temperature where ΔS is zero, and thus ΔG has its maximum value.

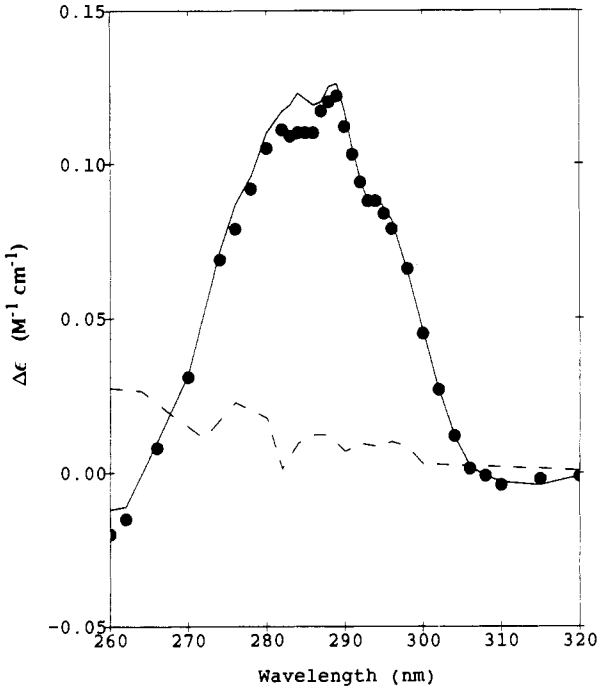

FIGURE 2: Near-ultraviolet CD spectra of wild-type kringle-2. The spectrum of the native protein is shown as the solid line, the spectrum of the renatured protein is represented by the filled circles, and the spectrum observed for reduced and alkylated kringle-2 is given by the dashed line. All spectra were recorded at 25 °C by using a 1-cm path length cuvette and protein solutions containing 100 mM citrate, pH 4.5, and having an A_{280} in a 1-cm cell of 1.0. A mean residue molecular weight of 110 was used in the calculation of $\Delta\epsilon$, the difference in extinction coefficient for left and right circularly polarized light. Curves shown are the average of 10 spectra.

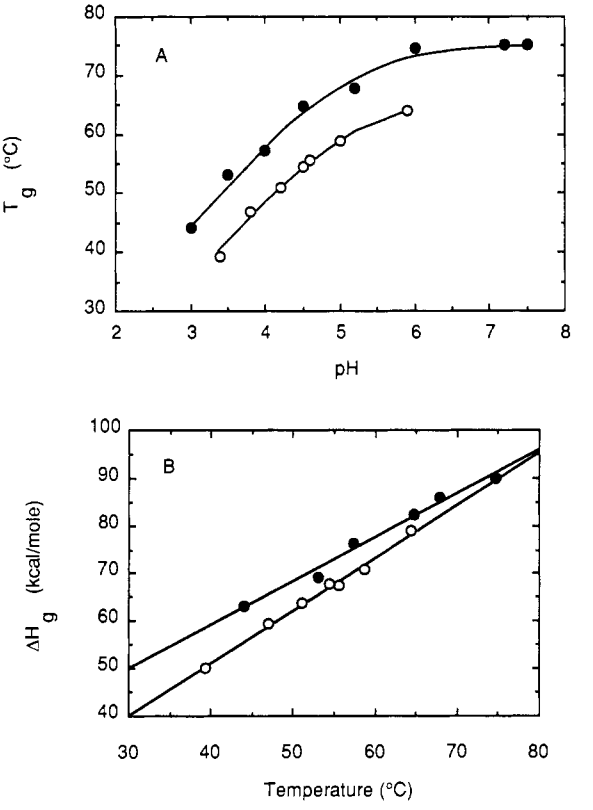

FIGURE 3: Dependence of kringle-2 stability on pH. Values determined for wild-type kringle-2 (●) and for V65M kringle-2 (○) are shown. (A) Dependence of T_g (melting temperature) on pH. For pH values between 3 and 6 protein solutions containing 100 mM citrate were used. Protein solutions containing 100 mM sodium phosphate were used for pH values 7.2 and 7.5. The protein concentration for all measurements was 1.5–2.0 mg/mL. (B) Dependence of ΔH_g on the observed melting temperature (T_g). The solid lines were obtained by least-squares analysis of the data giving a slope (ΔC_p) of 0.9 and 1.1 kcal/(mol·deg) for wild-type and V65M kringle-2, respectively.

Equilibrium dialysis experiments indicate that renatured kringle-2 has a K_D for binding L-lysine at pH 8 and ambient temperature of 150 ± 30 μM. This value compares favorably with a K_D of 100 μM previously reported for L-lysine binding to native kringle-2 (Cleary et al., 1989). Proper folding of the renatured protein is also demonstrated by near-ultraviolet circular dichroism measurements as shown in Figure 2. The near-UV CD spectrum of renatured kringle-2 is nearly superimposable with the spectrum of the native protein. In contrast, kringle-2 that had been reduced with DTT and in which the cysteines had subsequently been alkylated with iodoacetamide has a much diminished near-ultraviolet ellipticity. This result is consistent with the expectation that kringle-2 is not stably folded without disulfide bonds. However, the native form having disulfide bonds can be reversibly denatured.

pH Dependence of Wild-Type Kringle-2 Thermal Denaturation. The dependence of wild-type kringle-2 stability on pH was examined by performing thermal denaturation experiments using protein solutions having pH values in the range of 3–7.5. At all of the pH values used, thermal denaturation of kringle-2 is described by a single, two-state endothermic transition. The reversibility of thermal denaturation decreases as the pH is raised and is essentially irreversible in the pH range 7–7.5. (Thermal denaturation at pH values greater than 7.5 was not examined.) The decrease in reversibility coincided with increased aggregation of the heat-denatured protein. As shown in Figure 3A, T_g increases monotonically with pH to reach a limiting value of 75 °C at pH 6. Analysis of the pH dependence of T_g using eq 13 of Privalov (1979) suggests that the change in protonation accompanying denaturation, $\Delta\nu$, decreases from about 2.5 at pH 3 to 1.5 at pH 6. The magnitude of $\Delta\nu$ probably corresponds to titration of histidine residues that are not accessible in the native protein. Proton NMR measurements indicate that one of the three histidines of native kringle-2 titrates with a pK_a of 6, another histidine begins to titrate to pH 5, and the third histidine does not begin to titrate until pH 4 (R. Kelley, I.-J. Byeon, and M. Llinás, unpublished results).

ΔH_g was determined for pH values where reversibility was >80% (pH ≤ 6) and is plotted against the corresponding T_g value in Figure 3B. As observed for denaturation of a variety of globular proteins (Privalov, 1979), ΔH_g displays a linear dependence on temperature. The slope of this dependence gives a value of 0.9 kcal/(mol·deg) for the ΔC_p associated with unfolding of wild-type kringle-2 domain. The ΔH values shown in Figure 3B are equal to the sum of heat effects from the conformational transition (ΔH_{conf}) and from ionization (H_{ion}) of buffer and the aforementioned histidine residues. Since the ΔH_{ion} for citrate is small, and ΔH_{ion} for both protein and buffer are independent of temperature (Izatt & Christensen, 1976), then ΔC_p determined as described above reflects the conformational transition only and does not include ionization effects. Nonetheless, there is a small uncertainty in this determination of ΔC_p due to variation in $\Delta\nu$ between pH 3 and 6.

Thermal Denaturation of V65M Kringle-2. A typical excess heat capacity profile observed for a kringle-2 mutant having valine 65 replaced with methionine is shown in Figure 1. This transition is similar to that observed for the wild-type protein except for a decrease in T_g and decreased fidelity of the high-temperature base line. An abnormal high-temperature

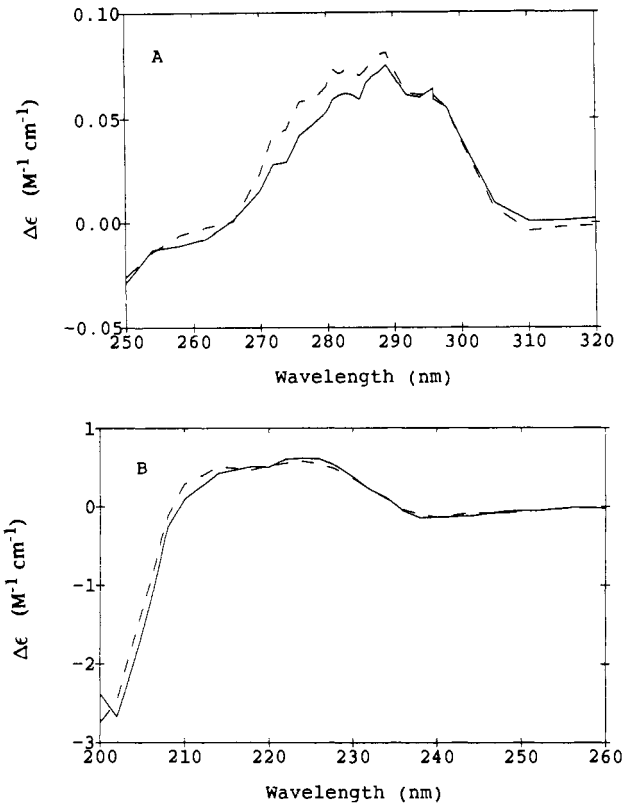

FIGURE 4: Comparison of CD spectra observed for V65M and wild-type kringle-2. Spectra were recorded on protein solutions containing 50 mM sodium phosphate, pH 7.0, by using a protein concentration of 36 μM for near-ultraviolet measurements (A) or 3.6 μM for measurements in the far-ultraviolet region (B). Spectra observed for wild-type (solid lines) and V65M (dashed lines) kringle-2 are shown. Each curve is the average of 10 spectra.

Table I: Lysine Affinity of Kringle-2 Mutants[a]

protein	K_D (μM)	protein	K_D (μM)
w-t	108 ± 33	V65A	114 ± 35
V65I	121 ± 28	V65M	158 ± 55
V65T	83 ± 25	V65S	75 ± 14
V65L	110 ± 19		

[a] Dissociation constant for L-lysine binding was determined by equilibrium dialysis at ambient temperature and pH 8. Values given are the mean of five or more determinations using an initial lysine concentration of 200 μM. The K_D was calculated by assuming a single binding site for lysine.

base line was observed for V65M kringle-2 in this particular experiment, probably because of aggregation of the heat-denatured protein, and thus ΔC_p was constrained to zero during deconvolution. A two-state analysis of the first melting curve provides values of 54.4 °C for T_g and 67.8 kcal/mol for ΔH_g. As observed for the wild-type protein, a second melting curve is similar to the first, indicating that thermal denaturation of V65M kringle-2 at pH 4.5 is reversible. These data show that replacement of valine 65 with methionine results in a large decrease in stability of kringle-2. This destabilization does not appear to result from a large perturbation of the native structure of kringle-2. The circular dichroic spectra of wild-type and V65M kringle-2 are nearly superimposable as shown in Figure 4. Replacement of valine 65 with methionine does not affect the affinity of the protein for L-lysine as shown by comparing the K_D values given in Table I.

The pH dependence of the melting temperature determined for V65M kringle-2 is compared with that of the wild-type protein in Figure 3A. Throughout this pH range, the variation

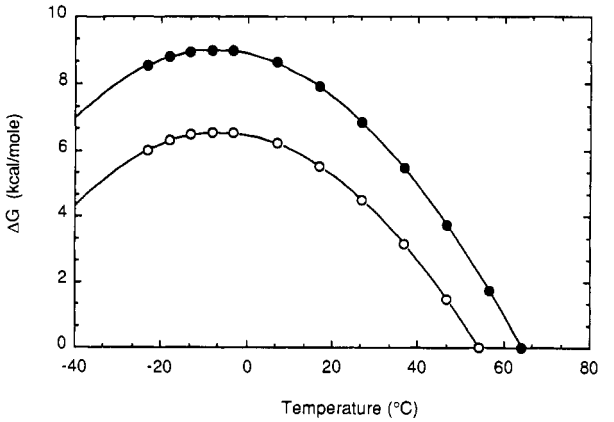

FIGURE 5: Temperature dependence of ΔG for folding of kringle-2. Stability profiles (Becktel & Schellman, 1987) for wild-type (●) and V65M (○) kringle-2 at pH 4.5 were calculated by using the data given in Table II and a ΔC_p of 1.0 kcal/(mol·deg).

in melting temperature with pH is equivalent for the two proteins and the T_g for V65M kringle-2 is always about 10 °C less than that measured for the wild-type protein. As shown in Figure 3B, the ΔH_g values determined for V65M kringle-2 also display a linear dependence on temperature with a value of 1.1 kcal/(mol·deg) determined for ΔC_p from the slope of this dependence. The difference in ΔC_p value between wild-type and V65M kringle-2, about 0.2 kcal/(mol·deg), may reflect a real difference between the two proteins in the relative change in exposure of hydrophobic residues accompanying denaturation. However, since the difference in ΔC_p between the two proteins is only about 2-fold larger than the error (ca. 10%) in determination of this parameter, we have chosen to assume that all the kringle-2 variants described here have the same ΔC_p for thermal denaturation [1.0 kcal/(mol·deg)].

Stability Profiles of Wild-Type and V65M Kringle-2. By use of a ΔC_p of 1.0 kcal/(mol·deg), the stability profiles (Becktel & Schellman, 1987) shown in Figure 5 were calculated for wild-type and V65M kringle-2 at pH 4.5. The temperature of maximum stability (T_s) for both proteins is about −8 °C. From the temperature dependence of ΔG shown in Figure 5, wild-type kringle-2 is predicted to undergo cold denaturation (Brandts, 1964; Privalov et al., 1986) at about −80 °C. The native conformation of the wild-type protein is stabilized by 9 kcal/mol free energy at T_s while the V65M mutant is considerably less stable, having a ΔG_s value of 6.5 kcal/mol. Both ΔG_s and T_g are pH dependent while T_s is independent of pH. The ΔG_s value calculated for wild-type kringle-2 increases from 6 kcal/mol at pH 3 to 11 kcal/mol at pH 6.

Effect of Other Residue 65 Replacements on Thermal Stability. In order to determine the nature of the destabilizing effect of the V65M mutation, we prepared a series of mutant proteins having Val 65 replaced with either Ala, Leu, Ile, Thr, or Ser. As shown in Table I, none of these mutations had a significant effect on the Lysine affinity of kringle-2. All of these mutant proteins except for V65S, which had a slightly reduced ellipticity in the near-ultraviolet region, gave CD spectra that were identical with the spectrum of the wild-type protein (data not shown). The effect of these mutations on stability was determined by performing DSC experiments on protein solutions containing 0.1 M citrate, pH 4.5. The protein concentration for all experiments, excluding studies of V65I, was 1.5–2.0 mg/mL. V65I has reduced solubility at pH 4.5, and thus a 0.5 mg/mL protein solution was used for calorimetry. Thermal denaturation of all the mutant proteins is described by a single, two-state transition. Thermal denatu-

Table II: Thermodynamic Parameters for Kringle Mutants[a]

protein	T_g (°C)	ΔH_g (kcal/mol)	ΔS_g [kcal/(mol·deg)]	$\Delta\Delta G$ (kcal/mol)[b]
w-t	64.3 ± 0.9	81 ± 6	0.240	0
V65I	64.8	67.5	0.200	+0.3
V65T	60.0	75.1	0.225	−1.0
V65L	55.8	70.1	0.213	−1.9
V65A	55.5	72.5	0.221	−2.0
V65M	54.4	67.8	0.207	−2.2
V65S	52.9	63.4	0.194	−2.4

[a] Results are from DSC experiments on protein solutions containing 0.1 M citrate, pH 4.5. [b] $\Delta\Delta G$ is the difference in stabilization free energy between wild-type and mutant protein calculated by extrapolating the stability profile for wild-type kringle-2 shown in Figure 5 to the melting temperature of the mutant protein. At this temperature the ΔG for folding of the mutant protein is zero.

ration of these variants is also reversible on the basis of a comparison of ΔH_g values calculated from consecutive heating cycles. Thermodynamic values obtained from a two-state analysis of these melting curves are given in Table II. The last column of this table shows the difference in stabilization free energy between the mutant and wild-type protein calculated at the melting temperature of the mutant protein. As observed for the wild-type protein, there is considerable variation in the ΔC_p values calculated from consecutive heating cycles while the variation in T_g is less than 0.5 °C (data not shown). As shown in Table II, replacement of valine 65 with Ala, Leu, or Ser has a similar destabilizing effect to that observed for the methionine substitution with V65S having the lowest T_g. Replacement with Thr results in a smaller destabilization while the Ile substitution has no effect on stability. The ΔH_g values measured for all the mutant proteins except for V65I are equivalent, within the experimental error, to the ΔH value calculated for the wild-type protein at the T_g of the mutant by using the temperature dependence of ΔH shown in Figure 3B. The unexpectedly low value of ΔH_g for V65I is probably the result of a larger error due to the lower protein concentration used for melting experiments on this variant. The heat-denatured form of V65I protein seemed to have a greater tendency toward aggregation, which would also lead to an underestimation of ΔH_g.

Discussion

Two studies of thermal denaturation of the kringle-4 domain of plasminogen, prepared by elastase digestion of human plasminogen, have been reported (Castellino et al., 1981; Novokhathy et al., 1984). Plasminogen kringle-4 and t-PA kringle-2 display similar thermal denaturation behavior as might be expected for homologous proteins. Both proteins undergo two-state, reversible denaturation, with t-PA kringle-2 having a slightly higher melting temperature than plasminogen kringle-4 at the same pH. Binding of the lysine analogue ϵ-aminocaproic acid to plasminogen kringle-4 results in an increase in the melting temperature of this domain. We have observed a similar effect for t-PA kringle-2 (unpublished results). The melting temperature and ΔH observed for the kringle-4 domain in intact plasminogen are conserved in the isolated domain, indicating that plasminogen kringle-4 is an independent folding unit. We suspect that this independence is also true for t-PA kringle-2; however, the low solubility of t-PA has prevented us from making reliable thermal denaturation measurements on the intact protein. Both kringles have a ΔC_p for denaturation of about 1 kcal/(mol·deg). The magnitude of ΔC_p is related to the change in exposure of hydrophobic groups accompanying denaturation (Kauzmann, 1959) and typically has a value between 1 and 2 kcal/(mol·deg) for globular proteins (Privalov & Khechinashvili, 1974). The ΔC_p measured for kringle denaturation is at the low end of this range but is consistent with the disulfide-bonded structure of the domain. For example, RNase A with four disulfide bonds has a similar ΔC_p value whereas metmyoglobin, which lacks disulfide bonds, has a ΔC_p of 2.0 kcal/(mol·deg) (Privalov et al., 1986). Disulfide bonds restrict the degrees of freedom available to the unfolded polypeptide, resulting in an apparent decrease in side-chain exposure relative to that expected for a random coil.

Park and Tulinsky (1986) have determined the structure of the kringle domain of prothrombin fragment 1 based on X-ray crystallographic measurements. The prothrombin kringle has a unique structure in that two of the disulfides stack at the center of the molecule and the rest of the polypeptide chain loops out from this central array. This kringle has no α-helix, a small amount of β-sheet, and a high fraction of residues in turns. Given the high sequence homology among kringle domains [cf. Tulinsky et al. (1988)], it is expected that the structure of t-PA kringle-2 is very similar to that determined for the prothrombin kringle. Despite this atypical structure, DSC studies indicate that the isolated kringle-2 domain of t-PA is a relatively heat stable protein having a melting temperature at neutral pH of 75 °C. Thermal denaturation at pH values less than 6 is described by a two-state, reversible process. As shown in Figure 5, the native conformation of t-PA kringle-2 has optimal stability at about −8 °C. The ΔG calculated for folding of the wild-type protein at this temperature varies from 6 kcal/mol at pH 3 to 11 kcal/mol at pH 6. The magnitude of ΔG is within the range observed for other globular proteins (Privalov, 1979). Thus, although kringles are somewhat lacking in the usual secondary structure elements, the folded protein is as stable as any other small protein. Of course, a large portion of the stabilization free energy of kringle-2 is due to the disulfide bonds since the fully reduced protein appears to be an unfolded molecule.

A proper comparison of the stabilities of a series of mutant proteins requires knowledge of the temperature dependence of ΔG for unfolding. For example, a decrease in melting temperature does not necessarily indicate decreased stability at temperatures below T_g since a mutation could affect the shape of the stability profile (Becktel & Schellman, 1987). The slope and maximum of the stability profiles shown in Figure 5 are dependent on ΔC_p, and thus it is important to accurately determine this parameter when ascertaining the effect of a mutation on stability. Although we have not precisely determined ΔC_p for all the mutant proteins described here, our results with V65M suggest that substitutions of residue 65 have only a small effect if any on the magnitude of ΔC_p. We conclude that the $\Delta\Delta G$ values shown in Table II calculated by using a value for ΔC_p of 1.0 kcal/(mol·deg) provide an accurate measure of the effect of the mutation on stability.

Residue 65 is in a segment that is strongly conserved between kringles and forms part of a β-sheet structure in the prothrombin fragment 1 kringle (Park & Tulinsky, 1986). This residue is a threonine in the prothrombin kringle and is situated on the surface of the molecule (A. Tulinsky, personal communication). The amide nitrogen of Thr 65 and the carbonyl oxygen of arginine 71 form one hydrogen bond of the β-sheet. In the structures modeled for the lysine binding sites of the plasminogen and t-PA kringles (Tulinsky et al., 1988) residue 65 is near the lysine binding site but the side chain points away from the binding pocket. This feature is

consistent with our observation that substitution of valine 65 has no effect on lysine affinity of t-PA kringle-2. Both the Oγ1 hydroxyl and Cγ2 methyl of the Thr 65 side chain appear to be at least partially accessible to solvent, and the hydroxyl does not appear to hydrogen bond with any protein partner. Modeling of side chains at this position having a δ substituent, such as Met or Ile, suggests that the longer side chain would protrude out into the solvent (A. Tulinsky, personal communication). This would be consistent with the low solubility observed for V65I kringle-2. The Thr 65 Cγ2 methyl of the prothrombin kringle seems to be positioned in a hydrophobic pocket formed by the side chains of Ile 20, Ser 67, and Leu 70. The Cγ2 methyl is in van der Waals contact with several atoms of the side chain of Ile 20. Ile 20 and Ser 67 are replaced by Ala and Asn, respectively, in t-PA kringle-2.

Our results suggest that a β-branched residue at position 65 is required for optimal stability of t-PA kringle-2 and that interactions with the Cγ2 methyl group are the primary determinant of this effect. Removal of the Cγ2 methyl results in a 1.5–2 kcal/mol decrease in the ΔG of folding as shown by comparing the effects of the Ile–Leu and Thr–Ser set of mutations (see Table II). Introduction of a hydroxyl is further destabilizing as shown by comparing the Val–Thr and Ala–Ser set of mutants. These two destabilizing effects are not quite additive as shown by comparing the $\Delta\Delta G$ values determined for the Thr, Ser, and Ala substitutions. However, we should caution that a rigorous structural interpretation of the thermodynamic data is not possible given the differences in sequence between t-PA kringle-2 and prothrombin kringle-1 and also because we do not know the effect of the substitution on the unfolded protein. For example, a β-branched residue could stabilize a protein by restricting the conformational freedom of the unfolded state, thus decreasing the entropic contribution favoring unfolding. Nonetheless, we can exclude explanations for destabilization based on changes in secondary structure. None of the substitutions results in a significant change in the far-ultraviolet CD spectrum, and the destabilization does not correlate with the propensities of the amino acids to be found in β-sheet structure (Chou & Fasman, 1974). Methionine has an equal propensity to valine and isoleucine for β-sheet formation, and yet the V65M mutant is less stable than wild type. The destabilization of kringle-2 by replacement of valine 65 does not appear to be related to the size of the side chain introduced since the alanine and leucine mutants have equivalent stability. The destabilization does not correlate with hydrophobicity of the side chain because methionine and leucine, which both have ΔG values for transfer from water to ethanol (Nozaki & Tanford, 1971) similar to valine, destabilize kringle-2 when substituted for valine 65.

We have shown that replacement of valine 65 with a non-β-branched residue results in a large decrease in the stability of the isolated kringle-2 domain. At present it is unclear what effect these destabilizing mutations might have when incorporated in t-PA since the domains of t-PA probably fold independently. We suspect that these mutations might increase the susceptibility of the kringle domain toward proteolytic cleavage. Thus, it might be expected that the t-PA variant described by Johnston and Berger (1987) having a methionine at 245 is more prone to irreversible inactivation via proteolytic cleavage than the t-PA with valine at 245 (Pennica et al., 1983).

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Registry No. t-PA, 105913-11-9; Met, 63-68-3; Leu, 61-90-5; Ile, 73-32-5; Thr, 72-19-5; Ala, 56-41-7; Ser, 56-45-1; Val, 72-18-4.

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Poly(ADP-ribosylation) of Histones in Intact Human Keratinocytes†

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ABSTRACT: The poly(ADP-ribosylation) of chromosomal proteins is an epigenetic consequence of clastogenic DNA damaging agents which affects chromatin structure and function. We studied the poly(ADP-ribosylation) of the major classes of histones in response to DNA breakage induced by an extracellular burst of active oxygen (AO) or the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the immortalized human keratinocytes HaCaT using a combination of affinity chromatography on phenylboronate resin and immunoblotting with polyclonal antibodies against histones H1, H2B, H2A, H3, and H4. The following findings characterized the poly(ADPR) reaction: (1) pretreatment of nuclear extracts with snake venom phosphodiesterase which removes poly(ADPR) chains strongly reduced the material which was retained by phenylboronate; (2) the ADPR transferase inhibitor benzamide (100 μM) suppressed AO-induced poly(ADP-ribosylation); (3) poly(ADP-ribosylation) reduced the electrophoretic mobility of the modified histones. Several histones were constitutively poly(ADP-ribosylated) in untreated controls: 0.03% of H2A, 0.04–0.06% of H2B, and 0.04% of H3.1 carried at least one poly(ADPR) chain of undetermined length. AO transiently increased the poly(ADPR) levels of all major histones with the exception of H1. The extent of substitution 30 min after exposure to AO generated by 50 μg/mL xanthine and 5 μg/mL xanthine oxidase was 0.8% for A24 > 0.3% for H4 > 0.1% for H3.1 = 0.1% for H3.2 = 0.1% for H2B.2 > 0.09% for H2A. Within 60 min, poly(ADPR) substitution had decreased to control levels for H3 and H4 and below control levels for H2A and H2B. The addition of benzamide (100 μM) prevented AO-induced poly(ADP-ribosylation) as well as the drop in cellular NAD levels (at 30 min). MNNG was more effective than AO in inducing histone poly(ADP-ribosylation), and to judge from the significant decreases in electrophoretic mobility, the lengths of the ADPR chains were longer. In increasing order, the extents of substitution 20 min after treatment with 5 μg/mL MNNG were 1.8% for H3 > 1.2% for H1 > 0.95% for H2B > 0.36% for H4. In addition, the variant form H2B.x and unidentified low-mobility H2B- and H4-related antigens were substantially poly(ADP-ribosylated). Our results indicate that AO and MNNG induce distinct patterns of histone poly(ADP-ribosylation). Consequently, different DNA strand breaking agents are expected to affect chromatin structure and function in a characteristic fashion.

The posttranslational modification of chromosomal proteins represents a mechanism for the structural and functional modulation of chromatin. Modification by poly(ADP-ribosylation) has been postulated to play a role in DNA replication, DNA repair (Durkacz et al., 1981; Sims et al., 1982), DNA amplification (Bürkle et al., 1987), and cell differentiation (Farzaneh et al., 1982; Althaus et al., 1982). It is a metabolic consequence of clastogens because DNA containing breaks activates the biosynthetic enzyme adenosine diphosphoribose (ADPR)[1] transferase which polymerizes NAD residues to poly(ADPR) chains (Althaus et al., 1985; Althaus & Richter, 1987). The rapid turnover of poly(ADPR) chains renders this protein modification particularly suitable for regulatory purposes. Three classes of chromosomal proteins have been found to serve as poly(ADPR) acceptors in intact cells: (1) nuclear enzymes, e.g., ADPR transferase and topoisomerase I (Singh et al., 1985; Adamietz, 1987; Krupitza & Cerutti, 1989); (2) transcription regulatory proteins, e.g., the protein encoded by the c-fos protooncogene (Krupitza and Cerutti, unpublished results); (3) structural proteins, e.g., proteins associated with the nuclear matrix (Cardenas-Corona et al., 1987; Adolph & Song, 1985) and histones (Kreimeyer et al., 1984; Adamietz & Rudolph, 1984). Enzymes are usually inactivated as a consequence of poly(ADP-ribosylation) while conformational changes result from the poly(ADPR) substitution of structural proteins (Althaus et al., 1985; Althaus & Richter, 1987). For example, the poly(ADP-ribosylation) of histones appears to relax nucleosomal cores (Poirier et al., 1982).

In the present work, we have concentrated on constitutive and clastogen-induced histone poly(ADP-ribosylation) in the intact, immortalized human keratinocytes HaCaT (Boukamp et al., 1988). Active oxygen generated extracellularly by xanthine/xanthine oxidase (Kellogg & Fridovich, 1975) and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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[1] Abbreviations: ADPR, adenosine diphosphoribose; AO, active oxygen; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; poly(ADP-ribose), poly(adenosine diphosphoribose); SVPD, snake venom phosphodiesterase; X/XO, xanthine/xanthine oxidase; NAD, nicotinamide adenine dinucleotide, oxidized form; AUT-PAGE, acid-urea-Triton-polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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