Construction, Expression, and Purification of Recombinant Kringle 1 of Human Plasminogen and Analysis of Its Interaction with ω-Amino Acids†

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Received September 28, 1990; Revised Manuscript Received November 27, 1990

ABSTRACT: An *Escherichia coli* expression vector, containing the alkaline phosphatase promoter and the stII heat-stable enterotoxin signal sequence, along with the cDNA of the kringle 1 (K1) region of human plasminogen (HPg), has been employed to express into the periplasmic space amino acid residues 82–163 ($E^{163} \rightarrow D$) of HPg. This region of the molecule contains the entire K1 domain (residues C^{84}–C^{162}) of HPg, as well as two non-kringle amino-terminal amino acids (S^{82}-E^{83}) that are present in their normal locations in HPg and a carboxyl-terminal amino acid, D^{163}, that results from mutation of the E^{163}, normally present at this location in the HPg amino acid sequence. After purification of r-K1 by chromatographic techniques, we have investigated its ω-amino acid binding properties by titration calorimetry, intrinsic fluorescence, and differential scanning microcalorimetry (DSC). The antifibrinolytic agent, ε-aminocaproic acid (EACA), possesses a single binding site for r-K1. The thermodynamic properties of this interaction, studied by calorimetric titrations of the heats of binding with this ligand, reveal a K_d of 12 ± 2 μM at 25 °C and pH 7.4, a corresponding ΔG of −6.7 ± 0.1 kcal/mol, a ΔH of −3.6 ± 0.1 kcal/mol, and a ΔS of 10.5 ± 0.8 eu. The intrinsic fluorescence of r-K1 decreases by approximately 44% when its binding site is saturated with EACA, and titrations of this perturbation with EACA lead to calculation of a K_d of approximately 13 μM, a value in good agreement with that obtained from titration calorimetric analysis. EACA represents the strongest binding ligand of a variety of simple aliphatic ω-amino acids examined. A cyclic analogue of EACA, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid, interacts with r-K1 with an approximate 12-fold tighter K_d (1.0 ± 0.2 μM). Investigations by DSC, at pH 7.4, demonstrate that a significant stabilization of the r-K1 structure occurs when EACA binds to this domain. The temperature of maximum heat capacity change (T_m) in the thermal denaturation of r-K1 increases from approximately 340.8 to 359.1 K as a consequence of EACA binding. These studies demonstrate that a fully functional EACA-binding kringle from HPg can be expressed and secreted in *E. coli*, purified by techniques that do not require refolding, and investigated as an independent structural unit.

Human plasminogen (HPg)[1] exists in plasma as the zymogen form of the serine protease, HPm, the major fibrinolytic and fibrinogenolytic enzyme. HPg is a single-chain protein, containing 791 amino acids in known sequence (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978; Malinowski et al., 1984; Forsgren et al., 1987), one site of N-linked glycosylation, present at Asn^{289}, and an additional O-linked oligosaccharide at Thr^{346} (Hayes & Castellino, 1979a–c). The cDNA for HPg has been cloned and sequenced (Malinowski et al., 1984; Forsgren et al., 1987; McLean et al., 1987), and a fully functional protein has been expressed in insect cells (Whitefleet-Smith et al., 1989; Davidson et al., 1990). The entire gene for HPg has also been cloned, and the exon–intron boundaries have been established (Petersen et al., 1990).

The non-protease region of HPm, comprising its amino-terminal 561 amino acid residues, has been proposed to exist in five separate domain regions, termed kringles (Sottrup-Jensen et al., 1978), along with peptides of various lengths that separate the kringles. These highly homologous triple disulfide-bonded kringle regions, of approximately 80 amino acids each, are present in a variety of proteins. Two such structures exist in human tPA (Pennica et al., 1983) and human prothrombin (Magnusson et al., 1975), and one each is present in human urokinase (Steffens et al., 1982) and human coagulation factor XII (McMullen & Fujikawa, 1985). Most interestingly, kringles appear 38 times in human apo-Lp(a) (McLean et al., 1987).

Kringle domains have been implicated in many nonproteolytic regulatory and recognition functions of HPg and HPm, especially their ability to interact with antifibrinolytic ω-amino acids, such as EACA (Sottrup-Jensen et al., 1978; Markus et al., 1978a,b; Sehl & Castellino, 1990; Thewes et al., 1990), with fibrin (Wiman & Wallen, 1977), and with plasmin inhibitors, such as α_2-antiplasmin (Wiman et al., 1978). HPg contains several classes of sites for EACA-like compounds situated on its latent noncatalytic chain (Markus et al., 1978a,b). In order to assign these sites to individual kringle regions, some of these latter domains have been isolated from

† Supported by Grant HL-13423 from the National Institutes of Health, Grant 89/833 from the American Heart Association, a NSERC of Canada Postdoctoral Fellowship (to N.M.), and the Kleiderer/Pezold Family Endowed Professorship (to F.J.C.).
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[1] Abbreviations: HPg, human plasminogen; HPm, human plasmin; tPA, tissue plasminogen activator; Lp(a), lipoprotein (a); 4-ABA, 4-aminobutyric acid; 5-APA, 5-aminopentanoic acid; EACA, ε-aminocaproic acid (6-aminohexanoic acid); 7-AHA, 7-aminoheptanoic acid; 8-AOA, 8-aminooctanoic acid; t-AMCHA, *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid; r, recombinant; K1, the kringle 1 region of human plasminogen, which consists of amino acid residues C^{84}–C^{162} of the intact protein (we have used this abbreviation interchangeably with our recombinant construct, which consists of amino acid residues S^{82}–[E^{163}→D] of human plasminogen); $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; phoA, alkaline phosphatase; DSC, differential scanning calorimetry; ES-MS, electrospray mass spectrometry.

0006-2960/91/0430-1948$02.50/0

limited proteolytic digests of HPg and their binding characteristics to EACA-type compounds evaluated. It has been determined by equilibrium binding methodology that isolated K1 contains a single site of K_d of approximately 17 μM (Lerch et al., 1980). Equilibrium dialysis (Lerch et al., 1980), NMR (DeMarco et al., 1987), and titration microcalorimetry (Sehl & Castellino, 1990) have demonstrated that isolated K4 possesses a slightly weaker binding site for EACA of K_d of approximately 25–50 μM, and NMR experiments led to the finding that proteolytically derived K5 displays a K_d for EACA of approximately 94 μM (Thewes et al., 1990). Investigations with chemical modifying agents (Lerch & Rickli, 1980; Hochswender & Laursen, 1981; Trexler et al., 1982) suggest that EACA binding to K4 is influenced by amino acid residues H^{31}, D^{54}, D^{56}, R^{69}, and W^{70} (the numbering is from the first kringle C most proximal to the amino terminus of HPg). Results from NMR investigations have demonstrated that K4 residues, W^{60}, F^{62}, and W^{70}, are perturbed in a major fashion by ligands that bind to the EACA site (Petros et al., 1989). Similar investigations with isolated K1 suggest that its interaction with EACA-like compounds involves, among others, residues H^{31} (Lerch & Rickli, 1980) and F^{35}, W^{61}, and Y^{71} (Motta et al., 1987).

While kringle domains from all proteins are highly homologous, functional differences are present among them, most notably in the presence or absence of EACA binding sites. Such sites are present in some of the kringle regions of HPg, tPA, and apo-Lp(a) but apparently absent in those from prothrombin, urokinase, and factor XII. The next major strides in elucidating the structure–function relationships of these important kringle domains, especially regarding the properties of the EACA binding site, will be accomplished with variant molecules produced by recombinant DNA techniques. To this time, the only recombinant kringle generated in this manner has been r-K2 from tPA, expressed in *Escherichia coli* cells (Cleary et al., 1989). With our long-term interests in plasminogen structure–function relationships, we wished to determine whether its K1 domain, which contains the EACA site most heavily implicated in its functional properties, could be expressed in relatively large amounts. Successes herein would allow evaluation as to whether the ligand binding properties of r-K1 are retained in the isolated domain and would lead to production of recombinant variants of K1 which would be most useful to investigations of the roles of amino acids involved in its structural determinants and its functional ligand binding properties. The expression, purification, and ligand binding properties of r-K1 are the subjects of this paper.

Materials and Methods

Proteins. Restriction endonucleases were purchased from Fisher Scientific (Springfield, NJ). HPg was purified from fresh plasma by affinity chromatography on Sepharose–lysine (Deutsch & Mertz, 1970; Brockway & Castellino, 1972). Polyclonal rabbit anti-HPg antibodies were generated according to standard methodology, and the monospecific pool was isolated by affinity chromatography on Sepharose–HPg.

Bacterial Strains and Plasmids. E. coli DH5α [F'Φ 80 d *lac*Z dM15 *rec*A1, *end*A1, *hsd*R17 (r_{K-}, m_{K+}), *sup*E44, λ-*thi*-1, *gyr*A96, *rec*A1] was purchased from Clontech (Palo Alto, CA). This strain was grown on LB media (Lech & Brent, 1987a) and supplemented with 40 μg/mL ampicillin (LB_{amp} media) in the cases of organisms harboring recombinant plasmids, all of which carried a β-lactamase-selectable marker. This bacterium was used for routine maintenance and manipulation of all plasmids. Plasmids phGH4R (Chang et al., 1987) and p119PN127.6 (McLean et al., 1987) were employed to construct the expression vector.

DNA Analytical Methods. Oligonucleotides were synthesized by phosphoramidite chemistry on a Biosearch (San Rafael, CA) Cyclone two column DNA synthesizer. The resulting oligonucleotides were purified with Applied Biosystems (Foster City, CA) oligonucleotide purification cartridges. cDNAs were sequenced by the dideoxy technique (Sanger et al., 1977) with the Sequenase reagent kit (U.S. Biochemicals, Cleveland, OH). Plasmid minipreparations were prepared by the alkaline lysate method (Lech & Brent, 1987b).

The cDNAs and cDNA fragments were purified by excising the appropriate bands after their electrophoretic separation on 1% agarose. Recombinant molecules were created by the method of Struhl (1985). Single-strand (ss) plasmid DNAs were generated as described (Vieira & Messing, 1987), and site-specific mutagenesis was conducted according to Kunkel et al. (1987). Plasmids were introduced into competent cells with the $CaCl_2/RbCl_2$ method (Kushner, 1978; Seidman, 1987).

Other Analytical Techniques. $DodSO_4$/PAGE was performed as published previously (Bothe et al., 1985). Western analyses were conducted as described (Davidson et al., 1990). Monospecific rabbit polyclonal anti-HPg was employed as the antibody to mark the presence of r-K1 on the Western-blotted nitrocellulose. FPLC was conducted on a system purchased from Pharmacia (Uppsala, Sweden).

Our procedures and equipment for amino acid compositions, by HPLC, and amino acid sequencing, using gas-phase sequencing with a Porton Instruments automated sequenator, have been previously described in detail (Chibber et al., 1990). For sequence analysis of r-K1, the sample was reduced with mercaptoethanol and blotted from $DodSO_4$/PAGE gells (3-μg load) onto Immobilon-P (Millipore, Bedford, MA). Here, the procedure described for Western analysis (Davidson et al., 1990) was employed, except that the blotting buffer was 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES)–NaOH/10% (v/v) methanol, pH 9.0. The blot was then stained with Coomassie Blue. The region of the Immobilon-P containing the r-K1 was then excised, inserted into the filter disk compartment of the sequenator, and subjected to automated sequence analysis, as described previously (Chibber et al., 1990).

Construction of the Expression Vector pSTIIK1. The plasmid p119PN127.6-M was produced by mutagenesis of the ssDNA of p119PN127.6 by employing two synthetic oligonucleotide primers. The first (boldface type indicates the mutagenic nucleotides), viz.

5'-CTTGC ACTCT **GATGC** ATACA CTTTC-3'

corresponds to the region preceding K1 and creates an *Nsi*I restriction endonuclease site located six nucleotides 5' of the codon for the first C of K1.

The second primer, viz.

5'-GCAAT **GCATG** CATTA **CTAGT** CACAC TCAAG-3'

corresponds to the region immediately following K1 and creates an E → D mutation at the amino acid residue immediately subsequent to the last C in K1. This is followed consecutively by two stop codons, a *Nsi*I restriction endonuclease site and a *Sph*I restriction endonuclease site, this latter site existing at a position six nucleotides 3' of the codon for the second stop signal. In close proximity, and 3' of this latter *Sph*I site, is another *Nsi*I site that was present in the original plasmid, p119PN127.6.

The resulting plasmid, p119PN127.6-M, was digested, first with *Sph*I and second with *Nsi*I. The resulting DNA frag-

ments were then isolated by agarose gel electrophoresis. The 0.3-kb band was excised and purified with Geneclean according to the recommendations of the manufacturer (Bio-101, La Jolla, CA). Plasmid phGH4R was treated with the same restriction endonucleases, and the 3.8-kb band was isolated and purified as above. The two DNA fragments were ligated with T4 DNA ligase (Promega, Madison, WI), in the usual manner, yielding plasmid pSTIIK1. The integrity of the r-K1 insert was verified by DNA sequence analysis.

After expression and release of the signal peptide, the resulting DNA coding for r-K1 from this construct would be predicted to produce r-K1 consisting of the amino acid sequence, S-E-[K1]-D, where K1 begins at the first C residue of the kringle and terminates at the final C of the same kringle. The two additional NH_2-terminal amino acids (S-E) are as they appear in the HPg amino acid sequence, and the additional COOH-terminal amino acid (D) represents a conservative mutation from the E that appears at this location (amino acid position 163) in the HPg sequence.

Expression of r-K1 in E. coli. After transformation of *E. coli* DH5α with the expression vector pSTIIK1, the phoA promoter was activated by growing the organism in a low-phosphate medium (Cleary et al., 1989). The medium described by Chang et al. (1987), without phosphate or micronutrients, was employed. Specifically, a quantity of 10 mL of a transformed overnight culture, grown in LB_{amp} media, was inoculated into 1600 mL of this minimal media, supplemented with ampicillin (40 μg/mL). Sterile air was bubbled through the media vigorously for approximately 13 h, or until the turbidity at 600 nm reached 1.4. The cells were harvested by centrifugation. Approximately 2.1 g of cells was obtained per liter of culture media.

Cell Fractionation. The transformed *E. coli* cells were collected by centrifugation and stored at −70 °C until use. After thawing, the cells (5 g) were stirred in 25 mL of a buffer containing 50 mM Tris-HCl/10% (v/v) mannitol, pH 8.0. After the cell mass was thoroughly resuspended, 5 mg of lysozyme was added and the mixture stirred for 30 min. Release of the soluble periplasmic fraction was effected by addition of 25 mL of H_2O at 4 °C and continued stirring for 30 min. After centrifugation (13000*g* for 30 min), the supernate was collected and saved as the periplasmic fraction.

Release of the cytosolic fraction from the remaining cell pellet was accomplished by addition of 50 mL of a buffer containing 25 mM Tris-HCl/5 mM $MgSO_4$/0.1% (w/v) ribonuclease and 0.1% (w/v) deoxyribonuclease. The resulting viscous slurry was shaken and then subjected to probe sonication (1 min, no. 7 power setting) in a N_2 atmosphere, employing a Heat Systems (Plainview, NY) Model W200R sonicator-cell disruptor, with cooling in an ice bath. The slurry was then allowed to stand for 30 min. After this time, the material was subjected to centrifugation at 13000*g* for 30 min and the supernate taken as the cytosolic fraction.

Refolding of proteins in the insoluble cell debris was attempted in a manner similar to that of Cleary et al. (1989). The pellet representing this fraction was resuspended in 20 mL of 25 mM Tris-HCl/6 M guanidine hydrochloride, pH 8.0, with shaking for 10 h. This solution was then diluted 5-fold, in three equal incremental additions, with 25 mM Tris-HCl/1.25 mM each of oxidized and reduced glutathione, pH 8.0, with 2-h intervals between additions. After centrifugation for 30 min at 13000*g*, the supernatant was collected and saved as the refolded fraction.

Purification of r-K1 from Cell Fractions. The crude cell lysate fractions obtained above were concentrated separately by collection of the pellet that precipitated between 20% and 80% (w/v) $(NH_4)_2SO_4$. This pellet was then dialyzed against a buffer of 50 mM Tris-HCl, pH 8.0. The dialyzate was applied to a column (2.5 cm × 30 cm) of Sepharose–lysine (Brockway & Castellino, 1972), equilibrated with the same buffer. This column was then washed with this same buffer, containing 500 mM KCl, until the absorbance of the eluate at 280 nm decreased to <0.05. Adsorbed r-K1 was then eluted with a buffer of 50 mM Tris-HCl/200 mM EACA, pH 8.0. After dialysis of the r-K1-containing pool of fractions against 25 mM Tris-HCl, pH 8.0, the material was applied to a Mono-Q HR10/10 column (1 cm × 10 cm, Pharmacia) equilibrated with the same buffer. Adsorbed r-K1 was then eluted at a flow rate of 2 mL/min with a gradient of this buffer containing 0 (start solvent) to 200 mM KCl (limit solvent), applied over a total volume of 40 mL. A peak of 280 nm absorbing material emerged at a position in the gradient corresponding to approximately 120 mM KCl. This was determined to be r-K1 by amino acid analysis, NH_2-terminal amino acid sequencing, $DodSO_4$/PAGE, and Western blotting with polyclonal rabbit anti-HPg.

Titration Calorimetry. Samples for titration calorimetry were dialyzed against a buffer of 150 mM sodium phosphate, pH 7.4. The titrations were performed by employing a Microcal (Northampton, MA) OMEGA titration calorimeter at 25 °C, with the latest software available from Microcal. Our experimental procedures and data analysis methods have been described in detail in an earlier publication (Sehl & Castellino, 1990), with the only change being that the curve-fitting plots in the current investigation were constructed by use of incremental heats accompanying binding, rather than total heats as previously described (Sehl & Castellino, 1990).

Differential Scanning Calorimetry. DSC experiments were conducted by employing a MC-2 scanning calorimeter (Microcal). Thermograms were obtained between the temperatures of 15 and 100 °C, at scan rates of 30 °C/h. The r-K1 solution was dialyzed against either 150 mM Hepes–NaOH, pH 7.4, or 100 mM Hepes–NaOH/50 mM EACA, pH 7.4, and added to the sample chamber of the calorimeter. An identical volume of dialyzate was placed in the reference cell. The chambers were temperature equilibrated at 10–15 °C, and the run was initiated. The base line for each run was determined by an identical experiment with the particular sample buffer placed in each chamber. The data obtained were recorded on an IBM-PC computer and permanently stored on a floppy disk.

Our methods for deconvolution of the traces and for obtaining the temperature of maximum heat capacity (T_m), the calorimetric ΔH, and the van't Hoff ΔH have been published in detail (Radek & Castellino, 1988).

Intrinsic Fluorescence. The steady-state intrinsic fluorescence (F) at 25 °C of solutions of r-K1, in the absence and presence of various concentrations of ω-amino acids, was obtained with an SLM Aminco SPF-500 spectrofluorometer. The excitation and emission wavelengths were 296 and 340 nm, respectively.

For titrations of the intrinsic fluorescence changes (ΔF) of r-K1 by the various ligands, a quantity of 0.8 mL of a 13.6 μM solution of r-K1, in a buffer consisting of 0.15 M sodium phosphate, pH 7.4, was placed in the cuvette and the arbitrary fluorescence intensity (F_o) measured. Aliquots of stock solutions of the various ligands in this same buffer were added incrementally until the ΔF did not undergo further change. Ligand stock solutions were prepared at concentrations in which no more than 30 μL was required for the entire titration.

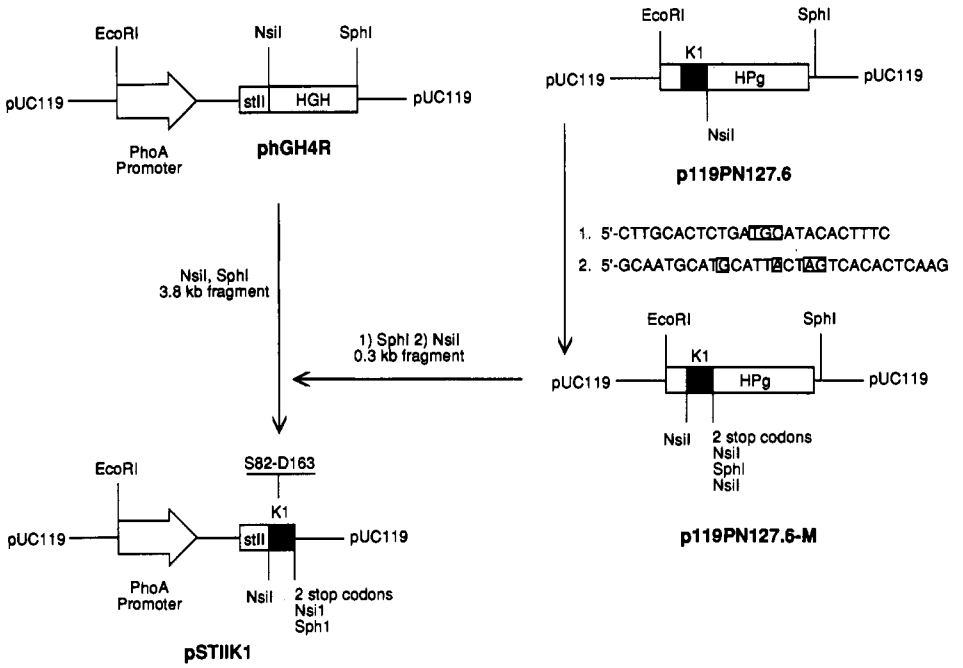

FIGURE 1: Construction of the *E. coli* expression vector for r-K1. Plasmid p119PN127.6 was mutagenized with oligonucleotide primers 1 and 2. The first primer served to place an *Nsi*I restriction endonuclease site six bases 5′ of the codon for the first C residue in K1. The second mutagenic primer resulted in an E → D mutation at the amino acid residue immediately subsequent to the last C in K1 (position 163 in the human plasminogen amino acid sequence), followed consecutively by two stop codons and *Nsi*I and *Sph*I restriction endonuclease sites, the latter positioned six nucleotides 3′ of the codon for the second stop signal. These changes yielded plasmid p119PN127.6-M. After excision of this latter plasmid, first with *Sph*I and second with *Nsi*I, the 0.3-kb fragment containing the r-K1-coding construct was purified and ligated to the purified 3.8-kb fragment of a *Nsi*I–*Sph*I digest of plasmid phGH4R. The resulting final plasmid, pSTIIK1, was employed as the expression vector in *E. coli* for r-K1. The r-K1 contained the sequence S-E-[K1]-D, with the COOH-terminal D representing a conservative mutation from E present at this sequence position of human plasminogen.

In these cases, control titrations of r-K1 with the buffer demonstrated that its initial fluorescence did not change over the entire volume range of the titration.

For evaluation of the K_d values, a computer program was written wherein the fluorescence titrations were analyzed by a nonlinear least-squares curve-fitting routine. A reversible two-state model of binding with a 1:1 molar stoichiometry was assumed, i.e.

$$[RL]/[R]_f = [L]_f/K_d$$

where RL refers to the r-K1–ligand complex, L_f to unbound ligand, R_f to unbound r-K1, and K_d to the dissociation constant for formation of RL. The total fluorescence (F_T) recorded was assumed to be linearly proportional to the weighted sum of the fluorescence values of each of the bound (F_B) and free (F_f) r-K1 species, i.e.

$$R_T F_T = F_f[R]_f + F_B[RL]$$

where R_T is the total [r-K1]. The parameters F_f, F_B, and K_d were allowed to float in an iterative calculation to the point at which the minimum sum of the squares of the differences between the modeled points and the experimental points was observed. This was taken to define the best fit curve. This procedure is essentially based on the Marquardt algorithm (Bevington, 1969) employed for the calculations of the calorimetric data (Wiseman et al., 1989).

^1H NMR of r-K1. Samples for NMR were prepared by dissolution of the r-K1 in 0.1 M phosphate, pH 7.4, in ^2H_2O, lyophilization of the sample, and redissolution in the same volume of ^2H_2O.

^1H NMR spectra were recorded at 25 °C on a Varian VXR 500S spectrometer in the Fourier mode at 500 MHz with quadrature detection. The spectral width was 8 MHz, and the number of data points was 30 K, providing a digital resolution of 0.27 Hz. Suppression of the residual $^1H^2HO$ was attained by gated pulse irradiation of this resonance at low decoupling power for 1.5 s between scans. The chemical shifts (ppm) reported are relative to an internal standard of dioxane, which is assumed to resonate 3.55 ppm downfield of tetramethylsilane. Enhancement of the resonances was achieved by Gaussian convolution.

ES-MS Analysis of r-K1. The electrospray mass spectrum of r-K1 was obtained on a Sciex (Thornhill, Ontario, Canada) API-III triple quadrupole mass spectrometer with the Ionspray interface. The instrument was calibrated with a solution of poly(propylene glycols). The spectrum analyzed represented a summary of four scans of the first quadrupole, acquired at 20 s/scan while the sample was infused at a rate of 5 μL/min from a 10% formic acid/30% acetonitrile solution.

RESULTS

The region of the cDNA for HPg containing the K1 domain has been engineered such that it could be excised intact from the gene and inserted into a bacterial expression vector for production in *E. coli*. The steps involved in this construction are summarized in Figure 1. The final material, obtained from expression with plasmid pSTIIK1, would be expected to produce the sequence S-E-[K1]-D, with the carboxyl-terminal D representing a conservative mutation from the E^{163} present in the HPg sequence. This latter mutation was fortuitously placed in the sequence by the nature of the construction of the gene, and we did not view it as a sufficiently serious consequence to alter it to the original sequence. The exact amino acid sequence of the r-K1 that was used throughout this study is provided in Figure 2.

We initially examined the various fractions of *E. coli* cells for the presence of r-K1, since it was found previously that expression of r-K2 from tPA in a similar system provided the majority of the material in the insoluble cell fraction (Cleary et al., 1989). We did not observe this phenomenon with r-K1

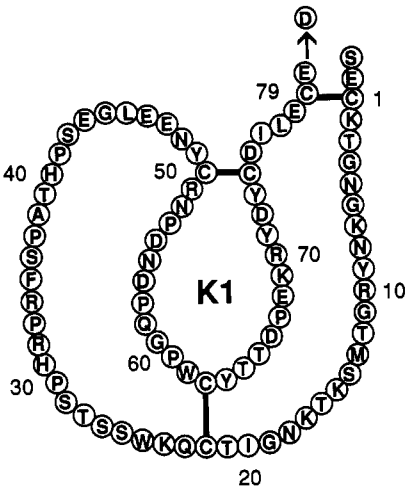

FIGURE 2: Amino acid sequence of the r-K1 employed in this study. This sequence was translated from the cDNA sequence (Forsgren et al., 1987). The sequence of the eight amino-terminal amino acids of the r-K1 was confirmed by direct sequence analysis.

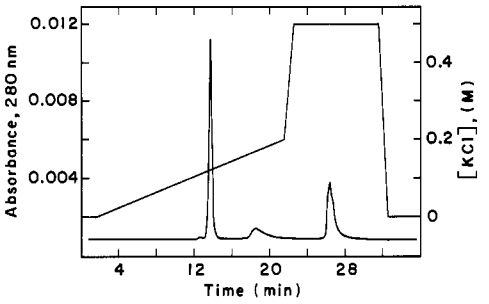

FIGURE 3: Preparative FPLC purification of r-K1. After adsorption and elution from a Sepharose–lysine column, as described under Materials and Methods, r-K1 was dialyzed against a solution of 25 mM Tris-HCl, pH 8.0, and placed on a column of Mono-Q HR10/10 (1 cm × 10 cm), equilibrated with the same solution. Adsorbed r-K1 was then eluted with a gradient of this buffer, containing 0 (start solvent) to 200 mM KCl (limit solvent), applied over a total volume of 40 mL. The fraction eluting at approximately 120 mM KCl contained highly purified r-K1.

from HPg and found that 88% of the peptide recognized by the anti-HPg antibodies was present in the periplasmic fraction of the *E. coli* cells. A further 12% was found in the cytosolic pool, while none was detected in the refolded fraction. Thus, for r-K1, it was not necessary to denature, refold, and reoxidize the disulfide bonds of this kringle, as was the case with tPA-r-K2 (Cleary et al., 1989).

Purification of the expressed r-K1 depended in the first step upon its specific adsorption to Sepharose–lysine and its elution with EACA (Deutsch & Mertz, 1970). The material obtained from this highly suitable purification method contained a small amount (20%) of other components. Final purification, concentration of the solution, and removal of any residual EACA were achieved with FPLC. An example of the elution profile of r-K1 from this latter chromatographic step is illustrated in Figure 3. The resulting material, obtained in a final yield of approximately 100 μg/g of cells (wet weight), reacted with polyclonal antibodies to HPg on Western blots, displayed a single band on $DodSO_4$/PAGE at the appropriate molecular weight, and possessed an amino acid composition within 5% of that expected for residues 82–163 ($E^{163} \rightarrow D$). Amino-terminal amino acid sequence analysis of the the purified and reduced r-K1 yielded the sequence SEX(indicates C at this location)KTGNG, with a single new amino acid residue appearing at each cycle, exactly as predicted from the expected

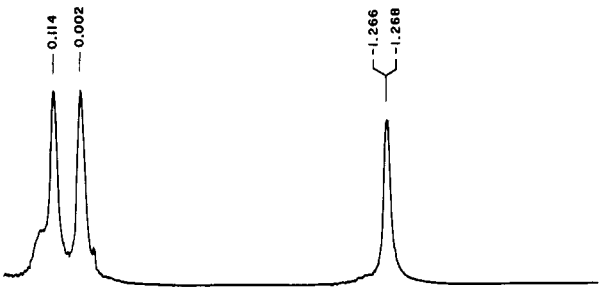

FIGURE 4: High-field methyl proton component of the ^1H NMR spectrum of r-K1 at 500 MHz, shown between 0.2 ppm and −1.6 ppm at 25 °C in ^2H_2O. The r-K1 concentration was 1.22 mM, and the buffer was 0.1 M sodium phosphate, pH 7.4. Labile hydrogen atoms were exchanged for deuterium.

sequence. This shows that the signal sequence has been properly cleaved from r-K1. The exact mass of r-K1 was obtained through ES-MS methodology. From analysis of the five independent m/z peaks obtained, the observed compound mass was 9389.44 ± 2.85, which compares very favorably with the expected mass of S-E-[K1]-D of 9390.2.

As an indication that proper folding of r-K1 has occurred, we have subjected the material to ^1H NMR spectral analysis at 25 °C, employing 500-MHz NMR spectroscopy. It has been amply demonstrated (DeMarco et al., 1982, 1985; Llinas et al., 1985; Ramesh et al., 1987; Thewes et al., 1987) that an important measure of proper kringle folding is the appearance of methyl proton doublet signals, present at approximately 0.2 and −1.0 ppm, arising from the protons of the δ,δ^1-CH_3 groups of L^{45}. The highest field methyl proton resonances (ca. −1 ppm) have been generally interpreted to result from interactions of methyl protons with aromatic rings (Wuthrich, 1976), and signals of this type have been observed in the ^1H NMR spectrum of trypsin (Perkins & Wuthrich, 1980). As seen in the relevant portion of the ^1H NMR spectrum of r-K1 shown in Figure 4, high-field doublet resonances (not clearly resolved) from L^{45} are present at −1.266 and −1.268 ppm and also at a lower field, of 0.114 ppm (unresolved doublet). These two sets of L^{45} methyl proton resonances, appearing at these locations, provide further important proof that our r-K1 is properly folded. The fact that we observe unresolved doublets for these resonances is due to the temperature of 25 °C chosen for collection of this spectrum. At this temperature, similar unresolved signals have been previously obtained for these same methyl protons (DeMarco et al., 1982). These investigators found that doublets appeared at 37 °C, due to sharpening of the spectral signals at this higher temperature (Thewes et al., 1987). We also observe this latter phenomenon. At a temperature of 37 °C, we find one resolved doublet with resonances at −1.257 ppm and −1.268 ppm and another with resonances at 0.115 ppm and 0.104 ppm, originating from the δ,δ^1-methyl proton groups of L^{45}. However, we have chosen to illustrate here the ^1H NMR spectrum obtained at 25 °C in order to remain consistent with the temperature employed for the ω-amino acid/r-K1 binding investigations.

Heat changes accompanying binding of EACA, and various structural analogues of this ω-amino acid, to r-K1 have been determined by titration microcalorimetry. A representative example of a such a titration with EACA and r-K1 is provided in Figure 5. Least-squares deconvolution of the titration data, with floating values of K_d and ΔH, led to the best-fit binding isotherm shown in Figure 6. In order to maximize the accuracy of the experimental values for these parameters, we set the stoichiometry of the interaction at 1.0. This value has

Table I: Thermodynamic Properties of the Binding of Various ω-Amino Acids to r-K1 at 25 °C

ligand	n (mol/mol)	ΔH (kcal/mol)	ΔS (kcal/mol·K)	ΔG (kcal/mol)	K_d (μM)
4-ABA[a]	1.0	−2.7 ± 0.6	6.7 ± 1.0	−4.7 ± 0.2	350 ± 150
5-APA[b]	1.0	−3.1 ± 0.1	10.7 ± 0.7	−6.3 ± 0.1	24 ± 4
EACA[c]	1.0	−3.6 ± 0.1	10.5 ± 0.8	−6.7 ± 0.1	12 ± 2
7-AHA[d]	1.0	−1.8 ± 0.2	10.5 ± 0.9	−4.9 ± 0.1	250 ± 40
t-AMCHA[e]	1.0	−4.7 ± 0.2	11.8 ± 0.9	−8.2 ± 0.1	1.0 ± 0.2

[a] 4-Aminobutyric acid. [b] 5-Aminopentanoic acid. [c] 6-Aminohexanoic acid (ε-aminocaproic acid). [d] 7-Aminoheptanoic acid. [e] *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid.

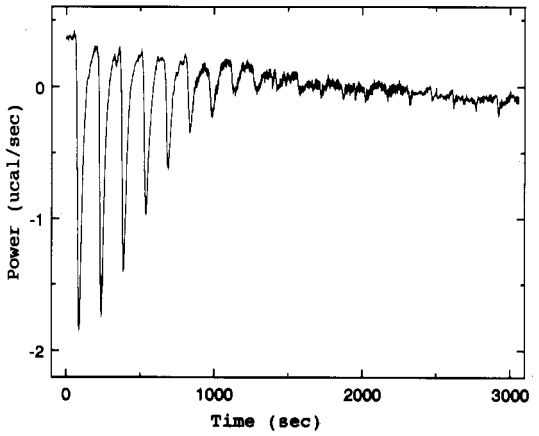

FIGURE 5: Heat change accompanying titration of r-K1 with EACA at 25 °C. An automated sequence of 2.5-μL injections, from a stock solution of 13.75 mM EACA, in 150 mM sodium phosphate, pH 7.4, was titrated into the sample cell containing 0.085 mM r-K1 in this same buffer. Each injection occurred over a 10-s time interval, with 2.5 min between injections.

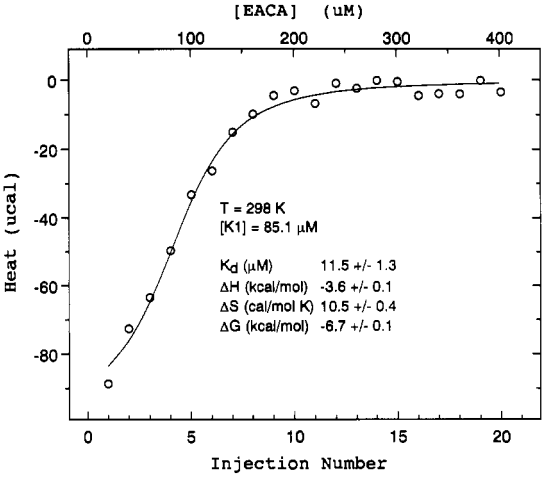

FIGURE 6: Best fit of thermodynamic parameters characterizing the incremental heat change (μcal) accompanying interaction of r-K1 with EACA. The data of Figure 4 were deconvoluted, with the value of n held constant at 1 mol/mol. The solid line represents the least-squares best fit to the data points obtained, with ΔH = −3.6 kcal/mol and K_d = 11.5 μM.

been experimentally determined for isolated r-K2 from tPA (Cleary et al., 1989) and for proteolytically derived K1 and K4 from HPg (Lerch et al., 1980). Thus, we believed that we were justified in proceeding in this fashion, especially since the initial purification step of our r-K1 relied upon its specific affinity for EACA. In addition to removing impurities, this procedure served to eliminate molecules of r-K1 (if any) that would not subsequently interact with EACA-type compounds. When the value of n was allowed to float, along with ΔH and K_d, in the iterative fitting of the thermodynamic binding constants to the titration data, its value was approximately 0.9–1.0 for the tighter binding ligands, 5-APA, EACA, and t-AMCHA, without significant differences from the above procedure in the values obtained for K_d, ΔH, and, consequently, ΔS. With this same procedure, the value of n was less reliable for the weaker binding ligands, 4-ABA and 7-AHA. In these latter cases, we found that the reproducibility of ΔH and K_d was greatly improved by having one less variable in the iterations, and therefore it was of value to preset n at 1.0 for these calculations. This was warranted since the same r-K1 samples were employed for these latter measurements and since n was found to be nearly 1.0 for similar ligands with this same sample.

It has been shown previously that the large intrinsic fluorescence changes originally found to accompany ω-amino acid binding to HPg (Violand et al., 1978) were observed in isolated K4 and K5 of HPg (Novokhatny et al., 1989). We demonstrate herein that a variety of ω-amino acids caused substantial changes in the intrinsic fluorescence of r-K1, ranging from −12% for 4-ABA to approximately −47% for 7-AHA. We have titrated these fluorescence changes with each of the ligands to obtain confirmation of the K_d values revealed by titration calorimetry, and in addition, we were able to employ this method to obtain a reliable K_d for 8-AOA, which was not possible, because of its high value, with the

Table II: Dissociation Constants (K_d) for the Binding of Various ω-Amino Acids to r-K1 at 25 °C As Determined by Titration of the Intrinsic Fluorescence Changes

ligand	ΔF_{max} (%)	K_d (μM)
4-ABA[a]	−12	380
5-APA[b]	−38	28
EACA[c]	−44	13
7-AHA[d]	−52	142
8-AOA[e]	−27	480
t-AMCHA[f]	−25	3

[a] 4-Aminobutyric acid. [b] 5-Aminopentanoic acid [c] 6-Aminohexanoic acid (ε-aminocaproic acid). [d] 7-Aminoheptanoic acid. [e] 8-Aminooctanoic acid. [f] *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid.

calorimetric titrations. Pertinent data are shown in Table II and demonstrate that the K_d values determined by titration of the intrinsic fluorescence changes and by titration microcalorimetry are quite similar.

Since we had previously demonstrated that kringle domains from HPg displayed large structural stabilizations when bound to EACA-like compounds (Castellino et al., 1981), we wished to examine whether the isolated r-K1 also showed this effect. In addition, this approach allowed us to compare the extent of this stabilization to the isolated K4 domain of HPg (Castellino et al., 1981) and the isolated r-K2 region of tPA (Cleary et al., 1989). DSC analysis is well-suited to revelation of this property, and we have employed this method for this purpose. Thermograms of r-K1 in the presence of a sufficient concentration of EACA to saturate its binding site, and in the absence of EACA, are illustrated in Figure 7. The T_m for r-K1 of 340.8 K is shifted to 359.1 K in the presence of EACA, demonstrating that a substantial stabilization of the native structure of r-K1 has occurred consequent to its binding to EACA. Unresolvable difficulties were experienced in obtaining

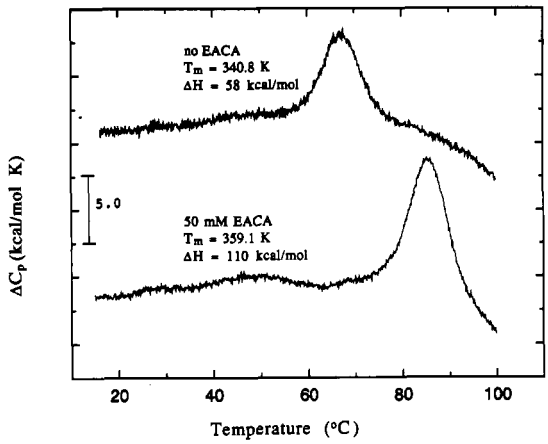

FIGURE 7: DSC thermogram of r-K1 illustrating the change in heat capacity at constant pressure (ΔC_p) against temperature. The buffers employed were 150 mM Hepes–NaOH, pH 7.4 (A), and 100 mM Hepes–NaOH/50 mM EACA, pH 7.4 (B).

a high-temperature base line for r-K1 in the presence of EACA, due to the high temperatures (>368 K) needed to attain this in aqueous solution. While this does not affect the value of the T_m, which is the major point of this experiment, it does provide a ΔH which is only an estimate of its true value.

Discussion

The results of this study show, for the first time, that r-K1 can be expressed in *E. coli* and secreted in a soluble cell fraction, with loss of the signal peptide. Chemically, r-K1 has the expected molecular size as revealed by ES-MS and Dod-SO_4/PAGE, as well as the predicted amino acid composition and amino-terminal amino acid sequence. Importantly, r-K1 can be purified by methods that do not require denaturation and oxidative refolding. This is significant because r-K1 contains three disulfide bonds. Apparently, their capacity to pair correctly, despite the reducing potential of the periplasm, shows that very strong conformational determinants are present in kringle-like structures and argues for preservation of the structural integrity of r-K1 expressed in *E. coli* cells. Other evidence for such conformational preservation is the ability of r-K1 to bind to Sepharose–lysine and, in solution, to interact with EACA-type compounds (Figures 5 and 6), its ability to bind to anti-HPg antibodies; and its DSC properties in the absence and presence of EACA (Figure 7), which are similar to those of the K4 domain of HPg isolated by limited proteolysis of HPg (Castellino et al., 1981). In addition, the substantial shielding from aqueous solvent of the protons of one of the δ,δ^1-CH_3 groups of L^{45}, resulting from their interaction with the aromatic rings of W^{25}, F^{35}, Y^{63}, and W^{61} (Motta et al., 1987), is observed in the ^1H NMR spectrum of r-K1 (Figure 4), and this provides a strong argument for the integrity of the folding of our r-K1. While proteins synthesized in *E. coli* cells are not glycosylated, this consideration is unimportant here, since the K1 domain of HPg does not have consensus sequences for N-linked glycosylation nor does this region contain O-linked oligosaccharide in intact HPg (Hayes & Castellino, 1979c). Therefore, given all of the above characteristics of r-K1, it is clear that we are conducting our studies with a properly folded molecule.

In this investigation, the typical yield of r-K1 was approximately 100 μg/g wet weight of *E. coli* cells, or 220 μg/L of cell culture fluid, at the cell densities (2.2 g/L) employed. This is below the value of approximately 250–400 μg/g wet weight of *E. coli* cells and approximately 43–68 mg/L of cell culture medium at the fermenter cell densities used (170 g/L) for tPA-r-K2 (Cleary et al., 1989). Whether the amount of r-K1 produced is a function of the chemical differences between the kringles, of the exact strain of *E. coli* cells used (DH5α for r-K1 versus 16C9 for tPA-r-K2), and/or of the cell culture conditions is not known. However, the lower cell mass density employed for expression of r-K1 may have been a factor in the lack of insoluble r-K1 in the system described herein, as compared to the 60% of the total tPA-r-K2 found in the insoluble fraction of the *E. coli* cells (Cleary et al., 1989).

The interaction of EACA with r-K1 is characterized by a K_d of approximately 12 μM (Table I), a value in excellent agreement with that of 17 μM found for K1 that had been produced by limited proteolytic treatment of HPg. The K_d for r-K1 is also in good agreement with the same value of 9 μM found for the single tight binding site for EACA to HPg (Markus et al., 1978a,b) and believed to be present on the K1 domain. The binding of EACA to r-K1 is tighter than that reported for interaction of EACA with proteolytically derived K4 and K5 of HPg, which displayed K_d values of 26 μM (Sehl & Castellino, 1990) and 64 μM (Novokhatny et al., 1989), respectively, and also tighter than the binding of EACA to r-K2 of tPA, which possessed a K_d value of approximately 96 μM (Cleary et al., 1989). The thermodynamic characteristics of the interaction of r-K1 with EACA show a significant entropic component, with approximately 40% of the ΔG contributed by the entropy of binding. If only the interaction between the ligand and r-K1 were considered, a negative entropy would be expected due to the decrease in the number of degrees of translational and rotational freedom of the free ligand and free r-K1 subsequent to their interaction. The contribution of the ΔS to this interaction is positive and approximately 2-fold greater than the entropic contribution to binding of EACA to K4 of HPg (Sehl & Castellino, 1990). Several different types of binding mechanisms can be responsible for the positive ΔS value observed. One major contribution to this net positive ΔS value likely involves changes in the hydration of the kringles and ligand as a consequence of ligand binding. This latter phenomenon could lead to a net loss of the more ordered water of hydration molecules from solvent-accessible regions of the uncomplexed components and a concomitant net increase in the less ordered bulk water molecules after binding, the effect of which would be an increase in the ΔS of the overall system. Such effects are possible through loss of ordered H_2O from solvated hydrophobic groups in the free ligand and r-K1 as a result from their interaction, as well as from the polar groups of the ligand and r-K1, resulting from this same interaction. Both of these effects are believed to be possible (Ross & Subramanian, 1981). Other possible more indirect contributions to the net positive ΔS involve potential ligand-induced conformational alterations induced in K4 and r-K1 upon complex formation. One such type of conformational alteration can result from the clustering of hydrophobic residues to form a hydrophobic binding pocket and consequent loss of structured H_2O bound to these groups in the absence of a complex. Evidence for such conformational changes in kringles upon ligand binding has not yet been clearly found, and in fact, examination of X-ray data suggests that the binding site may preexist to a major extent in the kringle.[2]

The response of thermodynamic binding properties to r-K1 of analogues of EACA is informative with regard to the nature of the binding pocket. From molecular modeling of kringles (Tulinsky et al., 1988), based on X-ray analysis of both K2

[2] A. Tulkinsky, personal communication.

from human prothrombin (Park & Tulinsky, 1986) and K4 from HPg (Mulichak et al., 1989), and NMR studies on HPg-K1 (Motta et al., 1987), HPg-K4 (DeMarco et al., 1987), HPg-K5 (Thewes et al., 1990), and tPA-r-K2 (Byeon et al., 1990), working models of the kringle binding site have been forwarded. For HPg-K1, the subject of this investigation, the amino group of the ligand likely interacts with D^{56}, perhaps reinforced by D^{54}, and the carboxyl moiety of the ligand binds to R^{34} and R^{70}. While R^{32} is in the neighborhood of R^{34}, it is apparently not important for interaction of K1 with lysine (Vali & Patthy, 1984). These polar interactions between the ligand and kringle domain can be stabilized by ion pairs, hydrogen bonds, and/or van der Waals interactions, and such interactions undoubtedly dominate the contributions leading to the negative ΔH values of the ligand/r-K1 binding interactions (Ross & Subramanian, 1981). Unless solvent shielding of the ions exists, ion pairs would not likely be important in aqueous solution, and the ΔH for binding (Table I) of EACA to r-K1 falls within the range expected for one to two net hydrogen bonds formed (or broken) as a consequence of complex formation (or dissolution). Also, as noted above, highly important to the binding site is the clustering of the hydrophobic backbone of EACA with a series of hydrophobic groups of r-K1, most probably including W^{25}, F^{35}, H^{40}, L^{45}, W^{61}, Y^{63}, and Y^{73}, some of which are needed for proper kringle folding and others for direct interaction with the ligand. The specificity of this binding pocket for ligands of the type considered herein is reasonably rigid with 4-ABA displaying the minimal structure required for binding and the EACA structure presenting the optimal straight-chain ligand. Binding rapidly diminished for structures longer than EACA, due to a large loss of favorable ΔH. The maximal distance between the amino and carboxyl groups on EACA is approximately 7.5 Å and may define the optimal geometry of the binding site. However, t-AMCHA interacts with r-K1 with a K_d of more than 1 order of magnitude lower than that of EACA. In this case, the maximum distance of separation between the amino and carboxyl groups of the ligand is approximately 6.8 Å. It is possible that the more hydrophobic nature of the cyclohexane ring in this latter compound is driving the stronger interaction with r-K1 and/or that the distance of 6.8 Å between the polar groups of the ligand, defined by t-AMCHA, is the optimal distance for interaction with their counterparts on r-K1. With 7-AHA, the greater separation between the amino and carboxyl groups may prevent formation of one of the charge interactions, since the ΔH that is observed for binding is reduced by approximately 50% from that same value for EACA. The ΔS for binding is essentially unchanged, which supports one hypothesis that displacement of highly ordered H_2O in the ligand and/or peptide hydrophobic groups is the driving force for the favorable entropic contribution of binding. The longer ligand, 7-AHA, may displace the maximum amount of rigidly bound H_2O from the binding pocket, as does EACA, but the limited amount of structured water in the these sites precludes any further release of H_2O by ligands of increasing size and hydrophobicity.

This same general trend has also been observed for interaction of this class of ligands with HPg-K4 (Sehl & Castellino, 1990). However, subtle differences do exist between these highly homologous kringles, as evidenced by the fact that interaction of these analogues with HPg-K4 displayed similar K_d values toward 4-ABA, 5-APA, and EACA. This was not the case with r-K1, where substantial differences were observed between 4-ABA and 5-APA (Table I). This suggests that there is more rigidity in specificity of the binding pocket of r-K1 than of K4, an observation in agreement with a conclusion reached earlier (Tulinsky et al., 1988). The molecular basis of the more open binding pocket of K4 has been proposed to be due to the replacement of F^{35} in r-K1 with a K at this sequence position in K4 (Tulinsky et al., 1988).

The intrinsic fluorescence of r-K1 displays a very large decrease as a result of ω-amino acid binding. The maximum extent of this decrease ranges from approximately 12% with 4-ABA to greater than 40% with 5-APA, EACA, and 7-AHA. Fluorescence changes upon such ligand interactions with K4 and K5 from HPg are also present, with K4 displaying a fluorescence increase upon ligand binding and K5 showing a decrease in intrinsic fluorescence consequent to ligand binding (Novokhatny et al., 1989). It is of interest that the alterations in intrinsic fluorescence, presumably resulting from changes in W-residue environments as a result of binding, are in the opposite direction, with a positive change in the case of K4 and a negative change for r-K1 and K5. The former phenomenon indicates that the net environmental shift of W residues is nonpolar, and in the latter cases the net W environmental shift is polar. This further reflects the subtle differences in the manner in which EACA-type ligands interact with various kringle regions. Regardless of the types of changes seen, the large intrinsic fluorescence decrease in r-K1 allowed the ready titration of this perturbation with the desired ligands, such that an independent check of the K_d for binding of these ligands was possible. The results, presented in Table II, were clearly very close to those determined in Table I from titration calorimetry.

DCS analysis of r-K1 demonstrates the presence of a single endothermic transition, which upon deconvolution provides a T_m of 340.8 K and a ΔH_{cal} of 58 kcal/mol. The value of the T_m is significantly higher than the same value obtained previously for HPg-K4, of 331 K (Castellino et al., 1981), and HPg K5, of 330 K,[3] and significantly lower than the T_m reported for tPA-r-K2, of 348 K (Kelley & Cleary, 1989). This indicates that r-K1 possesses greater structural stability than the two HPg kringles and less stability than the tPA kringle. The very significant increase in T_m of kringle domains, resulting from their respective interactions with EACA, first reported for HPg-K4 and inferred for HPg-K5 (Castellino et al., 1981) is also observed with r-K1 from HPg (Figure 7). These data demonstrate that a large stability in structure of these kringles accompanies binding of EACA. This may result from preferential binding of EACA to the native state of r-K1 and a consequent shift to the native state of the equilibrium between the native and heat-denatured states and/or from a ligand-induced conformational rearrangement of the kringle to a more stable structure. The ΔH_{cal} value obtained for r-K1 is close to that previously found for HPg-K4 (Castellino et al., 1981). It cannot be unequivocally stated that the differences in ΔH_{cal} observed between these kringles, and for r-K1 in the presence of EACA, are significant, given their very low absolute values and the uncertainty in the high-temperature base line in the r-K1 endotherms. Definition of the high-termperature base line in the thermogram for the r-K1/EACA complex (Figure 7) requires reliable data at temperatures above 95 °C in aqueous solution, and this is unlikely to be accomplished. However, the T_m values are extremely reliable and reproducible, and firm conclusions can be made regarding their differences.

In summary, we have shown that r-K1 from HPg can be successfully secreted into the periplasm of *E. coli* cells, with

[3] L. C. Sehl, and F. J. Castellino, unpublished data.

proper pairing of disulfide bonds, and can be purified without the need for denaturants and oxidative refolding. Its structure and ligand binding properties are reflective of those same properties of K1, when present in intact HPg. This lends further credence to the proposal that K1 exists as a structural domain in HPg (Castellino et al., 1981) and that studies of isolated K1 and other related kringles is a fruitful manner of extracting the properties of individual kringles in a multikringle protein. The fact that sufficient quantities of r-K1 can be generated in a facile manner by recombinant DNA technology provides impetus for the expectation that variant proteins will be generated that will be most revealing of structure–function relationships of these important structural units.

ACKNOWLEDGMENTS

We thank Ms. Tracey Colpitts for her assistance with the NMR experimentation.

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Isolation and Chemical Characterization of Two Structurally and Functionally Distinct Forms of Botrocetin, the Platelet Coagglutinin Isolated from the Venom of *Bothrops jararaca*†

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Received July 25, 1990; Revised Manuscript Received November 13, 1990

ABSTRACT: Two distinct forms of botrocetin, the von Willebrand factor (vWF)-dependent platelet coagglutinin isolated from the venom of the snake *Bothrops jararaca*, were purified and characterized structurally and functionally. The apparent molecular mass of the one-chain botrocetin was 28 kDa before and 32 kDa after reduction of disulfide bonds, while that of the two-chain botrocetin was 27 kDa before and 15/14.5 kDa after reduction. Amino acid composition of the two species revealed a similar high content of potentially acidic residues (greater than 60 Asx and Glx residues/molecule) but significant differences in the content of Cys and Phe residues. The NH_2-terminal sequence of the one-chain botrocetin was Ile-Ile/Val-Ser-Pro-Pro-Val-Cys-Gly-Asn-Glu-. Two constituent polypeptides of the two-chain botrocetin showed similar but different NH_2-terminal sequences, distinct from that of the one-chain species: (α) Asp-Cys-Pro-Ser-Gly-Trp-Ser-Ser-Tyr-Glu- and (β) Asp-Cys-Pro-Pro-Asp-Trp-Ser-Ser-Tyr-Glu-. The carbohydrate content of both species was less than 2% of the total mass, and the p*I* was 4.0–4.1 for the one-chain species, and 4.6, 5.3–5.4, and 7.7–7.8 for the two-chain species. No free sulfhydryl group was detected in each species. Both types of botrocetin were resistant to proteolysis at neutral pH. Incubation of ^{125}I-labeled one-chain botrocetin with the crude venom solution resulted in no detectable structural change. On a weight basis, the two-chain botrocetin was 34 times more active than the one-chain form in promoting vWF binding to platelets. A new experimental approach revealed that vWF and botrocetin form a soluble complex, and the binding of ^{125}I-labeled two-chain botrocetin to vWF was clearly inhibited in a dose–response manner by one-chain botrocetin as well as the unlabeled two-chain counterpart. Furthermore, when the concentration of botrocetin was not limiting, the parameters of vWF binding to platelets were identical with either species. These results clearly indicate the existence of two different forms of botrocetin which differ in their molecular structure and affinity for vWF, and provide their initial chemical characterization.

The von Willebrand factor (vWF)[1] mediates platelet adhesion to exposed subendothelium, and its interaction with the platelet GP Ib–IX complex is of critical importance in this process. To reproduce this event in vitro, the antibiotic ristocetin has

† This work was supported by grants-in-aid from the Japanese Ministry of Education, Science, and Culture for Scientific Research on Priority Areas (to K.T. and H.F.), a grant-in-aid from the Naito Medical Foundation (to Y.F. and K.T.), a grant-in-aid from the Fujita Health University (to K.T.), and Grant HL-15491 from the National Institutes of Health (to Z.M.R.). This work was presented at the XIIth Congress of the International Society on Thrombosis and Haemostasis (Abstract 1200), Aug 1989, Tokyo, Japan.

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; CAM, carbamoylmethyl; PE, pyridylethyl; CM, carboxymethyl; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; GP, glycoprotein; vWF, von Willebrand factor; HPLC, high-performance liquid chromatography; TBS, 0.05 M Tris-buffered saline, pH 7.4; TPCK, N^{α}-tosylphenylalanine chlorometyl ketone; PRP, platelet-rich plasma; BSA, bovine serum albumin; endo F, endo-β-*N*-acetylglucosaminidase F; DEAE, diethylaminoethyl; BM_{50}, concentration of botrocetin resulting in half-maximal binding to vWF; IC_{50}, concentration of unlabeled botrocetin inhibiting binding of radiolabeled botrocetin to vWF by 50%; K_d, dissociation constant; B_{max}, maximal binding at saturation; PVDF, poly(vinylidene difluoride).

0006-2960/91/0430-1957$02.50/0