The cystine knot promotes folding and not thermodynamic stability in vascular endothelial growth factor*

Yves A. Muller‡§¶, Christoph Heiring‡§║, Rolf Misselwitz**, Karin Welfle**, and Heinz Welfle**

From the ‡Forschungsgruppe Kristallographie, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, D-13092 Berlin, Germany; §School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1-9QG, UK; ‖Graduate school, Fachbereich Biologie, Chemie, Pharmazie, Freie Universität Berlin, D-14195 Berlin, Germany; **Arbeitsgruppe Biopolymerspektroskopie, Max-Delbrück-Centrum für Molekulare Medizin, D-13092 Berlin, Germany.

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¶ To whom correspondence should be addressed: Department of Biochemistry, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1-9QG U.K. Tel: +44-1273-678515; Fax: +44-1273-678433; E-mail: y.muller@sussex.ac.uk
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Other Footnotes:

1 The abbreviations and trivial names used are: VEGF, vascular endothelial growth factor; GdnHCl, guanidinium hydrochloride; r.m.s. root mean square.

2 The atomic coordinates and structure factors (accession codes 1MJV, 1MKG, 1MKK) of the structures described here, have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
SUMMARY

Cystine knots consist of three intertwined disulfide bridges and are considered major determinants of protein stability in proteins where they occur. We questioned this function and observed that removal of individual disulfide bridges in human vascular endothelial growth factor (VEGF) does not reduce its thermodynamic stability but reduces its unexpected high thermal stability of 108 °C by up to 40 °C. In wild-type VEGF ($\Delta G_{\text{u,25}}^0 = 5.1 \text{ kcal} \cdot \text{mol}^{-1}$) the knot is responsible for a large entropic stabilisation of $T\Delta S_{\text{u,25}}^0 = -39.3 \text{ kcal mol}^{-1}$, which is compensated for by a $\Delta H_{\text{u,25}}^0$ of $-34.2 \text{ kcal mol}^{-1}$. In the disulfide deficient mutants this entropic stabilisation disappears, but instead of a decrease, we observe an increase in the thermodynamic stability by about 2 kcal · mol⁻¹. A detailed crystallographic analysis of the mutant structures suggests a role of the cystine knot motif in protein folding rather than in the stabilisation of the folded state. When assuming that the sequential order of the disulfide bridge formation is conserved between VEGF and glycoprotein α-subunit, the crystal structure of the mutant C61A-C104A, which deviates by an r.m.s. deviation of more than 2.2 Å from wild-type VEGF identifies a true folding intermediate of VEGF.
INTRODUCTION

The cystine knot structural motif consists of three highly intertwined disulfide bridges and occurs in the small inhibitor cystine knot proteins, the cyclotides and the growth factor cystine knot proteins. The first two classes of proteins consist of small polypeptides ranging from 25 to 30 amino acids with functions as diverse as protease inhibition (squash family of inhibitors), ion channel blocking (toxins) and antimicrobial activity (cyclotides) (1-3). The third class consists of the growth factor cystine knot proteins, with members such as TFG-β, α-NGF, PDGF, VEGF, BMPs, IL-17s and many others. These physiologically important growth factors share a common monomer structure but differ in their mode of dimerisation (3), thus introducing molecular diversity into the structural mechanism by which these growth factors activate their cognitive receptors (4). Whole genome analyses hint that the cystine knot motif might be very common in extracellular signalling molecules (5).

The molecular function of the cystine knot motif is poorly understood. In this motif, two disulfide bridges connect two neighbouring chain segments and form a ring structure; a third disulfide bridge penetrates this ring segment and crosslinks two additional chain segments. By implication the cystine knot motif interlocks four separate chain segments. Considerations on the small size of the inhibitor cystine knot proteins and the lack of extended hydrophobic core regions in both the inhibitor and growth factor cystine knot proteins, lead to the assumption that this structural motif is a major determinant for the thermodynamic stability of these proteins. Thus, amide-exchange experiments estimated the thermodynamic stability of the toxin ω-MVIIA to be about 4 kcal mol⁻¹ (6). This equals the stabilisation often introduced by single disulfide bridges of 3 to 5 kcal mol⁻¹ (7,8) and therefore it is plausible that removal of single cystines impairs the stability of these proteins.

We challenged this assumption and studied the thermodynamic and structural properties of wild-type vascular endothelial growth factor (VEGF) and of three of its four
possible cystine deletion mutants. We produced the mutants C51A-C60A’ with the intermolecular disulfide bridge removed (named hereafter ΔI), and C57A-C102A (ΔII) and C61A-C104A (ΔIII) in which the disulfide bridges forming the outer ring of the knot motif have been removed (Fig. 1A). Unexpectedly we observe that none of these mutants appears to be thermodynamically destabilised. Moreover, deletion of the cystine knot disulfide bridges increases the thermodynamic stability by ~2kcal · mol⁻¹. In contrast we observe that individual disulfide bridges are to various extents crucial for the structural integrity and thermal stability of VEGF.

EXPERIMENTAL PROCEDURES

Protein production, sample preparation and circular dichroism measurements – The cystine deletion mutants C51A-C60’A (ΔI), C57A-C102A (ΔII) and C61A-C104A (ΔIII) of truncated human VEGF (residues 13 to 104) have been produced as previously described (9) and were studied in 20 mM Na-phosphate buffer, pH 7.5, 0.1 M NaCl (buffer A) or in buffer A supplemented with various amounts of guanidine hydrochloride (GdnHCl, 0-2.5 M). Protein concentrations were determined spectrophotometrically at 276 nm using absorption coefficients of A¹%,¹cm = 5.74 for wild-type VEGF and A¹%,¹cm = 5.64 for the VEGF variants ΔI, ΔII and ΔIII calculated from the amino acid composition (10). The CD spectra in the far ultraviolet region (190 to 260 nm) were measured with a Jasco-J720 spectropolarimeter and are characteristic for β proteins with a minimum at 212 nm. The far ultraviolet CD spectra (190-260 nm) of wild-type VEGF and ΔI are very similar; a 1 nm red shift of the zero transition point and higher ellipticity values near 190 nm of the spectrum of ΔII, and lower ellipticity of ΔIII in the 230 nm region were observed which might indicate small structural changes (data not shown).
**GdnHCl-induced unfolding monitored by circular dichroism** – Protein aliquots in buffer A with appropriate GdnHCl concentrations were incubated overnight at room temperature and unfolding was determined measuring the ellipticity at 220 nm at 25 (± 0.2) °C. For refolding experiments the proteins were unfolded overnight in buffer A, 6 M GdnHCl. Aliquots were transferred in buffer A with appropriate GdnHCl concentrations and incubated for 15 h at room temperature. Fractions of unfolding, \( f_u \), equilibrium unfolding constants, \( K_u \), and free energies of unfolding, \( \Delta G_{u,25}^0 \), were determined as described (11).

**Differential scanning calorimetry** – Excessive heat capacity curves were measured using an ultra sensitive scanning microcalorimeter VP-DSC (MicroCal Inc., Northampton, Ma.), a heating rate of 1 K min\(^{-1}\) and protein concentrations in the range of 0.15 to 0.30 mg/ml. The calorimetric enthalpy changes, \( \Delta H^{cal} \), and van't Hoff enthalpy changes, \( \Delta H^{vH} \), were calculated using the Origin for DSC software package (MicroCal Inc., Northampton, Ma.). The free energy at 25°C, \( \Delta G_{u,25}^0 \), and the \( \Delta G_u^0 \) versus T profile were calculated with the Gibbs-Helmholtz equation, the \( \Delta H_u^0 \) versus T and \( T \Delta S_u^0 \) versus T profiles were calculated with \( \Delta C_p \) and with \( \Delta H_u^0 \) and \( \Delta S_u^0 \) at reference temperatures \( T_H^* \) and \( T_S^* \), respectively, that were chosen close to the melting temperatures (12).

**Crystal structure determinations** – Crystals were obtained by the hanging drop method by mixing 1 µl of protein solution (10 to 12 mg/ml protein concentration, 20 mM Tris-HCl buffer, pH 8.0 and 100 mM NaCl) with 1 µl of reservoir solution and equilibrating the droplet over 750 – 1000 µl reservoir solution. In case of \( \Delta I \) the reservoir consisted of 0.1 M Na-citrate pH 5.6, 32 % (v/v) 2-propanol and 8 % PEG 4000. The \( \Delta II \) reservoir contained 2.0 M Na-formate and 0.1 M Na-acetate pH 4.6 and the \( \Delta III \) reservoir 0.1 M Na-citrate pH 5.6, 12 % (v/v) 2-propanol and 28 % PEG 4000. Datasets of \( \Delta II \) and \( \Delta III \) were collected at the synchrotron beamline BW7b of the EMBL outstation at DESY Hamburg, while the dataset of
ΔI was collected with Cu-Kα radiation from a rotating anode generator. Data were reduced with program XDS (13). The crystal structures of ΔI and ΔII could be solved by molecular replacement with program AMORE (14) using the wild-type VEGF structure (15) as a search model and refined to convergence with program CNS (16). In case of mutant ΔIII no unambiguous molecular replacement solution could be obtained. However, ΔIII crystallises in the same spacegroup and with similar cell dimensions than ΔI and therefore we assumed that the molecular packing in the ΔI and ΔIII crystals is similar. Phases for the ΔIII dataset were calculated to 4.0 Å with the ΔI model and extended in 1000 steps to 2.5 Å by two-fold non-crystallographic symmetry averaging and solvent flattening using program DM (14). The redefinition of the NCS-symmetry operator and the repeating of the density modification procedure yielded electron density maps, which could be readily extended to 1.32 Å using the free atom refinement protocol of program WARP (17). Conventional refinement together with manual inspection was performed with programs REFMAC (14) and ONO (18). In the final refinement round anisotropic thermal displacement factors were introduced. Consequently, the free R-factor dropped from 22.6 to 19.5 % and in parallel the crystallographic R-factor from 20.6 to 16.5 %.

RESULTS AND DISCUSSION

Thermodynamic and thermal stability of wild-type VEGF and mutants — The normalized transition curves of the guanidine-hydrochloride (GdnHCl) induced unfolding of wild-type VEGF and variants ΔII and ΔIII are shown in Fig. 2A. The refolding curve of wild-type VEGF coincides well with the unfolding curve in support of a fully reversible unfolding process. The half-transition GdnHCl concentration decreases from 4.3 to 2.5 and to 1.9 M for wild-type VEGF and mutants ΔIII and ΔII, respectively (Table 1). Concomitantly, the cooperativity of the unfolding increases in the same order and as a net result the free energies
\( \Delta G^{\circ}_{u,25} \) calculated from the equilibrium unfolding constants and after linear extrapolation to 0 M GdnHCl (11) are similar for mutants ΔII (7.5 kcal mol\(^{-1}\), Table 1) and ΔIII (7.3 kcal mol\(^{-1}\)), and close to wild-type VEGF (5.4 kcal mol\(^{-1}\)).

Similar \( \Delta G^{\circ}_{u,25} \) values are obtained from differential scanning calorimetry (DSC, Fig. 2B). The wild-type, ΔIII and ΔII melt at \( T_m \) values of 108.7, 83.1 and 69.8°C, respectively (Table 1). The heat capacity change \( \Delta C_p \) increases in this sequential order from 1.5 to 1.60 and 1.84 kcal mol\(^{-1}\)K\(^{-1}\). The free unfolding energies \( \Delta G^{\circ}_{u,25} \) calculated with the knowledge of \( T_m \), \( \Delta H^{\circ}_m \) and the assumption of a temperature invariant \( \Delta C_p \) are 5.1, 7.7 and 8.8 kcal mol\(^{-1}\). The temperature invariance of \( \Delta C_p \) is a necessary simplification difficult to overcome (19). The good agreement between the free energies obtained by DSC and GdnHCl-induced unfolding supports our approach (Table 1). This agreement and values close to one for \( \kappa = \Delta H^{\text{vH}}/\Delta H^{\text{cal}} \) (quotient between the van’t Hoff enthalpy \( \Delta H^{\text{vH}} \) and the calorimetric enthalpy \( \Delta H^{\text{cal}} \)) also corroborates the assumption of a two-state unfolding mechanism (20).

The discrepancy observed between the high thermal stability (\( T_m = 108.7 \) °C) and the moderate free unfolding energy (\( \Delta G^{\circ}_{u,25} = 5.1 \) kcal mol\(^{-1}\)) of wild-type VEGF is unusual because in most proteins high thermal stability correlates with large thermodynamic stability (21-24). Thus the \( T_m \)-value of wild-type VEGF is reminiscent of proteins from thermophilic organisms and unexpectedly high for a human protein. Removal of a single disulfide bridge from the cystine knot lowers the \( T_m \) value by as much as 38.9 °C (ΔII, Table 1) and 25.6 °C (ΔIII). In contrast the free unfolding energies do not vary and if calculated at 25 °C are even about 2 kcal mol\(^{-1}\) more elevated for the mutants (Table 1). A plot of \( \Delta G_u \) versus T illustrates this behaviour (Fig. 2C). The maxima of the unfolding free
energies occur at different temperatures, namely at ~55, ~30 and ~15° C for wild-type VEGF, ΔIII and ΔII, respectively. Above temperatures of 50° C, ΔG_u of wild-type VEGF is significantly larger than that of ΔIII and ΔII and remains positive up to 109°C. At the physiological important temperature of about 40°C, the free unfolding energy curves intersect and yield almost identical ΔG_u^0 values close to 7 kcal mol^{-1} (Fig. 2C).

Enthalpic and entropic contributions to ΔG_u^{0,25} – Although the free unfolding energies ΔG_u^{0,25} are similar, the enthalpic and entropic components differ considerably (Table 1). They contribute to the free energies through the Gibbs equation: ΔG_u = ΔH_u - TΔS_u. A positive ΔG_u^0, necessary to prevent spontaneous unfolding, is either achieved through a positive ΔH_u^0 large enough to exceed a destabilizing positive TΔS_u^0 (as observed for ΔII at 25° C, Table 1), a positive ΔH_u^0 together with a negative TΔS_u^0 (ΔIII), or by a negative TΔS_u^0 sufficiently large to exceed a negative ΔH_u^0 (wild-type VEGF). In wild-type VEGF, TΔS_u^{0,25} is negative and the absolute value large (-39.3 kcal mol^{-1}). The large positive contribution of - TΔS_u^{0,25} to the free unfolding energy is compensated by a negative ΔH_u^{0,25} (-34.2 kcal mol^{-1}). At elevated temperatures TΔS_u^0 and ΔH_u^0 change sign, TΔS_u^0 and ΔH_u^0 become positive above 52 and 48 °C, respectively. The positive ΔG_u^0 results above ~50°C from a positive ΔH_u^0 which is partly compensated by a positive TΔS_u^0. This compensational behaviour is frequently observed in proteins (25), by contrast the elevated temperatures at which TΔS_u^0 and ΔH_u^0 change sign in wild-type VEGF are unusual (12,26).

Wild-type VEGF is stabilised solely through entropic contributions – For every protein, the conformational entropy ΔS_u^{0,conf} and the solvation entropy ΔS_u^{0,solv} are the main components of the unfolding entropy change ΔS_u^0. ΔS_u^{0,conf} is always positive because the
number of possible conformations increases in the unfolded state, $\Delta S_{u,\text{solv}}^0$ is negative because upon unfolding water molecules are recruited from the solvent to surround the increased number of hydrophobic residues accessible on the surface in the unfolded chain. In wild-type VEGF the large negative value of $\Delta S_{u}^0$ suggests an unusually small $\Delta S_{u,\text{conf}}^0$ compared to $\Delta S_{u,\text{solv}}^0$. Because both the entropy change ($\Delta S_{u,25}^0$) and the enthalpy change ($\Delta H_{u,25}^0$) are negative, we conclude that at room temperature wild-type VEGF is stabilized entirely by entropic contributions.

Deletion of the cystines increases the conformational freedom of the unfolded state. Therefore a smaller negative $T\Delta S_{u,25}$ is observed for $\Delta$III and a value more common in proteins (26) is observed for $\Delta$II (Table 1). The observed increase in $\Delta C_P$ and the unfolding cooperativity $m$ of the mutants reflect this increase in the conformational freedom and the concomitant increase in the protein accessible surface upon unfolding (27,28).

We infer that none of the disulfide bridges increases the thermodynamic stability of VEGF – Our analysis of the thermodynamic role of the disulfide bridges in VEGF must appear incomplete. In case of mutant $\Delta$I, the breakdown of the two-state approximation paired with protein aggregation prevented its characterisation. Nevertheless from the fact that $\Delta$I can be refolded and crystallised at room temperature (see below) we estimate that its free unfolding energy can not be considerably smaller than the already small thermodynamic stability of wild-type VEGF. Indirect evidence hints that this might also be the case for the fourth possible mutant, which we failed to produce. The recent crystal structure of IL-17E (29) revealed that IL-17s belong to the cystine knot growth factor family with the particularity that the disulfide bridge penetrating the ring structure of the cystine knot is missing. The conclusion that this disulfide bridge is not a prerequisite for the thermodynamic
stability of IL-17s possibly extends to VEGF. From these considerations we anticipate that none of the disulfide bridges is required to thermodynamically stabilise VEGF.

The thermodynamic properties we observe for wild-type VEGF are unusual and possibly extend to other cystine knot proteins. Unfortunately, comparisons with other growth factors cannot be drawn, because to the best of our knowledge no thermodynamic characterisation of a cystine knot growth factor has been reported to date. Data on the function of the individual disulfide bridges in these growth factors is restricted to observations on impaired expression in mammalian cells (30), as well as to measurements of residual biological activity in mutants in which single cysteines have been removed (31,32).

Crystal structures of the mutants – The mutants show that the cystines do not provide additional stability at physiologically relevant conditions. To further investigate this, we determined the crystal structures of variants ΔI, ΔII and ΔIII at 2.1, 2.5 and 1.3 Å resolution, respectively (Table 2, Fig. 1). The r.m.s. deviations of the pairwise superpositions of the main-chain atoms of the mutants and the wild-type VEGF dimer (15) range from 1.19 to 1.72 and 2.25 Å for variants ΔII, ΔI and ΔIII, respectively. In all the variants a conserved core region can be defined formed by the residues of the 4 central β-strands of the monomer and including as much as 38 residues. This core superimposes with the wild-type VEGF monomer with an average r.m.s. deviation of as low as 0.45 Å. Therefore structural differences in the mutants are restricted to differences in loop structures or differences in the dimerisation mode (Table 3, Figs. 1B, C).

As an indicator for changes in the dimerisation of VEGF we studied the coplanar arrangement of the monomers in the dimer. For each variant we calculated the planarity angle. It is defined as the deviation from 90° of the angle enclosed between the line connecting the Cα’s of Glu30 and Ser74 in each monomer and the two-fold symmetry axis relating the monomers in the dimer (Table 3, Fig. 1C). In wild-type VEGF this angle is 2.44° (± 0.87°),
and is with the exception of the structure of a complex between wild-type VEGF and a peptide (6.2°, 33), extremely well conserved in the different crystal forms and in different VEGF-protein complexes (15,34,35). Different angles are observed for the mutants; here the deviations from planarity angles are –2.4, 6.3 and –10.5° for mutants ΔI, ΔII and ΔIII, respectively. The difference of 13° between wild-type VEGF and mutant ΔIII is significant; it translates into a displacement of the outer strand of VEGF (residues 73 to 83) by about 3.2 Å parallel to the two-fold axis, when comparing the wild-type structure to ΔIII (Fig. 1C). This appears a direct consequence of rearrangements in the side-chain orientations in the dimer interface in this mutant (see below).

In addition to global changes, we observe a number of local structural changes in the mutants (Table 3, Fig. 3). In ΔII only small changes occur. When replacing Cys57 and Cys102 with alanines, the distance between the equivalent Cα’s is slightly reduced from 3.97 to 3.59 Å; any additional changes lay well within the deviations observed for different wild-type VEGF structures (15). In ΔI the distance between the equivalent Cα’s is increased from 3.87 to 6.84 Å (Table 3, Fig. 3A). This reflects a widening of the monomer-monomer interface at the position where the intermolecular disulfide bridge occurs in wild-type VEGF. As a consequence the cystine-knot motif rotates as a rigid body away from the interface. The structure of the cystine knot itself is not perturbed.

More drastic changes occur when replacing the disulfide bridge formed between Cys61 and Cys104 with alanine residues (ΔIII). Superposition of the ΔIII monomer onto wild-type VEGF yields an r.m.s. deviation as large as 1.99 Å. This is mainly caused by a conformational change in the loop segment connecting Cys60 and Cys68. Ala61 points away from Ala104 and the distance between the Cα-positions is increased from 3.81 to 12.14 Å (Fig. 3C, D). In ΔIII the diameter of the VEGF dimer is reduced by about 2 Å whereas this distance does barely change in ΔI and ΔII (Table 3). This is only partly a consequence of a
change in planarity i.e. the inclination of the monomers with respect to the dimer two-fold symmetry axis (Table 3). More importantly, we observe a tighter packing in the dimer interface, reducing by 1.7 Å the distance between the two β-strands lining the centre of the interface (distance calculated between the equivalent Cα-positions of Glu30). Two novel symmetry equivalent salt bridges are formed across the interface between His27 and Glu30 and reorientations in the side-chains of residues Ile29 and Leu32 occur. This increase in compactness and in the packing of the interface is totally unexpected. The disulfide bridge, removal of which, causes these changes, is of all the disulfide bridges studied the most remote from the centre of the dimer.

It is not possible to directly correlate the observed structures with the thermodynamic unfolding data because with the exception of an increase in ΔC_p and m, which reflect an increase in the solvent accessibility of the unfolded state, we have not characterised the unfolded state structures. We only note that the mutant ΔII, which structure is the most similar to wild-type VEGF shows the most drastic changes in T_m, ΔC_p, m, ΔS^0_u,25. Therefore the altered thermodynamic properties of this mutant must result largely from the effect of these mutations on the unfolded state.

Conclusions – The data reported here shed light on the role of the cystine knot in VEGF and the observed properties might extend to other cystine knot growth factors. Previous studies have revealed the importance of the disulfide bridges for the folding of glycoprotein hormone α-subunit in vivo (30). Here we showed that in vitro it is possible to generate cystine deficient mutants of the cystine knot growth factor VEGF and to subsequently investigate their crystal structures. This was possible because, as we showed, individual disulfide bridges in VEGF only contribute to the thermal stability and structural integrity of the protein but they do not change its thermodynamic stability. This property might be of primary importance with respect to protein folding. The close spatial proximity of
the six cysteines in the cystine knot renders the temporarily occurrence of non-native-like disulfide bridged intermediates during the folding process very likely and indeed such intermediates have been observed in case of the smaller inhibitor cystine knots (6). If such intermediates would be significantly stabilised, folding would come to a halt; any reshuffling of the disulfide bridges, which does not change the absolute number of disulfide bridges, would be severely hampered if the folding landscape would have such pronounced local minima.

The structural differences and rearrangements we observe in mutant ΔIII, possibly, yield unprecedented insights into the folding pathway of VEGF. When assuming that the disulfide bridge formation in VEGF follows the same sequential pattern recently elucidated for the cystine knot growth factor glycoprotein hormone α-subunit (36) the disulfide bridge corresponding to mutant ΔIII would be formed last in VEGF. The structural differences could thus reveal a true folding intermediate. This folding intermediate would be characterised by a more compact structure than native VEGF and differs from the latter by an r.m.s. deviation of 2.2 Å.

The crystal structures show that the cystine knot is of primary importance for the structural integrity of VEGF. Although the knot appears to only affect the fine-structure of the protein, we anticipate that these changes have drastic consequences for the biological activity of VEGF. Loop regions adjacent to the knot participate in receptor binding and in particular residue Glu64 located on the surface loop formed between residues 61 and 68 determines VEGF receptor specificity (37,38). The conformation of this loop is drastically changed in mutant ΔIII (Fig. 3C, D) and the spatial separation between residues participating in receptor binding is also changed in ΔI (15,34). Therefore the biological activity profile should be altered in these mutants.
We conclude that at physiological conditions the disulfide bridges in the cystine knot growth factor VEGF are of no importance for the thermodynamic stability of the protein. However, we observe that to various extents the cystines are crucial for its ability to adopt the ‘right’ fold.

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Fig. 1. **Molecular structures of wild-type VEGF and mutants ΔI, ΔII and ΔIII.** A, ribbon representation of wild-type VEGF. The two monomers forming the dimer are highlighted in light and dark grey. The disulfide bridges are displayed in a stick representation. The disulfide bridges removed by double alanine mutations are colour coded in red, green and blue for the mutant ΔI, ΔII and ΔIII, respectively. No protocol could be established for the refolding of mutant C26A-C68A from inclusion bodies (disulfide bridge in yellow). B, superposition of the Cα-backbones of ΔI (in red, top), ΔII (in green, middle) and ΔIII (in blue, bottom) superimposed onto wild-type VEGF (in grey). C, illustration of the differences in planarity observed in wild-type VEGF (grey) and the mutants. The two-fold symmetry axis is indicated as a vertical line.

Fig. 2. **Unfolding behaviours of wild-type VEGF and mutants.** A, guanidine hydrochloride induced unfolding of wild-type VEGF and variants ΔII and ΔIII. Normalized GdnHCl-induced unfolding (black) and refolding (magenta) of wild-type VEGF, variant ΔII (green), and variant ΔIII (blue). Curves are sigmoidal fits of the experimental data. B, plot of melting temperatures, $T_m$ (red) and unfolding enthalpies $\Delta H^0_u$ (blue) of wild-type VEGF versus guanidine hydrochloride concentrations, determined from DSC measurements. $T_m$ and $\Delta H^0_m$ values are obtained by linear extrapolation to 0 M GdnHCl. Attempts to measure the excessive heat capacity curves for variant ΔI failed. In ΔI the two monomers are not linked by intermolecular disulfide bridges and the heat capacity curves do not show a cooperative unfolding transition. C, temperature dependence of the free energy of unfolding, $\Delta G^0_u$, of wild-type VEGF and variants ΔII and ΔIII. $\Delta G^0_u$ values were calculated from the $\Delta H^0_m$, $T_m$ and $\Delta C_p$ values given in Table 1.
Fig. 3. **Stereographic superpositions of the knot regions in the mutants.** A, the knot region of mutant ΔI and B, of mutant ΔII. C, D, in the mutant ΔIII (in blue) a novel interaction is introduced in the loop segment connecting Cys60 and Cys68. The side-chain of Asn64 turns inwards and the amide group makes hydrogen bonds to the main chain nitrogen and carbonyl oxygen of Leu66. This interaction replaces a main-chain main-chain hydrogen bond between residues 63 and 66 in wild-type VEGF. Clearly visible in the electron density of ΔIII at 1.32 Å resolution are the two conformations for the disulfide bridge between Cys26 and Cys68. No alternative conformations for any of the VEGF disulfide bridges have so far been observed in wild-type VEGF. Figure prepared with Molscript (39) and Raster3D (40).
Table 1. Thermodynamic data

| Parameter * | wild-type | ΔII   | ΔIII  |
|-------------|-----------|-------|-------|
| Mutation    | -         | C57A-C102A | C61A-C104A |
| $c_{1/2}$ [M] † | 4.32      | 1.86  | 2.51  |
| m [kcal mol⁻¹ M⁻¹] † | -1.26     | -4.02 | -2.92 |
| $\Delta G_{m,25}^0$ [kcal mol⁻¹] † | 5.4       | 7.5   | 7.3   |
| $T_m$ [°C] ‡ | 108.7     | 69.8  | 83.1  |
| $\Delta H_m^0$ [kcal mol⁻¹] ‡ | 91.4      | 110.3 | 96.3  |
| $\Delta C_p$ [kcal mol⁻¹ K⁻¹] ‡ | 1.50      | 1.84  | 1.60  |
| $\Delta H_{m,25}^0$ [kcal mol⁻¹] ‡ | -34.2     | 27.8  | 3.4   |
| $T \Delta S_{m,25}^0$ [kcal mol⁻¹] ‡ | -39.3     | 19.0  | -4.3  |
| $\Delta G_{m,25}^0$ [kcal mol⁻¹] ‡ | 5.1       | 8.8   | 7.7   |

* Thermodynamic unfolding parameters (denoted by subscript u) determined in buffers containing guanidine hydrochloride are extrapolated to 0 M GdnHCl and reported as molar quantities at 25°C (superscript 0 and subscript 25). $\Delta H_m^0$ is the molar unfolding enthalpy at transition temperature $T_m$. The absolute error of $T_m$ is ±0.3°C. The relative error of the molar enthalpy changes $\Delta H_m^0$ is ±5 %.

† Derived from CD-measurements.

‡ Derived from DSC-studies.
Table 2. Crystallographic data

|                                  | ΔI              | ΔII             | ΔIII             |
|----------------------------------|-----------------|-----------------|------------------|
| **Data collection**              |                 |                 |                  |
| Mutant                           | C51A-C60'A      | C57A-C102A      | C61A-C104A       |
| Space group                      | P2₁             | P6₅             | P2₁              |
| Cell parameters (a, b, c), [Å], [°] | 29.28, 77.20, 55.11 | 96.20, 96.20, 125.90 | 28.22, 75.47, 55.11 |
| Resolution [Å]                   | 2.1             | 2.5             | 1.32             |
| Completeness [%]                 | 99.5            | 98.6            | 99.2             |
| Redundancy                       | 3.4             | 4.7             | 3.8              |
| R_Merge [%]                      | 6.8             | 6.6             | 7.4              |
| **Model**                        |                 |                 |                  |
| Number of dimers in the asymmetric unit | one         | two            | one             |
| Number of residues               | 192             | 376             | 187              |
| Number of solvent molecules      | 211             | 195             | 208              |
| **Diffraction agreement and stereochemistry** |                 |                 |                  |
| Resolution [Å]                   | 19 - 2.1        | 38 - 2.5        | 28 - 1.3         |
| R-value [%]                      | 19.61           | 23.41           | 16.54            |
| Free R-value [%]                 | 24.81           | 28.82           | 19.51            |
| R.m.s.d. bond lengths [Å]        | 0.010           | 0.011           | 0.011            |
| R.m.s.d. angles [°]              | 1.50            | 1.51            | 1.40             |
| Anisotropy, mean †               | -               | -               | 0.655            |
| standard deviation               | -               | -               | 0.125            |

* Differences in resolution are reflected in the compilations of the main-chain dihedral angles, phi and psi. For mutant ΔIII 95 % of all residues lay within the most favourable regions of the Ramachandran plot (41), whereas this number drops to 90.2 % for ΔI and to 86.2 % for ΔII. In all cases the crystallographic unit contains more then one monomer. Thus, the medium resolution of ΔII was partially compensated through the application of non-crystallographic symmetry restraints during the refinement.
† defined as in Merritt (1999) (42).
| Mutant       | ∆I (mutant - wild-type) [Å] (± 0.17) * | ∆II (mutant - wild-type) [Å] (± 0.131) | ∆III (mutant - wild-type) [Å] (± 0.160) | wild-type |
|--------------|--------------------------------------|---------------------------------------|---------------------------------------|-----------|
| C51A-C60’A   | 1.72                                 | 1.19                                  | 2.25                                  | -         |
| C57A-C102A   | (± 0.18)                             | (± 0.131)                             | (± 0.160)                             |           |
| C61A-C104A   | 0.57                                 | 0.85                                  | 0.77                                  | 0.67 †    |
| (Ser74Ca-Ser74’Cα) | 35.80                                | 37.01                                 | 34.36                                 | 36.44     |
| Deviation from planarity [°] § | -2.4                                 | 6.3                                   | -10.5                                 | 3.6       |
| Cβ-Cβ distances [Å] | 51 – 60’ | 6.84                                | 3.92                                  | 3.85      | 3.87 |
|              | 26 – 68                              | 3.69                                  | 3.74                                  | 3.85      | 3.77 |
|              | 57 – 102                             | 3.80                                  | 3.59                                  | 3.77      | 3.97 |
|              | 61 – 104                             | 3.77                                  | 3.94                                  | 12.14     | 3.81 |

* Deviations from mean values are reported because both the mutants and wild-type VEGF crystallise with more than one molecule in the crystallographic asymmetric unit.

† Average r.m.s. deviation for all 16 possible pairwise superpositions of the four dimers in the asymmetric unit of the wild-type structure (Protein data bank accession code 2VPF).

‡ Diameter of the dimer, measured as the distance between the equivalent Cα positions of Ser74 in both monomers.

§ The deviation from planarity gives a measure for the coplanarity of the monomers.
Fig. 1. Muller et al.
Fig. 2. Muller et al.
Fig. 3. Muller et al.
The cystine knot promotes folding and not thermodynamic stability in vascular endothelial growth factor
Yves A. Muller, Christoph Heiring, Rolf Misselwitz, Karin Welfle and Heinz Welfle
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