Cotranslational Incorporation of a Structurally Diverse Series of Proline Analogues in an *Escherichia coli* Expression System

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A set of Escherichia coli *expression strains have been defined that are competent for the incorporation of a structurally diverse series of proline analogues under culture conditions that are compatible with high levels of analogue substitution within a proline-rich protein substrate. These bacterial strains have been employed to assay the efficacy of incorporation of noncanonical amino acids into a recombinant-protein test substrate and to create variant polypeptides in which native protein sequences have been globally substituted with imino acid analogues in response to proline codons. We envision that these methods may be used to interrogate the effect of imino acid substitution on protein structure and function and may be particularly informative in the context of structural comparison of a series of modified proteins with respect to the stereoelectronic differences between the incorporated proline analogues.*

Introduction

The imino acid proline occupies a unique niche in protein structural biology as a consequence of its distinctive structural and conformational properties vis-à-vis the other canonical amino acids.[1] In contrast to the latter, proline has an endocyclic amino group that narrowly limits its accessible conformational space, as well as that of surrounding residues when placed in the context of a polypeptide chain, and prevents participation of the prolyl amide group in the hydrogen-bonding interactions that normally act to stabilize protein structure.[2,3] Therefore, proline residues are not easily accommodated within periodic, hydrogen-bonded secondary structure and are often associated with aperiodic features such as turns and loops or with non-α/β periodic structures. Nonetheless, proline serves multiple roles of critical importance for protein structure and function;[4] these roles include delimitation of periodic secondary structure elements,[5] alteration of the polypeptide-backbone trajectory through the formation of reverse turns or helical kinks,[6,7] restriction of conformational entropy in the folded and unfolded states,[8] preferential destabilization of misfolded protein structures,[9] and conformational modulation through the thermodynamically accessible *cis/trans* isomerization of the Xaa–Pro peptide bond (Xaa = another amino acid).[10] In addition, proline residues are often observed within repetitive oligopeptide motifs[11] as central features in which the unique conformational characteristics of the imino acid largely determine the structural and functional properties of the repetitive domain. These proline-rich domains usually occur as either intrinsically unstructured elements[12] associated with protein–protein and protein–ligand recognition[13] or as the structural components of extracellular matrix proteins.[14,15]

Despite the structural importance of proline residues, few experimental methods are available to directly investigate local conformational effects that arise due to the presence of proline residues in polypeptide sequences on the structure and function of the corresponding native, folded proteins. Directed mutagenesis techniques have been applied routinely to replace proline with other canonical amino acids, however these studies are limited in that the intrinsic structural differences between proline and the other canonical amino acids must be carefully considered during interpretation of the experimental results. As a point of contrast, scanning proline mutagenesis has been employed to identify functionally critical regions of protein secondary structure through the disruptive effect of the proline residues on hydrogen-bonding interactions and local peptide conformation.[16] Recently, chemosynthetic[17] and biosynthetic[18] methods have been described that permit substitution of noncanonical amino acids into full-length, native protein sequences in place of canonical amino acids. In principle, these methods enable the substitution of proline residues in polypeptide sequences with noncanonical imino acids that more closely mimic the structural and conformational properties of the canonical amino acid. These investigations can provide important information on the role of proline residues within specific structural contexts, particularly under conditions in which the differences in protein structure and function can be interpreted on the basis of the often subtle stereoelectronic distinctions that are observed between the canonical amino acid and its analogues.[19–24] Of the available methods, the biosynthetic approach holds the most promise for large-scale synthesis of native proteins in which specific canonical amino acids, or sets of amino acids, have been replaced with structurally similar amino acid analogues.[18,25] Several bacterial expression systems have been described that enable global substitution of proline residues in protein sequences with imino acid analogues;[26–30] however, these expression systems have nei-

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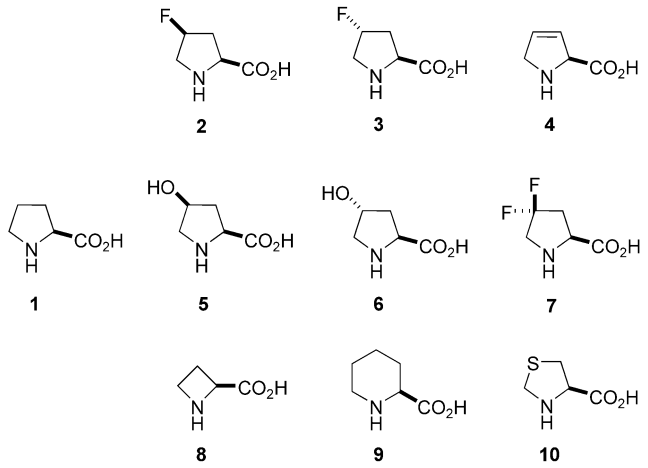
ther been optimized with respect to efficacy of analogue incorporation nor defined in terms of the accessible scope of structural analogues that might be accommodated in a conventional biosynthetic system. We describe herein methods for the multisite substitution of canonical proline residues with a structurally diverse set of noncanonical imino acids that differ substantively in stereoelectronic properties (Scheme 1). We envision that incorporation of these analogues may be employed to address specific structural questions regarding the multiple roles of proline residues in native protein sequences through comparison of the differential effect of the various analogues on the macromolecular structure and function of biosynthetic polypeptides.



Scheme 1. *Chemical structures of proline (**1**) and the imino acid analogues (**2**–**10**) that were tested for use as substrates for biosynthetic incorporation into the elastin-mimetic protein sequence, elastin-**1**: **1**, (2S)-proline; **2**, (2S,4S)-4-fluoroproline; **3**, (2S,4R)-4-fluoroproline; **4**, (2S)-3,4-dehydroproline; **5**, (2S,4S)-4-hydroxyproline; **6**, (2S,4R)-4-hydroxyproline; **7**, (2S)-4,4-difluoroproline; **8**, (2S)-azetidine-2-carboxylic acid; **9**, (2S)-piperidine-2-carboxylic acid; **10**, (4R)-1,3-thiazolidine-4-carboxylic acid.*

Results and Discussion

The elastin-mimetic polypeptide, elastin-**1**, was employed as a test substrate for assessing the efficacy of multisite incorporation of a wide range of proline analogues by using a high-level, IPTG-inducible expression system (IPTG = isopropyl-β-D-thiogalactopyranoside).[31] The repetitive domain of elastin-**1** comprises a concatenated series of pentapeptide repeats based on the consensus sequence (Val-Pro-Gly-Val/Ile-Gly), in which proline residues constitute 20% of the amino acids within the repetitive domain (80 prolines per polypeptide chain). The high density of proline residues and the uniform structural environment of these residues within the polypeptide sequence should provide a well-defined context for evaluating the efficacy of analogue incorporation in terms of its effect on protein yield and analogue substitution level. Moreover, biophysical studies of elastin and elastin-mimetic polypeptides have suggested a crucial structural role for the proline residues within the pentapeptide repeats that may be essential for the normal physiological function of the native polypeptide material.[32,33] Thus, substitution of proline analogues into the repetitive domain of elastin-**1** may provide critical information on the local structural parameters that influence the thermodynamics and kinetics of elastin assembly under physiologically relevant conditions. The construction of the synthetic gene encoding the elastin-**1** sequence has been previously reported in a pET-19b-derived expression system.[31] In order to maintain tighter control over the basal level of gene expression, this DNA cassette was subcloned into a pQE-derived expression vector, pAG2, under the control of a phage T5 promoter that was inducible with IPTG (Table 1). A copy of the over-producing lactose repressor allele *lacI*^{q} was incorporated into the plasmid to maintain transcriptional silencing of the target sequence prior to induction. The decahistidine tag was retained from the original construct to facilitate purification of the target protein from the endogenous host proteins by immobilized metal affinity chromatography.

Table 1. E. coli *strains and plasmids.*

Strain	Genotype	Comments
DG99	F$^-$, *thi*-1, *endA*1, *supE44*, *hsdR*17, *proC*::Tn10, *lacI*^{q}, Δ*(lacZ)M*15	
CAG18515	F$^-$, *proA*3096::Tn10 Kan, *rph*-1	ref. [47]
UMM5	*putA*1::Tn5, *proC*24, *metB*1, *relA*1, *spoT*1, *bglF*18::IS150	ref. [41]
UQ27	*proS*127(ts), *lacZ*4, lam$^-$, *argG*75	ref. [48]

Plasmids	Genotype	Comments
pRAM2	pET-19b/elastin-**1**	ref. [31]
pAG1	pQE60/elastin-**1**	
pAG2	pQE80 L/elastin-**1**	*lacI*^{q}
pME1	pPROTetE.133/pPROLarA.231	Cm^R, p15A ori
pCS-364	pQE30/*proS*	ref. [45]
pWK1	pME1/*proS*	
pWK2	pME1/*proS*(C443G)	

$$MGH_{10}S_2GHID_4KHM[(VPGVG)_4VPGIG]_{16}V \qquad \text{elastin} - \mathbf{1}$$

The facility with which amino acid analogues are incorporated into native proteins in place of a canonical amino acid often depends on the degree of structural similarity between the two molecular species.[18] Native aminoacyl-tRNA synthetases must discriminate between closely related canonical amino acids and have evolved selective editing mechanisms that operate at both the pretransfer and posttransfer stages to ensure high fidelity aminoacylation of tRNA substrates with the cognate amino acid.[34,35] However, structurally similar amino acids can often be incorporated into proteins in place of a canonical amino acid under selective pressure in which the host bacterium is depleted of the canonical amino acid and supplemented with the analogue. As the structural divergence increases between a cognate amino acid and an analogue, the efficiency of incorporation of the analogue decreases, as judged by reduced protein yield and/or appearance of products associated with

amino acid starvation. Given the structural diversity of the proline analogues in Scheme 1, a single technique is unlikely to suffice for incorporation of the entire set of analogues. Three different methods were explored to assess the efficacy of incorporation of various proline analogues into the target polypeptide, in which the host-cell physiology was altered to an increasing degree to accommodate more structurally divergent proline analogues. The result of this process was the definition of conditions for the biosynthesis of a series of elastin derivatives, elastin-**2**–elastin-**10**, based on substitution of the canonical proline residues encoded in the elastin-**1** sequence with the respective imino acid analogues **2**–**10** (Scheme 1). The simplest experimental method employed the auxotrophic *Escherichia coli* strain DG99 (*proC*::Tn10, Tc^R) as a host organism under conditions of proline depletion and analogue supplementation.[26–30] This approach worked well for incorporation of the mono-4-fluoroprolines, **2** and **3**, and 3,4-dehydroproline, **4**, as the native genomic background activity of prolyl-tRNA synthetase within the host bacterium was probably sufficient to recognize and charge these structurally similar proline analogues to the $tRNA^{Pro}$ at a level of efficiency approaching that of the canonical amino acid (Figure 1 a). In contrast, structurally less similar analogues, that is, most of the imino acids in Scheme 1, were not readily incorporated into the elastin sequence with this approach.

An alternative method that has been successfully employed for the multisite substitution of weakly recognized amino acid analogues in response to sense codons involves the coexpression of the wild-type aminoacyl-tRNA synthetase[36] or, in particularly difficult cases, a mutant synthetase that displays more permissive substrate selectivity.[37] Both of these strategies were investigated to facilitate incorporation of the more recalcitrant proline analogues into the target protein substrate. An expression vector was constructed in which variants of *E. coli* prolyl-tRNA synthetase (ProRS) could be expressed under the control of an orthogonal promoter system. This vector system was derived from the modular series of bacterial plasmids reported initially by Lutz and Bujard.[38] Plasmid pME1 contained the transcriptional/translational control elements, multiple cloning site, and chloramphenicol resistance marker of plasmid

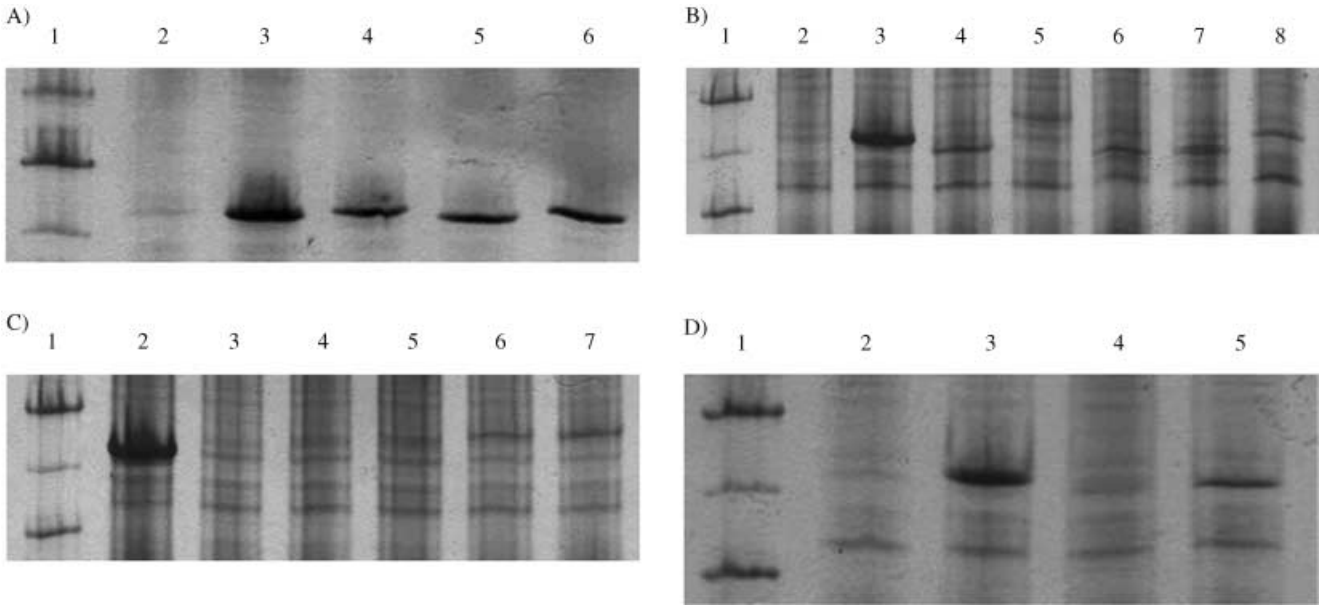


Figure 1. *Sodium dodecylsulfate (SDS) PAGE analysis of whole-cell lysates derived from expression cultures for production of elastin analogues in the presence of proline derivatives* ***1***–***10****. A) Incorporation of proline analogues by using the genomic background activity of prolyl-tRNA synthetase. Expression cultures were derived from* E. coli *strain DG99[pAG2] after 3 h induction with 1 mM IPTG. Lane 1, molecular-weight standards (25, 35, and 50 kDa); lane 2, proline-deficient (negative) control; lane 3, proline-supplemented (positive) control; lane 4, with (2S,4S)-4-fluoroproline (**2**); lane 5, with (2S,4R)-4-fluoroproline (**3**); lane 6, with (2S)-3,4-dehydroproline (**4**). B) Incorporation of proline analogues under conditions of coexpression of wild-type prolyl-tRNA synthetase. Expression cultures were derived from* E. coli *CAG18515[pAG2][pWK1] after 3 h induction with 1 mM IPTG in hyperosmotic (600 mM NaCl) media. Lane 1, molecular-weight standards; lane 2, negative control; lane 3, positive control; lane 4, with (2S,4S)-4-hydroxyproline (**5**); lane 5, with (2S,4R)-4-hydroxyproline (**6**); lane 6, with (2S)-4,4-difluoroproline (**7**); lane 7, with (4R)-1,3-thiazolidine-4-carboxylic acid (**10**); lane 8, (2S)-azetidine-2-carboxylic acid (**8**). Note that the expression culture for analogue* **10** *(lane 7) was performed by using* E. coli *strain UMM5[pAG2][pWK1] under 3 h induction in media supplemented 800 mM sucrose rather than 600 mM NaCl. In addition, the incorporation of proline analogues* **6** *and* **8** *into the target polypeptide resulted in noticeable shifts in the gel mobility as detected by SDS PAGE analysis (lanes 5 and 8). A similar correspondence between hydroxyproline substitution level and decreased electrophoretic mobility has been observed previously for recombinant glutathione-S-transferase expressed under conditions of hydroxyproline supplementation in the auxotrophic* E. coli *strain JM108 (Δ(lac-proAB)).[26] C) Dependence of target-protein accumulation on osmolyte (NaCl) concentration for expression cultures derived from CAG18515[pAG2][pWK1] after 3 h induction with 1 mM IPTG. Lane 1, molecular-weight standards; lane 2, positive control; lanes 3–7, expression in the presence of 0.5 mM (2S,4R)-4-hydroxyproline (**6**) under conditions of increasing hyperosmolarity in culture medium supplemented to final concentrations of 8.5, 100, 200, 400, and 600 mM NaCl, respectively. D) Effect of wild-type and mutant prolyl-tRNA synthetase expression on proline-analogue incorporation. Expression cultures were derived from* E. coli *strain CAG18515[pAG2] after 3 h induction with 1 mM IPTG. Lane 1, molecular-weight standards; lane 2, negative control; lane 3, positive control; lane 4, supplementation with 0.5 mM (2S)-piperidinyl-2-carboxylic acid (**9**) in 600 mM NaCl medium with coexpression of wild-type ProRS from pWK1; lane 5, supplementation with 0.5 mM (2S)-piperidinyl-2-carboxylic acid (**9**) in 600 mM NaCl medium with coexpression of the C443G mutant ProRS from pWK2.*

pPROTetE.133 and the compatible p15A replicon of plasmid pPROLarA.231 (Table 1). Duplex DNA cassettes encoding variants of *E. coli* prolyl-tRNA synthetase were amplified by PCR and cloned as *Kpn* I/*Xba* I fragments into the polylinker of pME1. The ProRS expression cassettes were placed under the control of the $P_L tet$ promoter and were expressed constitutively from the respective plasmids in *E. coli* strains that lacked the *tet* repressor. The incorporation of proline analogues into the elastin-**1** sequence was assayed in the auxotrophic *E. coli* strain CAG18515 (*proA*::Tn10 Kan, Kn^R) under conditions in which recombinant wild-type ProRS was constitutively expressed from plasmid pWK1 (Table 1). Electrophoretic analysis of whole-cell lysates derived from these expression cultures indicated that most of the proline analogues, with the exception of **9** and **10**, supperted a detectable level of target protein expression with respect to the proline-deficient negative control (Figure 1 b). Moreover, hyperosmotic concentrations of osmolytes such as sucrose or sodium chloride in the culture media enhanced the level of accumulation of the target polypeptides in a concentration-dependent manner (Figure 1 c). Hyperosmotic expression cultures have been employed previously to facilitate cotranslational incorporation of (2*S*,4*R*)-4-hydroxyproline, **6**, into test proteins in response to proline codons in an *E. coli* host system.[26] This approach relies on the hyperosmotically induced up-regulation of the biosynthesis of endogenous low-affinity proline transporters (the *putP*, *proP*, and *proU* gene products) in *E. coli*.[39] These proteins can recognize and transport proline analogues into the bacterium, thereby enhancing their cellular concentration and, thus, their functional efficacy as substrates for endogenous prolyl-tRNA synthetase.[26]

However, neither **9** nor **10** could be incorporated into elastin-mimetic polypeptide substrate by using expression strain CAG18515[pWK1], even under conditions of hyperosmolarity. The inability of the latter substrate to be incorporated into elastin-**1** is particularly puzzling as cotranslational incorporation of **10** into proteins has been reported for in vivo[28] and in vitro[40] bacterial expression systems derived from *E. coli*. Recent experimental evidence has suggested that several proline analogues, particularly **4** and **10**, are subject to intracellular oxidative degradation through the action of endogenous *E. coli* enzymes such as L-proline dehydrogenase (*putA*) and Δ^1-pyrroline-5-carboxylate reductase (*proC*).[41] The cotranslational incorporation of **10** into the elastin sequence was explored in *E. coli* strain UMM5, which contains genetic mutations in the chromosomal loci associated with these two enzymes that impair their activity towards oxidative degradation of **4** and **10** (Table 1). Despite the use of this mutant strain, expression of elastin-**10** could only be detected under conditions in which the wild-type ProRS was coexpressed from plasmid pWK1 in hyperosmotic media, with the best yields of isolated protein resulting from culture in media supplemented with 800 mM sucrose (Figure 1 b). However, the yield of isolated elastin-**10** under these conditions was comparable to that of a thiaproline-substituted analogue of recombinant human annexin V,[28] which contained a much lower content of proline residues than the elastin test substrate did. In addition, *E. coli* strain UMM5 improved the isolated yield of the elastin-**4** derivative by approximately an order of magnitude in comparison to the *E. coli* strain DG99, even though the level of target-protein accumulation appeared to be comparable between the two strains as judged by SDS PAGE analysis of whole-cell lysates from expression cultures conducted under similar conditions. These observations emphasized the important effect of host-cell physiology on target-protein accumulation, particularly under conditions in which chemically reactive amino acid analogues are employed as substrates for in vivo ribosomal protein synthesis.

In contrast to the other analogues, (2*S*)-piperidine-2-carboxylic acid, **9**, could not be incorporated into elastin-**1** by using any of the auxotrophic strains, even with coexpression of the wild-type ProRS under hyperosmotic conditions. Previous reports indicated that **9** accumulated intracellularly in *E. coli* under hyperosmotic conditions as a consequence of the action of the osmotically regulated proline transporters derived from expression of *proP* and *proU*.[42] However, analogue **9** did not act as an effective substrate to activate ATP-PP_i exchange in the presence of *E. coli* ProRS in an in vitro assay,[40c] neither did the analogue inhibit ATP-PP_i exchange of *E. coli* ProRS in the presence of the cognate amino acid, proline (ATP-PP_i = adenosine triphosphate–inorganic pyrophosphate). These data suggested that this analogue could not serve as an effective substrate for the aminoacylation reaction, which correlated with the inability of **9** to support the biosynthesis of an elastin-**9** derivative from in vivo expression systems that utilized wild-type *E. coli* ProRS activity. We hypothesized that the larger, six-membered piperidinyl ring of **9** could not be accommodated within the activation site of native *E. coli* ProRS. If this were the situation, then analogue **9** would be effectively precluded from engaging in the aminoacylation reaction with $tRNA^{Pro}$ isoacceptors, and, therefore, could not be cotranslationally incorporated into the target polypeptide in response to proline codons.

A representative steric model for the environment surrounding proline in the activation site of *E. coli* prolyl-tRNA synthetase can be estimated from the crystal structures of the corresponding ProRS enzymes from *Thermus thermophilus* (PDB file: 1H4T) and *Methanothermobacter thermautotrophicus* (PDB file: 1NJ5).[43,44] Both structures have been determined with proline or proline-derived substrate analogues bound in the activation site, and, in both cases, relatively close contacts are observed between the *exo* face of the proline ring and the terminal heavy atom of the side chain belonging to the amino acid that resides within the activation site of the prolyl-tRNA synthetase, that is, Ser288 of *T. thermophilus* ProRS and Cys265 of *M. thermautotrophicus* ProRS. The positioning of this residue may inhibit the ability of the prolyl-tRNA synthetase to accommodate more sterically demanding proline analogues such as **9** within the activation site. The homologous amino acid within the sequence of *E. coli* ProRS (Cys443) has been implicated as a structurally critical residue within the activation site, as chemical derivatization of the sulfhydryl side chain abrogates aminoacylation activity.[45] However, site-directed mutagenesis experiments indicated that this cysteine residue may be substituted by glycine, alanine, or serine with relatively minor attenuation of the aminoacylation activity. In particular, the C443G mutant

of *E. coli* prolyl-tRNA synthetase maintained near-native levels of aminoacylation activity ($k_{cat}/K_M \approx 26\%$ of wild-type ProRS; k_{cat} = rate of catalysis, K_M = Michaelis constant),[45] although the available volume within the activation site should be significantly increased in the mutant as a consequence of the large difference in steric demand between the Cα substituents of glycine and cysteine.

The C443G mutant of *E. coli* prolyl-tRNA synthetase was prepared by site-directed mutagenesis and cloned into the expression plasmid pME1 (Table 1). The ability of analogue **9** to support protein biosynthesis of elastin-**9** was examined in *E. coli* strain CAG18515 under conditions in which wild-type and mutant ProRS variants were expressed from the plasmids pWK1 and pWK2, respectively (Figure 1 d). Electrophoretic analysis of whole-cell lysates from these expression cultures indicated the accumulation of a new protein that migrated at the expected molar mass versus the molecular weight standards, *but only under conditions in which the mutant ProRS was co-expressed within the bacterial culture*. To our knowledge, this represents the first example of a bacterial expression system that is competent for incorporation of the ring-expanded proline analogue **9**. The expression level of the elastin-**9** derivative in the presence of analogue **9** approaches that observed for other proline analogues within the series and suggests that the substitution process is relatively efficient despite the high density of encoded proline sites in the coding sequence of elastin-**1**. Hyperosmotic conditions (600 mM NaCl) within the expression media provided the highest accumulation levels for the target protein, elastin-**9**. This result coincided with the previously reported observation that the intracellular transport and accumulation of **9** was facilitated in the presence of high concentrations of extracellular osmolytes.

Elastin derivatives elastin-**1**–elastin-**10** could be purified to homogeneity from the endogenous proteins of the bacterial host by using immobilized metal affinity chromatography. The isolated yields of the polypeptides (Table 2) ranged from approximately 15–50 mg L^{-1} for fully induced expression cultures in modified minimal medium (NMM) under conventional batch fermentation conditions in a shake-flask culture. The protein yields are quite respectable, especially in consideration of the high density of proline residues in the target polypeptide sequence. These synthetic elastin derivatives have some of the highest effective concentrations of amino acid analogues that have been incorporated into a test substrate by using a biosynthetic approach. Since the average frequency of occurrence of proline residues in a typical protein sequence is significantly lower than that encoded within the amino acid sequence of elastin-**1**, the methods described herein should be sufficient to effect global substitution of proline residues with analogues **2**–**10** in high expressed-protein yield for more conventional polypeptides. Amino acid compositional analysis (Table 2) and MALDI-TOF mass spectrometry (Table 3 and Figure 2) of the elastin derivatives suggested virtually complete substitution of proline with the respective imino acid analogue. Little residual proline content was detected in the recombinant target proteins elastin-**2**–elastin-**10**, and the molecular ions within the mass spectra corresponded well with the calculated masses for elastin derivatives in which the encoded proline residues were completely substituted with the respective analogues (Table 3). Spectroscopic analyses of selected elastin derivatives demonstrated structural features that were commensurate with high levels of incorporation of the respective imino acid analogues (Figure 3) in comparison to the canonical amino acid within the same structural context.

Table 2. *Isolated yields and proline-analogue incorporation levels for purified elastin derivatives under optimized expression conditions for cotranslational incorporation of imino acid analogues **1**–**10**.*

Elastin derivative	Mass yield [mg L^{-1}]	Percentage of proline analogue
Theoretical	–	18.9
elastin-**1**[a]	46.0	19.9
elastin-**2**[a]	50.8	21.6
elastin-**3**[a]	45.0	21.4
elastin-**4**[b]	34.4	18.6
elastin-**5**[c]	41.0	21.3
elastin-**6**[c]	26.0	21.3
elastin-**7**[c]	15.2	23.1
elastin-**8**[c]	16.5	19.4[f]
elastin-**9**[d]	16.0	13.3[g]
elastin-**10**[e]	27.3	20.1

[a] Expression from *E. coli* strain DG99[pAG2]. [b] Expression from *E. coli* strain UMM5[pAG2]. [c] Expression from *E. coli* strain CAG18515[pAG2][pWK1] at 600 mM NaCl. [d] Expression from *E. coli* strain UMM5[pAG2][pWK1] at 800 mM sucrose. [e] Expression from *E. coli* strain CAG18515[pAG2][pWK2] at 600 mM NaCl. [f] The computed value for the proline analogue (2*S*)-azetidine-2-carboxylic acid reflects the sum of peaks in the chromatogram that resulted from hydrolytic cleavage of the analogue under the experimental conditions employed in the amino acid analysis. The major product eluted at the position expected for homoserine, which could have arisen from hydrolytic cleavage of the azetidine ring under the acidic conditions employed in the amino acid analysis. [g] The peak in the chromatogram associated with (2*S*)-piperidine-2-carboxylic acid could not be effectively integrated due to elution during the buffer change between methionine and valine. The residual proline content (0.12%) could be computed from the chromatogram as an effective upper limit on the canonical amino acid substitution, which suggested that the majority of the encoded proline sites were occupied by the amino acid analogue.

Table 3. *MALDI-TOF mass spectrometric data for purified elastin derivatives incorporating proline analogues **1**–**10**.*

Elastin derivative	Calculated *m/z*[a]	Observed *m/z*	$\lvert\Delta m/z\rvert$ (% error)
elastin-**1**	35866.36	35851.85	14.51 (0.04)
elastin-**2**	37305.60	37224.26	81.34 (0.22)
elastin-**3**	37305.60	37225.46	80.14 (0.21)
elastin-**4**	35705.10	35726.39	21.29 (0.06)
elastin-**5**	37146.31	37121.18	25.13 (0.07)
elastin-**6**	37146.31	37115.23	31.08 (0.08)
elastin-**7**	38744.84	38690.87	53.97 (0.14)
elastin-**8**	34744.22	34716.04	28.18 (0.08)
elastin-**9**	36988.50	36950.08	38.42 (0.10)
elastin-**10**	37309.02	37351.87	42.85 (0.11)

[a] Molar masses for the elastin derivatives were calculated based on complete substitution of proline with the corresponding amino acid analogue. The calculated molar mass assumes proteolytic cleavage of the N-terminal methionine residue as a consequence of the endogenous activity of *E. coli* methionyl-aminopeptidase.[51]

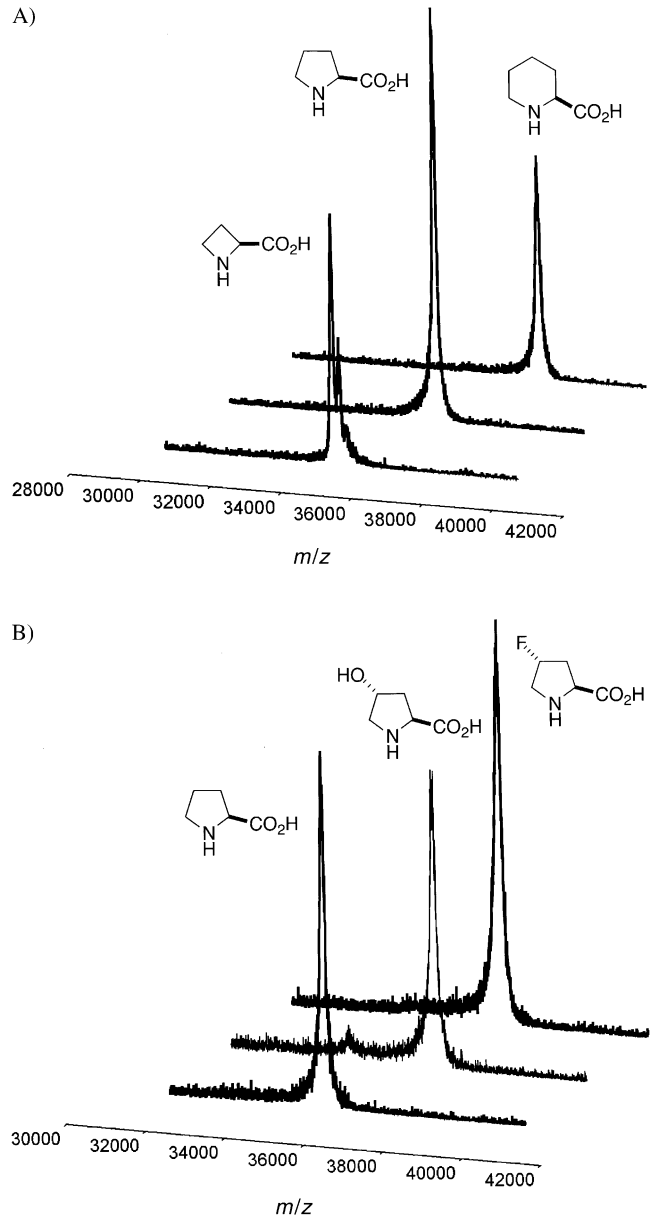


Figure 2. Comparative MALDI-TOF mass spectrometric analysis of selected elastin derivatives. A) MALDI-TOF mass spectra of elastin polypeptides incorporating contracted- and expanded-ring analogues in place of the canonical proline residues in the repetitive pentapeptide domains. B) MALDI-TOF mass spectra of elastin polypeptides incorporating functionalized analogues of proline in place of the canonical proline residues in the repetitive polypeptide domains. Note that the observed differences in molar mass between the elastin derivatives are commensurate with a high level of substitution of the canonical amino acid with the corresponding analogue.

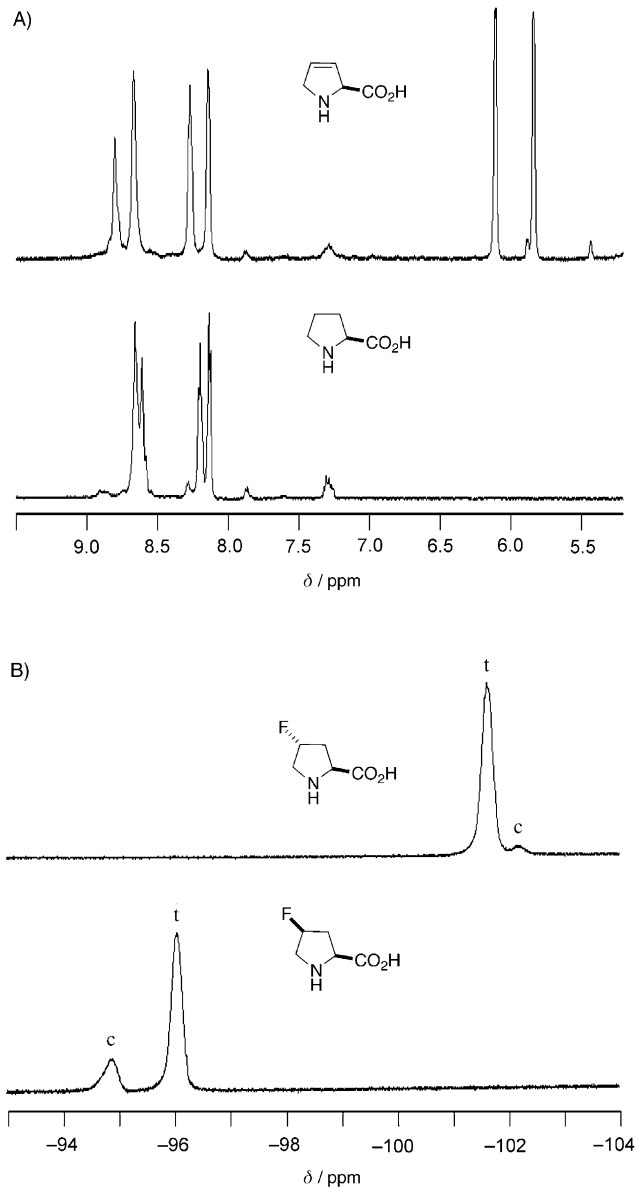


*Figure 3. NMR spectroscopic analyses of selected elastin derivatives. A) ^{1}H NMR spectra of elastin-**1** (lower spectrum) and elastin-**4** (upper spectrum) in the downfield region encompassing the amide proton and olefinic proton resonances. Note the presence of the olefinic protons in the upper spectrum that are consistent with incorporation of (2S)-3,4-dehydroproline at a level commensurate with that of the amide protons of the repeat unit. The minor resonances within the spectrum can be attributed to the presence of the* cis *configuration of the Val–Pro peptide bond. B) ^{19}F NMR spectra of elastin-**2** (lower spectrum) and elastin-**3** (upper spectrum) indicating the incorporation of (2S,4S)-4-fluoroproline and (2S,4R)-4-fluoroproline, respectively, into the recombinant polypeptide. The symbols t and c refer to the* trans *and* cis *isomers, respectively, of the Val–Pro peptide bonds within the repeat units. These spectroscopic assignments were based on comparison with ^{19}F NMR chemical shift values for 4-fluoroproline model compounds as described in the literature.[19,46] The greater relative abundance of the* cis *peptidyl-bond isomer in elastin-**2** versus elastin-**3** reflects the increased thermodynamic preference for this configuration in (2S,4S)-4-fluoroproline derivatives.[19,27]*

In summary, we have defined a series of *E. coli* expression strains that are competent for the incorporation of a structurally diverse set of proline analogues and developed culture conditions that are compatible with high levels of analogue substitution within a test-protein substrate. These bacterial strains may be employed to assay the efficacy of incorporation of novel proline analogues into recombinant proteins or to create variant polypeptides in which native protein sequences have been globally substituted with imino acid analogues in response to proline codons. Moreover, these methods may be used to investigate the effect of imino acid substitution on protein structure and function and may be particularly infor-

mative in the context of structural comparison of a series of substituted proteins with respect to the stereoelectronic differences between the incorporated imino acid analogues. Preliminary studies of the elastin derivatives have indicated that the incorporation of imino acid analogues into the polypeptide can have quite dramatic effects on the thermodynamic and conformational properties associated with the self-assembly of elastin-mimetic polypeptides. The results of these studies will be reported in due course.

Experimental Section

Materials and methods: All chemical reagents were purchased from either Fisher Scientific, Inc. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Proline analogues **2**, **3**, **4**, **8**, and **9** were purchased from Bachem Bioscience, Inc. (King of Prussia, PA), and **5**, **6** and **10** were obtained from Sigma Chemical Co. (2*S*)-4,4-Difluoroproline, **7**, was synthesized from *N*-tert-butoxycarbonyl-(2*S*,4*S*)-4-hydroxyproline methyl ester (Bachem Bioscience, Inc.) by using a modification of the method of Demange et al.[46] IPTG was purchased from Research Products International Corp. (Prospect, IL). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA), shrimp alkaline phosphatase was obtained from Roche Applied Science (Indianapolis, IN), and Platinum *Pfx* DNA polymerase was obtained from Invitrogen Corp (Carlsbad, CA). Plasmid pPROTetE.133 was obtained from BD Biosciences, Inc. (Palo Alto, CA), and plasmids pQE-60 and pQE-80L were obtained from Qiagen, Inc (Valencia, CA). Plasmid pCS-364, which encodes the native *E. coli* prolyl-tRNA synthetase as an N-terminal hexahistidine fusion in plasmid pQE-30,[45] was a gift from Professor Karin-Musier Forsyth of the University of Minnesota and plasmid pPROLarA.231 was obtained from Professor Rik Myers of the University of Miami. *E. coli* strain DG99 was purchased from the American Type Culture Collection (ATCC no. 47041) and strains CAG18515[47] and UQ27[48] were obtained from the *E. coli* Genetic Stock Center at Yale University. *E. coli* strain UMM5[41] was provided by Professor Charles Deutch of Arizona State University. Synthetic oligonucleotides were purchased from either Sigma–Genosys, Inc. (The Woodlands, TX) or Integrated DNA Technologies (Coralville, IA) and were used as received. TALON metal affinity resin was purchased from BD Biosciences, Inc. NMM medium was prepared according to the protocol of Budisa et al.,[49] with the exception that proline was not added to the medium prior to cell culture. Procedures for the manipulation of DNA, the transformation of competent cells, and the growth and induction of bacterial cultures were adapted from the published literature[50] or instructions supplied by manufacturers. Reagents for the manipulation of bacteria and DNA were sterilized by either autoclave or passage through a 0.22-μm filter. Enzymatic reactions were performed in the reagent buffers supplied by the manufacturers. Site-directed mutagenesis was performed by using the Quik-Change mutagenesis technique from gene-specific oligonucleotide primers.

Physical and analytical measurements: DNA sequence analyses were performed at the Emory University Core DNA Sequencing Facility on a Perkin–Elmer ABI Prism model 377 DNA sequencer. Amino acid compositional analyses were performed at the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University. Protein electrophoresis was performed on 10–15% gradient, discontinuous SDS polyacrylamide gels on a PhastSystem apparatus from Amersham Pharmacia Biotech and visualized with a silver staining procedure.

The molar masses of elastin analogues were determined by MALDI-TOF MS on an Applied Biosystems Voyager System 428 mass spectrometer in the positive linear mode. The matrices, 2-(4-hydroxyphenylazo)benzoic acid (HABA) or 4-hydroxy-3-methoxycinnamic acid, were used at a concentration of 10 mg mL^{-1} in water/2-propanol (50:50). The protein solution (1 mg mL^{-1} in distilled water) was mixed with the matrix solution in a ratio of 1:10 and dried under vacuum or air. Bovine serum albumin was used as a standard for external calibration.

Solution NMR spectra were acquired with either a Varian INOVA 600 instrument (599.742 MHz, ^{1}H) or a Varian UNITY 600 instrument (564.044 MHz, ^{19}F) equipped with a 5-mm ^{1}H/^{19}F probe. Spectra were collected at 4 °C on specimens consisting of protein (10 mg) dissolved in sterile H_2O/D_2O (70:30) in which the pH value was adjusted to 2.7 to retard amide proton exchange rates. Chemical shifts for ^{1}H NMR spectra were referenced and reported relative to internal sodium 2,2-dimethyl-2-silapenta-5-sulfonate (0.0 ppm). Standard solvent-suppression techniques were employed to reduce the signal due to the residual protons of H_2O in the ^{1}H NMR spectra of aqueous solutions. Chemical shifts for the ^{19}F NMR spectra are referenced and reported relative to an external sample of aqueous (10% v/v) trifluoroacetic acid (0.0 ppm).

Plasmid construction: The plasmid pRAM2 was employed as a source of the gene encoding the elastin-**1** sequence.[31] Double digestion of pRAM2 with *Nco* I and *Bam*H I afforded a duplex DNA cassette of approximately 1300 bp, which was inserted into the compatible *Nco* I/*Bgl* II sites of plasmid pQE-60 to generate plasmid pAG1. An *Eco*R I/*Hin*D III cassette derived from pAG1 was excised, isolated, and cloned into the compatible sites of plasmid pQE-80L to afford pAG2, which incorporated a copy of the overproducing repressor allele *lacI^q* to ensure tight control of the basal level of transcription prior to induction with IPTG. Plasmid constructs encoding variants of the *E. coli* prolyl-tRNA synthetase were derived from plasmid pCS-364. The internal *Kpn* I restriction site within the *E. coli* ProRS gene was removed by site-directed mutagenesis, which introduced a silent mutation into the coding sequence of the prolyl-tRNA synthetase. Mismatch primers were employed to introduce the C443G mutation within the activation site of the wild-type *E. coli* ProRS gene in a *Kpn*I-negative mutant of pCS-364. The identity of the plasmid constructs was confirmed by double-stranded DNA sequence analysis. The wild-type and mutant ProRS genes were amplified from the plasmid constructs by PCR in which unique *Kpn* I and *Xba* I restriction sites were incorporated at the 5′- and 3′-termini, respectively, through gene-specific oligonucleotide primers. The upstream PCR primer incorporated a new ATG initiation codon and annealed downstream of the sequence encoding the hexahistidine tag (encoded within the DNA target) to prevent inclusion of this sequence within the PCR products. An acceptor plasmid for the ProRS genes was constructed from ligation of the *Avr* II/*Spe* I fragment of pPROTetE.133, containing the transcriptional/translational control elements, multiple cloning site, and chloramphenicol resistance gene, to the corresponding fragment of pPROLarA.231, containing the p15A origin of replication. The ProRS genes were cloned as *Kpn* I/*Xba* I DNA cassettes into the corresponding sites within the polylinker of the acceptor plasmid pME1 to generate plasmids pWK1 and pWK2, which encoded wild-type ProRS and the C443G mutant, respectively, under control of the $P_{L}tet$ promoter. These synthetic plasmid constructs were screened for prolyl-tRNA synthetase activity by measurement of their ability to rescue a temperature-sensitive ProRS phenotype in *E. coli* strain UQ27. The plasmids were transformed into competent cells of *E. coli* UQ27 and cultured at 30 °C. Luria–Bertani (LB) solid media

(34 μg mL^{-1} chloramphenicol) were then streaked with single colonies of the transformants and identical plates were incubated at permissive (30 °C) and nonpermissive (42 °C) temperatures. The acceptor plasmid pME1 lacking a ProRS gene was employed as a negative control. Plasmids that displayed the appropriate growth phenotype were characterized by DNA sequence analysis to ascertain the identity of the ProRS sequence.

Bacterial growth and expression: The expression vector encoding elastin-mimetic protein sequence pAG2 was transformed into *E. coli* strain DG99 or cotransformed with either pWK1 or pWK2 into *E. coli* strains CAG18515 or UMM5 to generate the expression strains employed for these studies. Single colonies of the expression strains were inoculated into sterile LB broth (50 mL) supplemented with the appropriate antibiotics (100 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol) as required for plasmid maintenance. The overnight culture was centrifuged at 4000 *g* for 10 min to isolate the cells, which were resuspended in sterile NMM medium (1 L) supplemented with the appropriate antibiotics. The proline concentration was adjusted to 0.3 mM from a sterile 100× stock solution. The culture was incubated with agitation (225 rpm) at 37 °C until the optical density at 600 nm (OD_{600}) reached between 0.8 and 1.0 absorbance units. The cells were then collected by centrifugation at 4000 *g* for 10 min. The cell pellet was washed with cold (4 °C), sterile 0.9% aqueous NaCl twice (2×100 mL) and resuspended in sterile NMM medium containing antibiotics but without proline supplementation. After incubation at 37 °C for 30 min, the proline analogues were added to a final concentration of 0.5 mM from sterile 100× stock solutions. For expressions involving proline analogues **5**–**10**, the osmolarity of the culture was adjusted prior to induction by addition of the appropriate osmolytes to a final concentration of either 600 mM for NaCl or 800 mM for sucrose. An aliquot of aqueous 1.0 M IPTG was added to the cultures to a final concentration of 1 mM to induce synthesis of the elastin-mimetic protein. After a 3 h induction period, the cells were harvested by centrifugation at 4000 *g* and 4 °C for 20 min. The cell pellet was resuspended in lysis buffer (50 mL, 50 mM sodium phosphate, 300 mM NaCl, pH 7.0) and stored at −80 °C.

Protein purification: The frozen cells were lysed by three freeze/thaw cycles. Lysozyme (1 mg mL^{-1}), ethylenediaminetetraacetate-free protease inhibitor cocktail, benzonase (25 U mL^{-1}), and $MgCl_2$ (1 mM) were added to the lysate and the mixture was incubated with shaking at 4 °C overnight. The cell lysate was centrifuged at 40000 *g* for 30 min at 4 °C. Supernatant and pellet were separated and analyzed by SDS PAGE to determine the location of the target protein. For soluble elastin-mimetic proteins derived from **1**, **2**, **3**, **5**, **6**, and **8**, the supernatant was loaded onto cobalt-charged TALON resin (5 mL) and washed with lysis buffer (50 mL) containing 20 mM imidazole. The target protein was eluted with elution buffer (20 mL, 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.0) and dialyzed (molecular weight cut-off = 10 kDa) against distilled deionized water (5×4 L). The dialysate was lyophilized to produce a white spongy solid. For elastin-mimetic polypeptides derived from **4**, **7**, **9**, and **10**, the insoluble target protein was resuspended in denaturing lysis buffer (100 mL, 50 mM sodium phosphate, 300 mM NaCl, 6 M urea, pH 7.0) and shaken at 4 °C overnight. The resulting mixture was centrifuged at 40000 *g* and 4 °C for 30 min. SDS PAGE analysis indicated that the majority of the target protein dissolved under these conditions. The soluble fraction was loaded onto cobalt-charged TALON resin (10 mL) that had been previously equilibrated with denaturing lysis buffer. The target protein was washed with denaturing lysis buffer (100 mL) containing 20 mM imidazole and eluted with denaturing lysis buffer (40 mL) containing 250 mM imidazole. The eluted target protein was dialyzed (molecular weight cut-off = 10 kDa) against a diminishing urea step gradient from 6–1 M and, subsequently, against distilled water (5×4 L). Lyophilization of the dialysate produced the elastin-mimetic polypeptides as white spongy solids.

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