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Residue-specific bioincorporation of non-natural, biologically active amino acids into proteins as possible drug carriers: Structure and stability of the *per*-thiaproline mutant of annexin V

(amino acid analogs/protein mutants/protein folding/proline/drug delivery)

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ABSTRACT **Residue-specific bioincorporation of 1,3-thiazolidine-4-carboxylic acid [thiaproline, Pro(S)], a non-natural amino acid analog of proline, into human recombinant annexin V was achieved with a proline-auxotrophic *Escherichia coli* strain by fermentation procedures in minimal medium. Quantitative replacement of proline with thiaproline was confirmed by mass-spectrometric, amino acid, and x-ray crystallographic analyses. The wild-type protein and its *per*-Pro(S) mutant were found to crystallize isomorphously and to show identical three-dimensional structures in crystals. In solution the dichroic properties of the wild-type and *per*-Pro(S) protein confirmed nearly identical overall folds. From thermal denaturation experiments, however, a reduced T_m (−4.5 K) value was determined whereas the van't Hoff enthalpy and entropy were not significantly affected. Therefore, protein mutants containing bioactive amino acid analogs like thiaproline at multiple sites would be expected to fully retain their functional properties, including immunogenicity, and thus could serve as promising vehicles for targeted drug delivery.**

Bioincorporation of non-natural amino acids into proteins has become an increasingly emerging field, not only because of the applications in structural research, but also for investigating function and stability because this approach allows for coupling of chemical modification with labeling of target proteins during their translation. In general, there are two approaches for bioincorporation of noncoded amino acids into proteins. The *in vitro* suppression procedure (1) exploits nonsense suppression phenomena where chemically misacylated and modified suppressor tRNAs are used in a cell-free translational system. The gene encoding the protein to be studied with the introduced nonsense (e.g., amber) mutation is translated in this system, which results in incorporation of the amino acid into the growing polypeptide chain at the specified site. Conversely, the *in vivo* approach requires auxotrophic expression hosts. This method is much simpler, and incorporation works under the strong selective pressure (selective pressure incorporation) obtained by a stable auxotrophism and a controllable strong expression system, which leads to a residue-specific labeled protein (2, 3).

The imino acid proline has a special position among the natural amino acids with its ability to restrict protein chain conformations because *cis/trans* isomerization (caused by the high energy barrier) acts as a rate limiting step in protein folding. The rotational barrier of *cis/trans* isomerization is crucial for the kinetic control of stability, reactivity, and recognition of peptides and proteins containing proline (4).

The non-natural proline analog thiaproline [1,3-thiazolidine-4-carboxylic acid, Pro(S)] belongs to the group of pseudoprolines on oxazolidine and thiazolidine basis, which are used as masked serine, threonine, and cysteine residues in peptide synthesis (5, 6). It has the same number of chemical bonds and very similar bond lengths and angles and therefore can be considered as isosteric to proline (Scheme 1). Proline analogs often are incorporated into synthetic peptides for different purposes, e.g., increased bioavailability (4), although there is no report about efficient bioincorporation of pseudo-proline analogs into proteins.

To follow up our previous studies on the expression of protein mutants with quantitative replacement of the methionine residues by the chalcogen analogs methoxinine, norleucine, selenomethionine, and telluromethionine by using the selective pressure incorporation method (2, 3), in the present study we have investigated the bioincorporation of proline analogs into human recombinant annexin V and the effects of such mutations on structure and stability of the protein. Annexin V contains five surface-exposed and thus solvent-accessible proline residues (7). Pro-13 is located near the N terminus in a flexible tail; Pro-87, Pro-119, and Pro-248 are in two short loops that connect helices, and Pro-163 is positioned in a larger loop consisting of nine residues. The Pro residues are in neighboring positions to aromatic amino acids, e.g., Pro-13 is very close to two Phe residues (Phe-12 and Phe-15), Pro-87 is connecting two helices that contain a cluster of three tyrosines, whereas Pro-163 and Pro-248 are located near a cluster of tyrosines and phenylalanines where additionally the only tryptophan residue (Trp-187) is positioned.

It is well known that several amino acid-like natural and non-natural compounds act as drugs or represent potentially interesting new ones; e.g., thiaproline acts as thiol antioxidant, free radical scavenger, and effective nitrite-trapping agent in the human body. It is also known as a drug in the treatment of different diseases, although its role in reversal of cancer still remains controversial (8). Correspondingly, synthetic or biosynthetic incorporation of such pharmacologically active natural or non-natural amino acids into peptides or proteins as carriers and vehicles could well represent an interesting new delivery and targeting system in human medicine.

MATERIALS AND METHODS

Growth, Bacterial Strains, and Culture Conditions. Proline auxotrophic *Escherichia coli* DSM 1563 (F^- λ^s *thr*$^-$ *leu*$^-$ *arg*$^-$

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Abbreviations: CD, circular dichroism; wt, wild type.

Data deposition: Structure Asav reported in this paper has been deposited in the Protein Data Bank, Biology Department, Brookhaven National Laboratory, Upton, NY 11973 (reference Asav).

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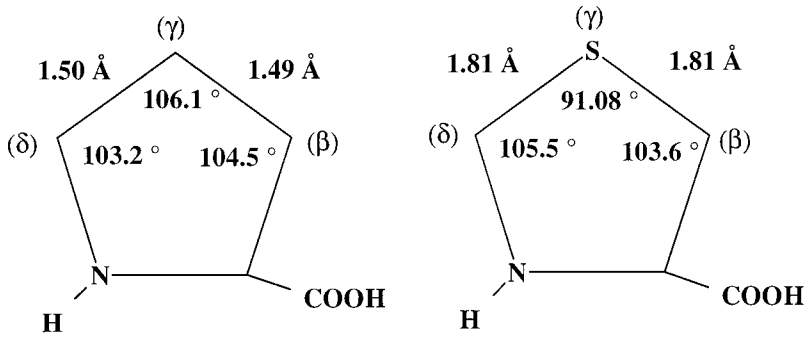

Scheme **1**

The structures of proline (*Left*) and its non-natural analog thiaproline (*Right*). The values for thiaproline were taken from the Cambridge database for small molecules, the data for proline are from ref. 18.

pro$^-$ *his*$^-$ *thi*$^-$ *SmA*^{r} *ara*$^-$ *xyl*$^-$ *mtl*$^-$ *gal*$^-$) was used for all expression experiments. The cells are transformed with two plasmids: ampicillin resistant pRSET-PP4 (2) (which contains the annexin V gene sequence under the control of T7 promoter) and kanamycin resistant pGP1–2 (which contains a gene for T7 polymerase) (9). Cultures were grown under the selective pressure of 100 mg/liter of ampicillin and 70 mg/liter of kanamycin. All growth experiments were performed in New Minimal Medium (2, 3).

Bacterial growth and expression experiments were performed as follows: 5 ml New Minimal Medium (NMM) with 0.3 mM L-Pro were inoculated with 5 μl of transformed *E. coli* DSM 1563 glycerol culture and left to grow overnight. One milliliter of overnight culture then was used as inoculum for 100 ml of prewarmed (30°C) NMM. Cultures were grown in the presence of limiting concentrations of proline (0.05 mM). They were induced by heat jump to 42°C for 30 min after 8, 10, or 12 hr of fermentation (after cessation of growth, i.e., when all proline is exhausted in the media). Immediately after the induction, the proline analog has been added to a final concentration of 0.3 mM. Cells then were allowed to express the mutant protein in the 3- to 6-hr time course. The proline analogs 1,3-thiazolidine-4-carboxylic acid (commercial sample, Fluka AG, Neu-Ulm), 1,3-oxazolidine-4-carboxylic acid (6), and 1,3-selenazolidine-4-carboxylic acid (10) were supplied as free amino acids or in the acetylated form to facilitate their cellular uptake (11). The mutant annexin V was isolated and purified as described elsewhere (2). The purity of the recombinant protein was analyzed by SDS/PAGE (silver staining), and by HPLC profile and electrospray mass spectra analysis. Concentrations of protein samples were determined spectrophotometrically by using an extinction coefficient of 22,500 M^{-1} cm^{-1}.

Biophysical Measurements. Fluorescence spectra were recorded with a Perkin–Elmer Luminescence Spectrometer (LS50B) equipped with digital software. Protein samples (2.5 μM) in PBS (12) were excited at 280 (slit 2.5 nm), and emission spectra were recorded in the 310- to 400-nm range.

Circular Dichroism (CD). CD spectra were recorded on a dichrograph Mark IV from Jobin Yvon (Longjumaeu, France) equipped with a thermostated cell holder and connected to the data station for signal averaging and processing. All spectra are averages of five scans and are reported as mean residue molar ellipticity ($[\Theta]_R$) in degrees $\times$ cm^2 $\times$ $dmol^{-1}$. Quartz cells of 0.1 cm optical path length and concentrations of 0.08 mg protein/ml in PBS containing 10% glycerol were used.

CD Melting Curves. CD melting curves were determined by monitoring the changes in dichroic intensity at 222 nm in function of temperature. For thermal denaturation experiments in the range of 5–95°C a heating rate of 0.5°C/min was applied with a Lauda RKS thermostat by using oil-jacketed cylindrical cells (Hellma, Forest Hills, NY).

Van't Hoff Analysis. The van't Hoff plots of the thermal denaturation are linear through the T_m region, thus allowing an estimation of the enthalpy (ΔH_m) of unfolding at T_m. Because at T_m $\Delta G = 0$ the entropy of unfolding (ΔS_m) at the transition region can be calculated from $\Delta S_m = \Delta H_m/T_m$. Thermal denaturation curves were fitted by using the Gibbs–Helmholtz equation (13) where the ΔC_p value (2,500 cal/mol) was determined empirically from the amino acid composition (14). Data for the unfolding transitions were fitted by using the nonlinear regression analysis program KaleidaGraph (version 3.0, Synergy Software, Reading, PA).

X-Ray Analysis. *per*-Pro(S) annexin V crystallized under the same conditions as the wild-type (wt) protein and the crystals were of the same space group (R3) exhibiting nearly the identical unit cell constants as crystals of wt annexin V (7). The crystals were harvested by using the reservoir solution and mounted in thin-walled glass capillary tubes. X-ray diffraction data were collected on a 300-mm MAR-Research image plate detector attached to a Rigaku RU200 rotating anode x-ray generator providing graphite monochromatized CuK_α radiation. Data were processed by using the DENZO and SCALEPACK programs (15), and loaded, scaled, and merged with CCP4 suite (16). Crystallographic refinement was done in one cycle

Table 1. Data collection and refinement parameters

Diffraction data	
Space group	R3
Cell constants	$a = b = 99.6$ Å, $c = 97.0$ Å
Limiting resolution	2.5 Å
No. of measured reflections	33,698
No. of unique reflections	12,532
R_{merge}*	4.9%
Completeness (25.0–2.5 Å)	99.3%
(2.55–2.5 Å)	95.8%
I/s(I)	9.5
Refinement and final model	
Resolution range	8.0–2.5 Å
Reflections used	12,015
Crystallographic *R* factor† (free *R* factor‡)	18.6% (25.9%)
R m.s. deviations bond lengths	0.010 Å
bond angles	1.411°
No. of non-hydrogen protein atoms	3,070
Calcium ions	5
Solvent molecules	152

*$R_{merge} = \sigma[I(h)_i - \langle I(h)\rangle]/\sigma\langle I(h)\rangle$; $I(h)_i$ is the observed intensity of the ith measurement of reflection h, and $\langle I(h)\rangle$ the mean intensity of reflection h calculated after loading and scaling.
†*R* factor $= \sigma[|F_{obs}| - F_{calc}|]/\sigma|F_{obs}| \times 100$.
‡R_{free} was calculated randomly omitting 10% of the observed reflections from refinement and *R* factor calculation.

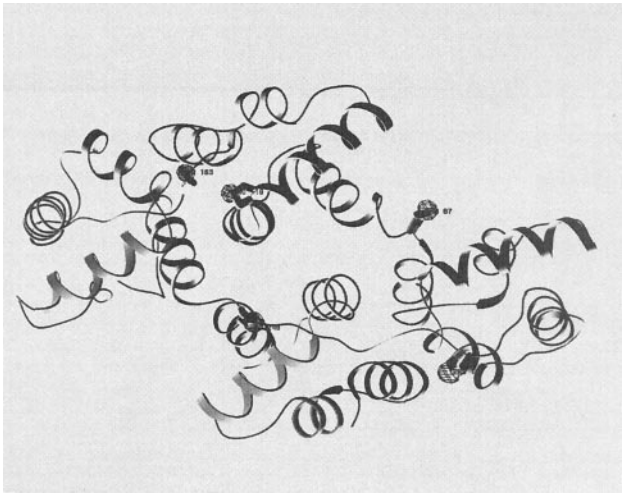

FIG. 1. Ribbon plot of human recombinant annexin V (top view) with difference densities at Cγ for all proline residues successfully substituted with thiaprolines (contouring level is 1.5 σ).

of gradient minimization by using the previous model of annexin V (7), the program X-PLOR (17), and target values of Engh and Huber (18). The new F_o-F_c Fourier map showed extra electron density in the Cγ position of all the prolines. These extra densities did not appear when parameters for thiaproline were used. The model and maps were observed on the graphic station (Silicon Graphics, Mountain View, CA) by using the program TURBO-FRODO (19). The stereochemistry of the model is correct as checked with the program PROCHECK (20). The final *R* factor was 18.6%. The data collection and refinement statistics are summarized in Table 1.

RESULTS

Expression of the annexin V mutants was performed in the proline-auxotrophic *E. coli* strain DSM 1563 containing two plasmids with the T7 expression system under efficient temperature control and by using an optimized minimal medium as reported previously (3). The *per*-Pro(S) mutant was produced at levels comparable to those of the wt annexin V independently of whether thiaproline was supplied in the free or acetylated form. At its isolation the quantitative replacement of Pro by Pro(S) was confirmed by x-ray crystallographic methods (Fig. 1) and electrospray mass spectrometry (expected molecular mass: 35,897 Da; found: 35,894 ± 4.02 Da) (Fig.

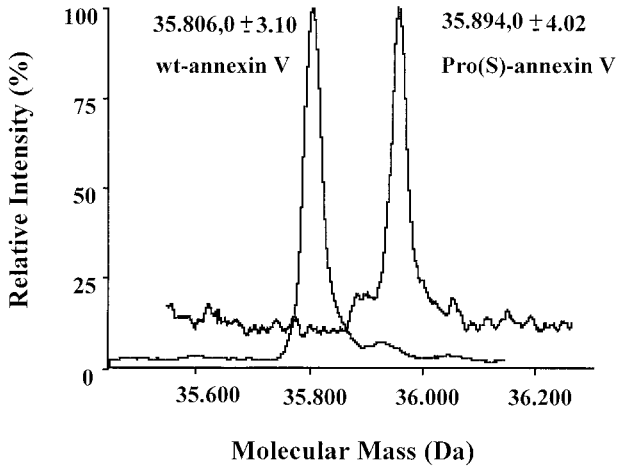

FIG. 2. Electrospray mass spectra of wt and *per*-Pro(S) annexin V. The deconvoluted spectra of separate measurements were superimposed at the same mass scale.

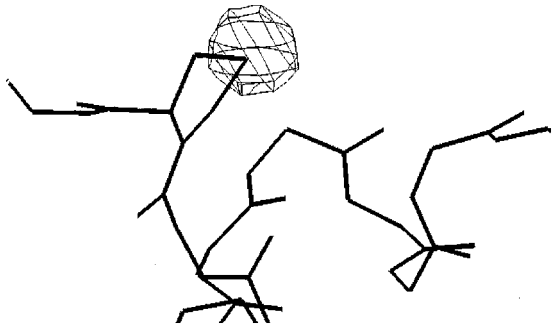

FIG. 3. Electron density difference between -CH_2- and sulfur at the site of replacement (Cγ) shown by the difference Fourier map ($F_{Pro(S)}$–F_{Pro}) contoured at 2.8 σ for the residue Pro-87.

2). Amino acid analysis revealed the absence of Pro and an equal increase in the content of Ser because of identical t_R values of Ser and Pro(S) as determined in separate experiments.

Bioincorporation of the oxa-analog 1,3-oxazolidine-4-carboxylic acid [Pro(O)] failed as previously observed in the case of the oxa-analog of methionine (2). Apparently Pro(O) is not activated and loaded onto $tRNA^{Pro}$ by the prolyl-tRNA synthetase. However, chemical stability could play a decisive role in this context as the stability of Pro(O) is known to be 10^4 times lower than that of Pro(S) (21). On the other hand, it has been reported that selenaproline [Pro(Se)] is activated by the prolyl-tRNA synthetase and incorporated into the growing polypeptide chain, but that apparently it impairs further chain elongation (10). This report was fully confirmed in the present study because the *per*-Pro(Se) mutant of annexin V could not be detected even in trace amounts by gel electrophoresis.

The crystals of wt annexin V and its *per*-Pro(S) mutant were found to be fully isomorphous, and the presence of sulfur in all of the proline residues was confirmed by the difference electron density F_o-F_c map resulting from the extra density in the Cγ position (Fig. 3). Similarly, a comparison of the CD spectra of wt and *per*-Pro(S) annexin V in PBS at 20°C revealed no effect of the mutations on the overall three-dimensional fold of the protein (Fig. 4). Therefore, both methods (CD spectroscopy and x-ray crystallography) excluded significant effects of the local geometry of the thiaproline residues (Scheme 1) on the secondary structure of the mutant.

The thermal unfolding, however, resulted in different denaturing profiles of the wt and *per*-Pro(S) protein (Fig. 5). The analysis of the transition curves indicated a decrease of the

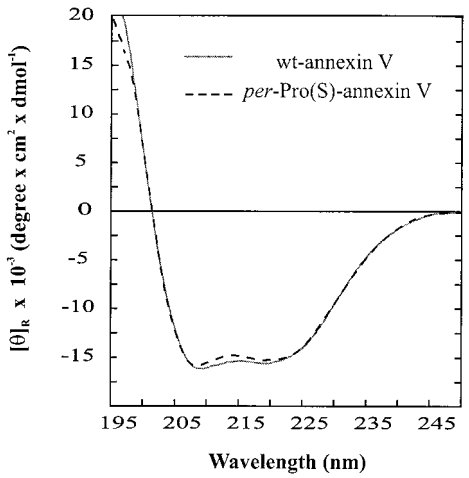

FIG. 4. CD spectra of wt and *per*-Pro(S) annexin V in PBS containing 10% glycerol at 20°C.

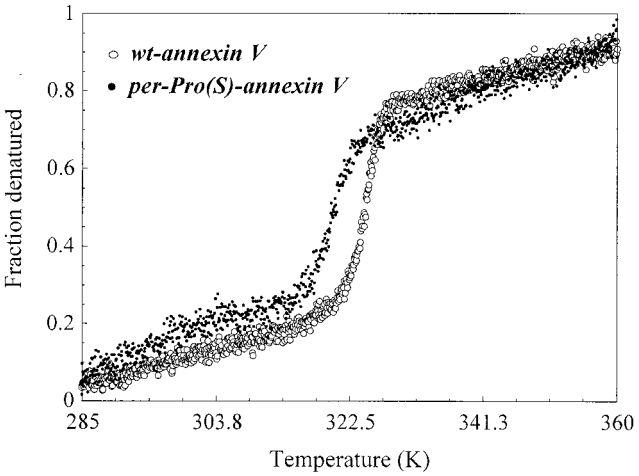

FIG. 5. Thermal denaturation of wt (●) and *per*-Pro(S) annexin V (○) in PBS containing 10% glycerol. Fractions of denatured protein are calculated from CD data monitored at 222 nm.

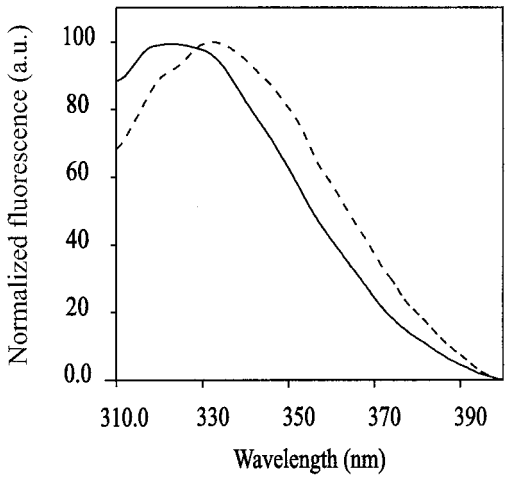

FIG. 6. Fluorescence emission spectra for native (solid line) and *per*-Pro(S) annexin V (dashed line) at 20°C in PBS containing 10% glycerol; samples were excitated at 280 nm.

melting temperature by 4.5 K whereas the observed changes of van't Hoff enthalpy and entropy were in the range of the standard deviations of the measurements as shown in Table 2. These findings strongly suggest that the folding cooperativity of *per*-Pro(S) annexin V is similar to that of the wt protein, but that the mutations lead to a less stable protein.

As shown in Scheme 1, the differences in bond lengths and angles might suggest a slightly enhanced flexibility of the thiazolidine ring if compared with proline. This suggestion is supported by the comparative studies on model peptides containing Pro and Pro(S), which clearly revealed that the rotational barrier of *cis/trans* isomerization of X-Pro(S) is about 30% lower than that of X-Pro (4). Although in crystals of *per*-Pro(S) annexin V all prolines were found to be in the *trans*-conformation, the faster rate constant of X-Pro(S) *cis/trans*-isomerization and thus the locally enhanced dynamics of the polypeptide chain could reasonably explain the lower T_m value of the mutant. Taking into additional account that all Pro residues are solvent accessible (Fig. 1) the observed differences in the denaturing profile also may be partly attributed to the solvation effects caused by the more hydrophilic and polar character of Pro(S) (6). Indeed, the fluorescence emission maximum of *per*-Pro(S) annexin V (Fig. 6) at 20°C in PBS was found to be red-shifted, indicating a less hydrophobic environment of the aromatic groups.

In conclusion, the observed differences in the stability of the *per*-Pro and *per*-Pro(S) protein can be attributed to the differences (*i*) in solvation properties between Pro and Pro(S), (*ii*) in their overall dynamics behavior, (*iii*) in the rotational barrier for *cis/trans* isomerization, and (*iv*) in bond angles and lengths or to a combination of all of them.

Despite the experimentally observed slightly reduced stability of the *per*-Pro(S) mutant, full retainment of the three-dimensional structure at room temperature on replacement of all Pro residues by Pro(S) would imply unchanged functional properties, possibly even including immunogenicity at least for annexin V. In fact, *per*-Pro(S) annexin V was found to exhibit the identical biological properties as the parent protein, i.e., binding to liposomes in the presence of Ca^{2+} and mediation of Ca^{2+} influxes into liposomes.

DISCUSSION

In the present study we could confirm that thiaproline is a potent inhibitor of cellular growth (10) as we previously observed for the methionine analogs norleucine and ethionine (2). In a defined culture medium containing an excess of Pro(S) and trace amounts of Pro, bacterial cells were growing until consumption of the proline, whereas in the absence of the natural amino acid growth of the cells was not taking place at all. Nevertheless bioincorporation of the thiaproline into annexin V occurred when the basic prerequisites of the selective pressure incorporation (SPI) method were fulfilled. The SPI method requires cell growth in a defined medium with a limited concentration of the natural amino acid while keeping the target gene transcriptionally silent. After depletion of the natural amino acid and the onset of the stationary growth phase, the natural amino acid is replaced by the appropriate analog. Induction of biosynthesis of the recombinant protein then will result in the accumulation of a protein containing exclusively the non-natural amino acid analog instead of the encoded amino acid. The method works because full selective pressure can be obtained when (*i*) the host strain exhibits a strong auxotrophism during the fermentation procedure, (*ii*) the cloned gene is under efficient control of the promoter, and (*iii*) the competitive expression system is able to use cytosolic enzymes to express mainly target DNA after induction of protein synthesis (3). Although this simple method usually results in high amounts of recombinant protein, it is limited to those non-natural amino acid analogs that are accepted by the aminoacyl-tRNA synthetases and ribosomes, i.e., by the cellular protein translational machinery. In fact, with the host cells used in the present study the other proline analogs Pro(Se) and Pro(O) were not incorporated into annexin V. However, we are confident that even this problem can be bypassed with an appropriate selection procedure for suitable mutant genotypes that exhibit an editing range even for these proline analogs. Selenium and the heavier tellurium atom at the γ-position of proline ring would represent helpful tools for x-ray analysis of the protein. Moreover, the isosteric character of these chalcogen analogs would lead to "atomic mutations,"

Table 2. Van't Hoff analysis for denaturation of wt- and *per*-Pro(S)-annexin V

Thermodynamic parameters	wt-annexin V	*per*-Pro(S)-annexin V	Difference
T_m (K)	324.940 ± 0.028	320.470 ± 0.063	4.470
ΔH_m (kcal mol^{-1})	165.570 ± 3.160	160.630 ± 6.270	4.940
ΔS_m (kcal mol^{-1} degree^{-1})	0.528 ± 0.012	0.501 ± 0.031	0.027

which could be of great interest in protein folding studies where altered protein properties could be attributed solely to these fine alterations. In this way the analysis of distinct contributions of various local interactions to the protein fold such as hydrogen bonding, van der Waals, and hydrophobic interactions should be possible (22).

The replacement of five proline residues in annexin V with Pro(S) led to a less stable protein with a T_m value lowered by 4.5 K, but without clearly detectable enthalpic or entropic effects (Table 2.). Conversely, a similar multiple (seven residues) replacement of the methylene group in norleucine by the sulfur atom of methionine produced in annexin V an increase in the T_m value by about 1 degree and concomitantly about a 20% increase in both the van't Hoff enthalpy and van't Hoff entropy (22). The effects of the subtle $CH_2 \rightarrow S$ exchanges are obviously strongly dependent on the topological environments of mutated side chains. In fact, the methionine residues mostly are located in hydrophobic interiors of the protein core, whereas the prolines are surface-exposed in the loops of annexin V. Moreover, these atomic mutations may lead to changes in nonbonded interatomic distances that are far below the level of detectability by x-ray crystallography.

The bioincorporation of non-natural amino acids into proteins in purposely selected host strains containing optimized expression systems and using the selective pressure method should allow for tailoring protein structures in a more rational way than by the current random mutagenesis approaches. Thereby the goals could be alterations of acidity or nucleophilicity of the enzyme active site residues, introduction of electron acceptors or metal chelators into proteins, alteration of the hydrogen bond donor or acceptor functionalities, or introduction of amino acids with altered or restricted torsion angles (1). Thus, engineering of proteins by a combination of both site-directed mutagenesis and non-natural amino acid bioincorporation could offer an attractive new approach.

Moreover, in view of the results obtained in the present study with the *per*-Pro(S) annexin V and previously with the *per*-Met(Se) mutant (2) such variants could represent promising vehicles for a targeted drug delivery of bioactive amino acid analogs like thiaproline and selenamethionine. The rationale of this approach is to replace naturally occurring amino acids in the polypeptide sequence with non-natural amino acids that, however, resemble the encoded amino acids in the shape, steric complementarity, and volume to avoid significant sterical clashes in the given molecular environment. By this procedure the resulting proteins should retain their characteristic folds and functional activities. Similarly, the immunological or antigenic properties of such mutants should remain largely unchanged, thus making it possible to avoid the potential risk of unintended and deleterious immune-system interactions during introduction into clinical settings. Pharmacologically active amino acids delivered by this system should be slowly released during protein metabolic digestion by cytosolic enzymes, thus supplying the target tissue with constant levels of the drug over shorter or prolonged periods of time. The main drawback of this approach could derive from the possible reincorporation of the newly released amino acids into proteins. However, the large preference of the translational machinery for the encoded amino acids should prevent such recycling. Bioincorporation of e.g., selenamethionine into ingestible recombinant proteins is one way of increasing the tissue levels of selenium and, therefore, such protein form certainly would represent a nutrient bioavailability enhancing compound. In addition, only L-configurated amino acids become available because the bioincorporation process is strictly stereospecific avoiding in this way the presence of even trace amounts of D-enantiomers as potential hazards to human health. Indeed, the efficient bioincorporation of non-natural amino acids into proteins may represent not only a promising approach for drug or nutrient conjugation into recombinant proteins, but also for their labeling with compounds for the preparation of potentially valuable clinical diagnostic tools.

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