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Towards New Protein Engineering: In Vivo Building and Folding of Protein Shuttles for Drug Delivery and Targeting by the Selective Pressure Incorporation (SPI) Method

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Abstract—In vivo incorporation of non-canonical amino acids enables an expansion of the amino acid repertoire beyond the standard set of 20 canonical amino acids prescribed by the genetic code. Such an expansion is demonstrated here by reassignment of the tyrosine codons TAT or TAC to the non-canonical amino acids 3-fluoro-tyrosine and 3-chloro-tyrosine, without change of the cellular translation machinery. This is achieved during fermentation with tyrosine–auxotrophic *E. coli* host strain AT 2471 under the efficient selective pressure. Since these halo-tyrosines show pharmaceutical activities, proteins substituted with these amino acid analogues might serve as shuttles for their specific delivery into target tissues. Such 'second' coding level in the frame of the genetic code represents a novel form of protein engineering.

Introduction

Proteins are large heteropolymeric molecules optimally folded into compact and specific functional units, made up of 20 canonical amino acids, specified by the universal genetic code. Most of their functional requirements can be fulfilled by this set of conserved building blocks encoded by the genes. Appropriate ligands, cofactors or metal bindings after ribosomal translation can gain additional functional diversification. Some of the canonical amino acids are further modified chemically usually via multiple biosynthetic pathways. Examples are the co-translational introduction of selenocysteine[1] or enzyme-catalysed chemical modifications among which the most important are phosphorylations, glycosylations, acetylations and oxidative modifications, i.e. hydroxylations of aspartate, proline, lysine and tyrosine.[2]

In the laboratory, artificial functionalisation of proteins and peptides can be achieved by pure chemical approaches like protein modifications,[3] peptide synthesis,[4,5] total chemical synthesis of small proteins.[6] Another possibilities include semisynthesis of synthetic protein fragments, which can be inserted into recombinantly expressed proteins in a previously developed active intermediates, such as homoserine lactones[7] or via newly developed protein splicing-based ligation methods.[8] However, to date this chemistry is still most efficiently performed by the translation machinery of intact living cells (in vivo) or by suitably prepared cellular extracts (in vitro).

The concept of 'new' protein engineering

The discovery of site-directed mutagenesis more than two decades ago[9] allowed for the in vivo permutation of any existing gene-encoded protein sequence by codon manipulations, e.g. AUG→AUA, at the DNA level. This classical protein engineering approach is definitively limited to the replacement of one standard and conserved amino acid with another. On the other hand, the delivery of new, non-canonical (unnatural) amino acids into the existing amino acid repertoire prescribed by the genetic code, is certainly a novel form of protein engineering. This approach leads to further protein functional diversifications, e.g. enhanced polarity, altered pK_a values, often without significant structural disturbance of the local environment in the protein structure. Such redefinition of codon meanings without modifications of translation machinery components, is proposed to be a 'second' coding level in the frame of the universal genetic code.[2,10]

Substitution of canonical amino acids with non-canonical ones is a relatively old approach in protein chemistry.[11–13] A revival of these approaches in recent years is a renaissance of the pioneering work and ideas from the fifties and sixties. At present, this emerging new field can be roughly divided into two methodologically different approaches: (i) in vitro

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suppression based on an exceptional natural phenomena of *suppression* using various cellular extracts, and (ii) in vivo approaches based on the use of intact host expression cells that are forced to make 'misincorporations' by externally imposed strong and efficient selective pressure (selective pressure incorporation, SPI).

The basis of the in vitro *suppression* approaches are from early observations that chain terminating mutations in some bacterial strains are correctable by specific suppressor tRNA molecules, which have altered reading capacities caused by mutations in their anticodons. These tRNAs can insert different canonical amino acids in response to the *nonsense* or *missense* codons or *frameshift* mutations in the parent gene.[14,15] Discovery that misacylated tRNAs participate in peptide bond formation at ribosomes[16,18] in a fashion consistent with the adapter hypothesis[17] was the second main prerequisite for the in vitro *suppression* methodology. Finally, after the establishment of cell-free translation systems,[19] all necessary prerequisites for in vitro *suppression* methodology were fulfilled. It is therefore not surprising that first results were reported by few groups almost simultaneously[20,21] or a few years later.[22] At the present stage of development, the in vitro *suppression* methodology allows a rather large number of various non-canonical amino acids to be introduced.[23–25] However, the major drawbacks for wider practical applications are extremely low protein yields as well as difficult, expensive and complicated working protocols. On the other hand, the possible practical applications after bypassing these shortcomings could be virtually limitless.

In contrast, the SPI-methodology is based on a traditional approach that uses auxotrophic mutants of cells to circumvent problems associated with non-canonical amino acid toxicity and preferential incorporation of the canonical amino acids.[11] Almost all non-canonical amino acids are toxic for living cells.[10] Thus, addition of non-canonical amino acid analogues into the growing medium affects not only cellular vitality, but also levels of incorporation that are usually low. For that reasons, several strategies were employed to enhance the degree of incorporation.[26] A general approach to achieve high, almost quantitative levels of incorporation was possible only after the advent of the recombinant DNA technology. It enables the target gene to be inserted into a suitable plasmid and to be placed under the control of an artificial promoter. With this technology, the efficient use of auxotrophs by the SPI-method becomes possible, since an artificial bias is established that favours incorporation of the non-canonical analogue over the natural amino acids as outlined in Fig. 1. This SPI-method has been proven to be an efficient in vivo incorporation approach giving rather high substitution levels in a residue-specific manner.[27–32]

In early work with non-canonical amino acids, the focus was on the elucidation of the mechanisms of protein biosynthesis and metabolism,[11] whereas later, and most recently the emphasis was centred on protein structural studies. For example, ^{19}F-aromatic amino acids have proved to be useful for protein NMR studies.[33] More recently, isosteric replacement of methionine by selenomethionine and even telluromethionine[27,34,35,47] were shown to be useful tools for

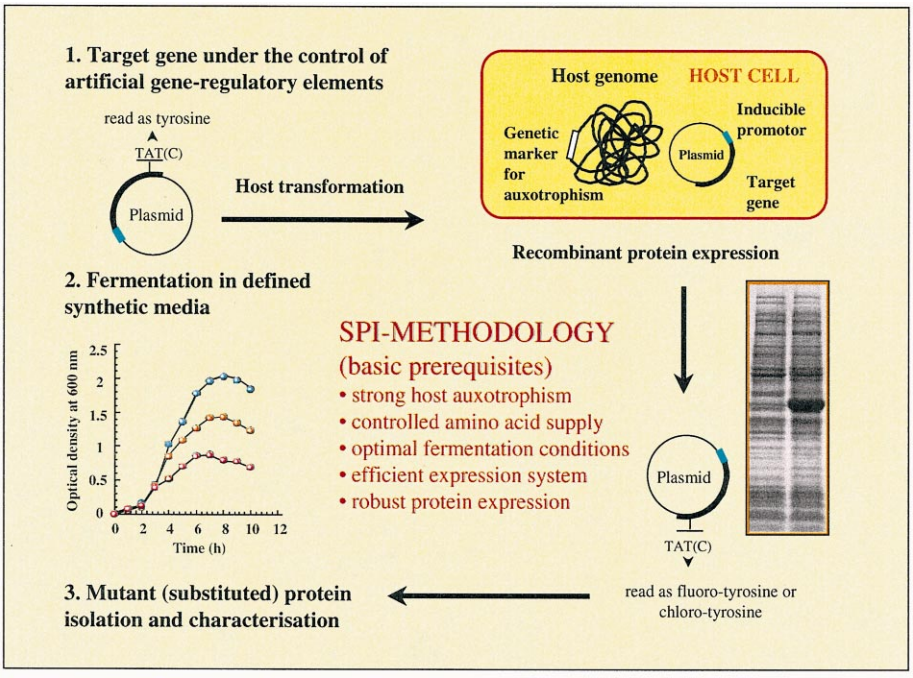

Figure 1. The concept of 'new' protein engineering by the use of SPI-methodology. This novel form of protein engineering is not performed by codon manipulation at the DNA-level (oligonucleotide-directed mutagenesis), but by codon reassignments at the level of ribosome-directed protein transition. For example, as a response to tyrosine TAT or TAC codon-containing DNA-template (plasmid), fluro- and chloro-tyrosines can be translated into the protein sequence. In the context of SPI-method this works by applying a strong selective pressure on genetically engineered but intact host expression cells in combination with manipulation of the amino acid supply in a defined synthetic medium. It is important that the metabolic pathway that supplies the expression host cells with the particular canonical amino acid (e.g. tyrosine) is switched off. This makes host cells auxotrophic, i.e. dependent on the amount of the tyrosine present in a defined minimal medium. The fermentation with growth at limiting concentrations of tyrosine as a natural substrate leads to its depletion from the medium. At this point, the halo-tyrosines are added and the protein synthesis induced resulting in the accumulation of substituted proteins.

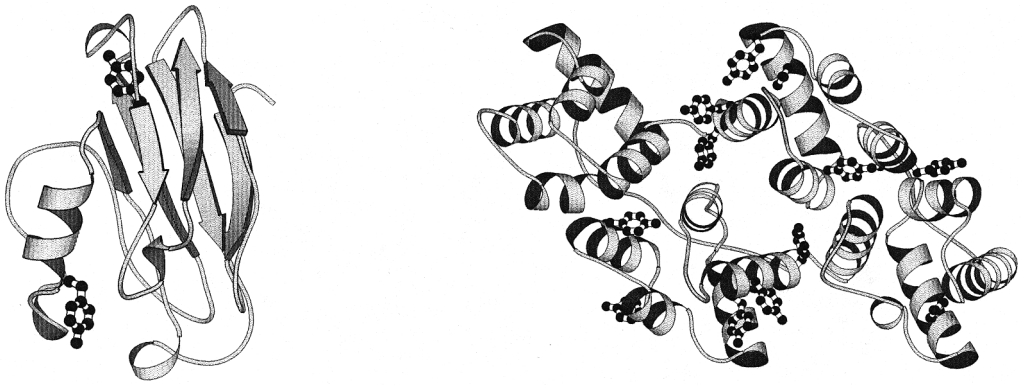

Figure 2. The three-dimensional structures of recombinant azurin (2 Tyr) from *Pseudomonas aeruginosa* (left side) and human recombinant annexin V (right side) represented as ribbon plots. Tyrosine residues in both molecules are marked as balls-and-sticks. Annexin V (12 Tyr) is shown from the top view (convex side) of the molecule.

routine crystallographic structure determination of proteins. In recent years, an expanded amino acid repertoire in vivo allowed the manipulation of protein activity and spectral properties,[29,36] whilst novel applications with this methodology can be envisaged in drug targeting and delivery,[31] studies of protein stability, folding and dynamics[32,37] and even in theoretical studies of the genetic code.[2]

The main drawback of the SPI-method is that only a limited number of amino acids can be introduced into recombinant proteins. This is due to the stringent proof-reading and editing mechanisms ('quality control') in protein translation that ensures the accuracy of protein synthesis i.e. the exclusive use of 20 standard amino acids in correct sequential order.[10] On the other hand, the SPI methodology is simple, cheap and easily reproducible, and often resulting in yields of labelled proteins in the range of their native counterparts. Thus, given the potential for production of substituted proteins at wt-level it is not difficult to imagine SPI as a straightforward method for 'new' protein engineering technology in industrial scale production of such proteins.

Halogenated amino acids in nature and their in vivo incorporation into recombinant proteins

One notable set of natural occurring post-transitionally modified amino acids are halogenated derivatives of histidine, tryptophan and tyrosine. Antibiotics from various marine algae and some higher plants contain chlorinated derivatives of aliphatic and aromatic amino acids.[38] Examples of post-translational iodinations, brominations and chlorinations of tyrosine were first described in vertebrate tyroglobulines[39] and invertebrate scleroproteins[40] while they are recently found in a variety of marine organisms.[41] Since iodine, bromine and chlorine are abundant in the marine environments, many organisms use them for modification of their amino acids into pharmacologically active substances for different purposes usually for chemical defences from predators.[42] Conversely, naturally occurring organofluorine compounds are quite rare. Despite this scarcity, they are as metabolically active substances very interesting, particularly in medicinal chemistry.[43] This is due to the fact that fluorinated amino acids were recognised as inhibitors of specific enzymes, substrates for incorporation into proteins and peptides, or as precursors of other critical biomolecules like aminergic neurotransmitters or lethal substances as fluoroacetate and fluorocitrate.[44]

In this study, we report an extension of our previous work on the production of protein mutants with methionine, proline and tryptophan-like non-canonical amino acids by using the SPI-methodology[27,31,33,47] with studies of bioincorporations of 3-fluoro-tyrosine (3-FTyr) and 3-chloro-tyrosine (3-ClTyr) and their effects on protein structure and stability of two recombinant proteins. As a main model protein we have chosen predominantly the α-helical human recombinant annexin V (Fig. 2) since its biochemical, biophysical and structural studies are well established.[32] The other model protein is the recombinant blue-coloured copper-containing azurin from *Pseudomonas aeruginosa* where β-sheet structural elements are dominating (Fig. 2). In fact the most attractive aspect of these investigations are the pharmacological properties of such tyrosine halo-derivatives. Namely, 3-FTyr is a potent metabolic toxin and 3-ClTyr is known to exhibit neuroactive properties. In this context, their full or even partial incorporations into recombinant proteins, which can serve as carriers and vehicles to target tissues, might represent an interesting new delivery and targeting model system in human medicine.

Results

Amino acid toxicity and fermentation conditions

Both chlorinated and fluorinated tyrosines, when presented in defined minimal medium as a sole Tyr-replacement source, do not support the growth of the *E. coli* Tyr-auxotrophic strain AT2471. In the presence of 3-ClTyr in the medium the cellular growth was supported until native Tyr is depleted while no significant growth was observed when Tyr and 3-FTyr were supplied together (Fig. 3). This is not surprising since fluorinated tyrosines were reported to be a strong kinetic tyrosine competitors in cellular metabolism.[45] Indeed, it is conceivable that 3-FTyr can be initially introduced into cellular proteins, but then compromises their function or structure which in turn inhibits any further cellular growth. Since a long time ago it is well known that small

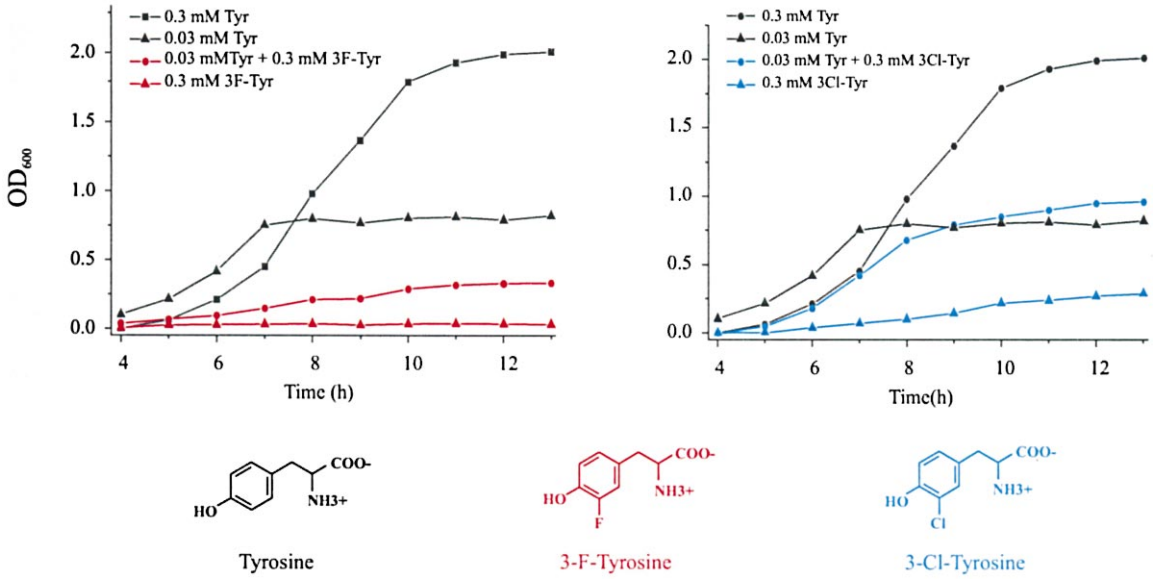

Figure 3. The growth curves at 30°C of the transformed tyrosine auxotrophic *E. coli* strain AT2471 in NMM medium under the different fermentation conditions as described in the Experimental. The cells were transformed with two plasmids: pRSET-PP4 (annexin V) and pGP1-2 as a source for T7 polymerase.[67] Note that the growth of cultures is efficiently limited with 0.03 mM Tyr, and this was used as the limiting concentration in all standard labelling experiments. The growth was followed by measuring the changes in the optical density at 600 nm (OD600) by UV-spectrometry.

amounts of 3-FTyr (0.06–0.2 mg L^{-1}) strongly antagonises tyrosine utilisation in several biological systems.[46] In our experiments 3-FTyr antagonises Tyr utilisation competitively in the ratio of 1:30 in *E. coli* strain AT2471 which is similar to the findings for several other microorganisms.[45] Increasing inhibitory effects of the individual halo-tyrosines are found to be F>Cl>Br (at least for factor 100 in *E. coli*[45]). This is also in agreement with our finding that 3-ClTyr (when compared with 3-FTyr) almost does not interfere with cellular growth in culture as long as the natural substrate Tyr is present (Fig. 3).

Fermentation protocols where 0.03 mM Tyr was a limiting natural substrate concentration in the minimal medium, worked well in our hands for an almost quantitative replacements of Tyr with 3-FTyr. On the other hand, such conditions made possible reproducible but only partial incorporation of 3-ClTyr in both proteins, i.e. 1 to 5 additional chlorine atoms, as judged from mass spectrometric analysis of the expressed protein mixture (Fig. 4B).

To increase the incorporation of 3-ClTyr ('enhanced selective pressure') the fermentation protocol was modified. Since 3-ClTyr does not act as a strong competitive inhibitor of Tyr (Fig. 3) it was possible to supply the cultures with 3-ClTyr and with a very low initial amount of tyrosine at the beginning of fermentation. After exhaustion of Tyr from the medium, protein synthesis is induced. In the case of annexin V, such fermentation indeed resulted in biosynthetic incorporation of chlorinated tyrosines into all 12 tyrosine sites (Fig. 4C), and 2 sites in azurin were labelled to higher extent (Fig. 4D) in comparison with initial attempts. Although in such conditions, Tyr should be theoretically absent from the culture medium at the point when protein synthesis is induced, wt-protein forms are always found in purified protein samples (Fig. 4). There are few explanations for these findings. Firstly, it is well documented that even the best controlled expression systems are not perfectly stringent.[47] In other words, the target gene is not perfectly 'silent' before induction of protein synthesis. Such 'gene leakage' will almost always induce the presence of wt-protein form to certain extents. The main factors responsible for that are probably the nature of the expression system itself, as well as the initial amounts of the natural substrate present from the beginning of the fermentation.[32] Secondly, for the residual tyrosines incorporations the protein turnover could be the source of traces of tyrosine which are preferably incorporated into the target protein.

Protein folding of 3-ClTyr-containing mutants could be affected due to the difficulties to accommodate such a bulky amino acid into the protein core. However, more than 2/3 of the annexin V Tyr-side chains are partially or fully solvent accessible as well as both Tyr-side chains of azurin (Fig. 2). Although inclusion bodies of our model proteins after fermentations were not detectable, protein yields were strongly reduced in all fermentation experiments with 3-ClTyr.

Analytical quantifications of incorporation levels

By routine bio-expression protocols in the context of the SPI-method, high-level introduction of fluorinated tyrosines in annexin V and azurin were easily achieved (Fig. 4A). Under the same conditions, 3-ClTyr incorporations are indeed possible, but only to limited extent. Mass spectrometric analysis (Fig. 4B) reveals a gradual decrease in the intensity of the protein signal with an increase of the number of introduced chlorine atoms. Further increases in the extent of substitution levels was possible by modified protocols as described in Experimental. As shown in Fig. 4C the whole range of replacements with the chlorine-derivative is possible from one (35848±4.2 Da) up to all Tyr-residues (36228±7.2 Da) in annexin V as well as in azurin (azurin

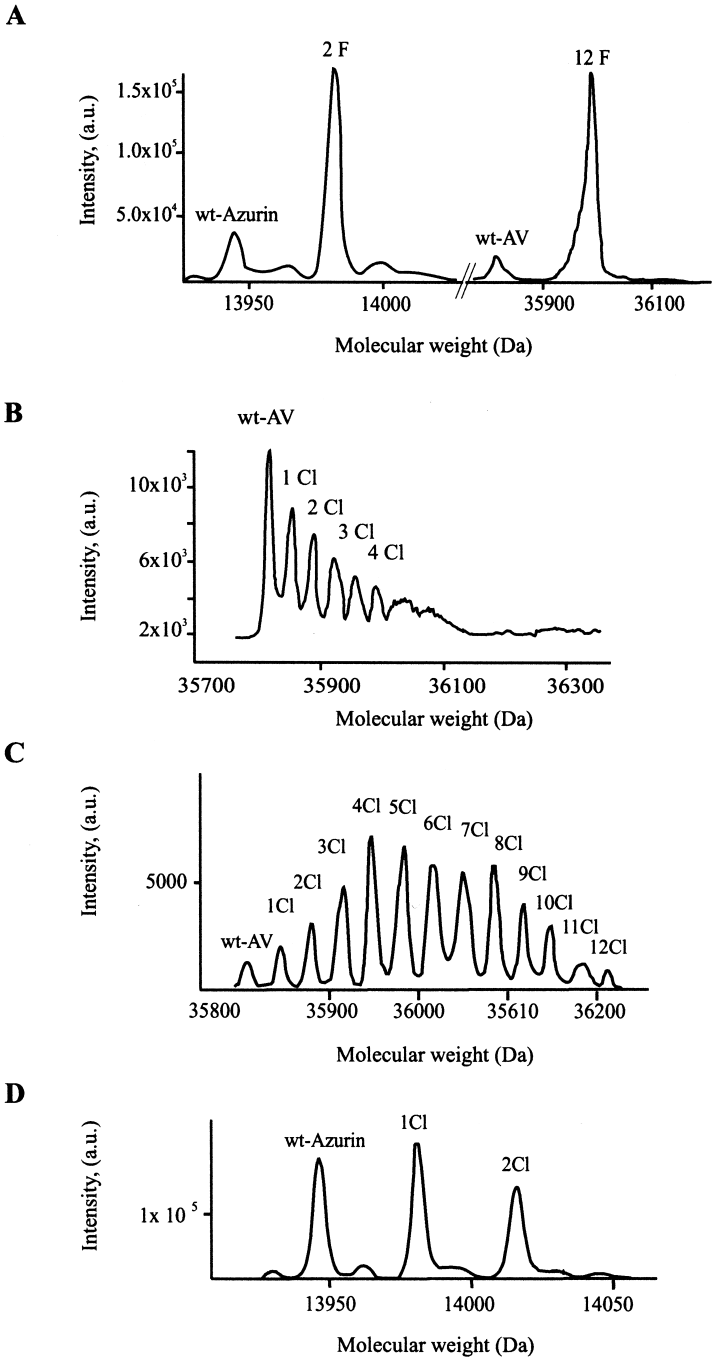

Figure 4. Electrospray-ionisation mass-spectrometric (ESI-MS) analyses for 3-FTyr and 3-ClTyr incorporation into human recombinant annexin V (12 Tyr residues) and recombinant azurin from *Pseudomonas aeruginosa* (2 Tyr residues). (A) Deconvoluted mass-spectra for fluorinated protein variants. Smaller peaks in both cases correspond to the wt-protein forms (annexin V: 35810±6.4 Da, azurin: 13946±3.0 Da); while dominant peaks correspond with the expected masses due to the fluorination of Tyr-residues (annexin V: 38021±3.5 Da, azurin: 13965±2.0 Da). (B) 3-ClTyr incorporation into annexin V under the standard fermentation conditions. The peak-intensities are considerably reduced in comparison with those from panel A indicating very small amount of detected protein species.[48] Nevertheless, beside wt-protein it is possible to detect an additional 5 protein species with 1- 5 chloro atoms (35845±3.5–35986±6.5 Da) in the mass-spectrum. (C) Introduction of the 3-ClTyr into recombinant annexin V using modified fermentation protocols (see Experimental). Deconvoluted mass spectra shows the distribution of different annexin V species containing 1 to 12 chlorine atoms in the range from 35848±4.2 to 36228±7.2 Da with a maximum intensity of the signal for protein species with 4–5 chlorine atoms. Although the intensities of signals are very low, their standard deviations never exceeded ±8.4 Da. (D) Introduction of the 3-ClTyr into recombinant azurin where three signals are clearly distinguishable: wild-type azurin (13946±2.5 Da), azurin with one additional chlorine atom (13980±2.1 Da), and azurin variant with two additional chlorine atoms (14015.8±3.2 Da).

with one 3-ClTyr:13980±2.1 Da; azurin with two 3-ClTyr: 14015.8±3.2 Da). However, mass-spectrometric analyses have to be interpreted with caution. It has been documented that regular and reproducible multiple charging of the protein in vacuum by the electrospray-ionisation method is dependent upon the retention of the 3D-structure of the measured protein.[48] According to these criteria, the fraction of annexin V that contains twelve 3-ClTyr-side chains (Fig.

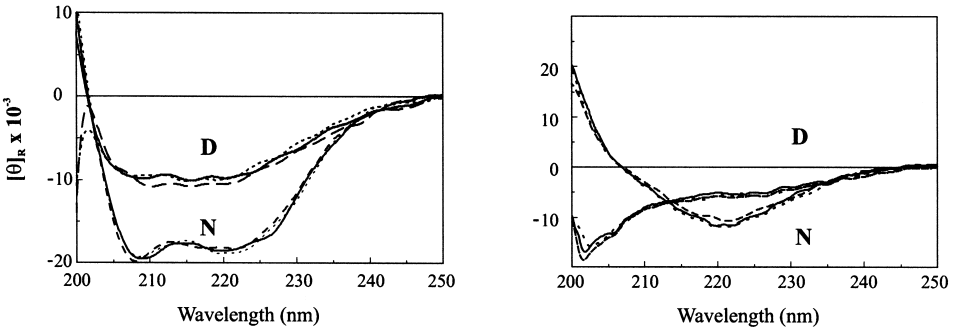

Figure 5. Far UV CD profiles of the wild-type and substituted variants of annexin V (left side) and azurin (right side) at 20°C (set of native states: **N**) and at 90°C (set of denatured states: **D**). (A) annexin V: wt-protein (full line), 3-FTyr-protein (dotted line), and 3-ClTyr-protein (dashed line). (B) wt-azurin (full line) 3-FTyr-azurin (dotted line) and 3-ClTyr-azurin (dashed line). The mean residual ellipticity ($[\theta]_R$) is expressed in $degree \times cm^2 \times dmol^{-1}$.

4C) should be in the folded form. Secondly, the intensities of the peaks in the mass spectrum do not correlate directly with the quantitative presence of these species in the whole protein sample. Thus, amino acid analysis for these purpose may represent a more sensitive method for evaluations of the extent of non-canonical analogue incorporations.

The mutant annexin V and azurin exhibits a reduced tyrosine and a increased leucine signal in the amino acid analyses composition chromatograms. Substitution levels by 3-FTyr in both proteins were over 93%. On the other hand, the amino acid analysis indicated the presence of only 5% 3-ClTyr of the sample from Fig. 4B and 35% for sample from Fig. 4C. Conversely, analyses of recombinant azurin sample from Fig. 4D indicated 50% incorporation for 3-ClTyr. Unfortunately, chromatographic resolutions of the protein species with a different number of chlorine atoms failed.

Conformational analysis of native and mutant proteins in solution

The far UV CD spectra of annexin V shows a curve shape typical for α-helical proteins with the two characteristic minima at 222 nm and at 208 nm of similar intensity (Fig. 5). The signal ratio between these two minima $([\Theta]_R)_{222}/([\Theta]_R)_{208}$ for the wild-type protein form is 0.96; 3-FTyr-annexin V 0.94, and for 3-ClTyr-annexin V 0.97. The far UV CD spectra of the wt- and mutants of azurin where β-sheet secondary structure elements dominate, reveals a set of nearly identical curves with the same shape having a characteristic minima at 220 nm (Fig. 5). These dichroic properties clearly demonstrates the identity of secondary structures of the native proteins and their mutants in solution within the experimental error. Similarly, fluorescence spectra are not changed upon tyrosine substitutions in comparison with the wt-protein forms (C. Minks, PhD Thesis). Annexin V show a fluorescence emission maximum at 320 nm and azurin at 308 nm when excited at 280 nm.[31,32,37]

In addition, far UV CD curves for both proteins were measured at two different temperatures. Spectra measured at 20°C represents the native state, whilst those measured at 90°C the denatured state of the probes. In the case of annexin V, the CD curves for wt- and mutant proteins of both the native and denatured state intersect in the spectral region at 203–205 nm (Fig. 5). This is the isodichroic point characteristic for many denaturing processes of α-helical proteins and thus are indicator of helix-to-coil transition.[37] It is worth noting, that the denatured state of both proteins and their mutants is not completely random coiled at 90°C; considerable amounts of residual secondary structure elements still exist at this temperature. Such phenomena are generally observed for a large number of monomer globular proteins.[37]

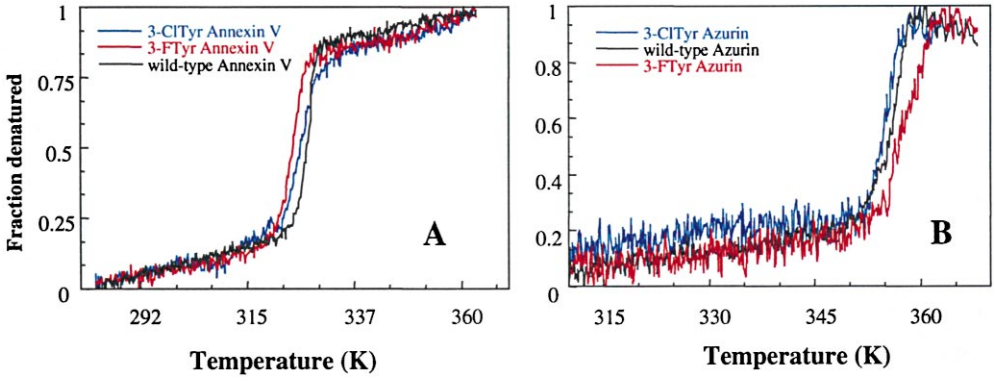

Figure 6. Thermal denaturation curves of native and mutant proteins. Fractions of unfolded protein are calculated from CD data monitored at 222 nm as described elsewhere.[32,37] (A) Annexin V and its substituted variants. Note that the transition of the 3-ClTyr annexin V is less steep, indicating less cooperative unfolding process. (B) Azurin and its mutants. Note that the 3-Ftyr-azurin has higher T_m value and a less steep transition part of denaturing curve in comparison with wt-protein.

Thermal denaturing experiments

The characteristic T_m value (melting temperature) of annexin V is 325.9±0.11 K, while for azurin it is T_m 358.1±0.81 K (Fig. 6). In comparison to wt-protein forms, the mutants of both proteins show opposite trends in thermal stabilities upon fluorination and chlorination. For example, 3-FTyr harbouring annexin V is 1.7 K (T_m 324.2±0.12 K) less stable, while the heterologous 'chloro-protein' is only by 1.0 K less stable (T_m 324.9±0.21 K) then the wt-protein. Surprisingly, opposite effects are observed for azurin: 3-ClTyr azurin exhibit reduced stability by 1.1 K (356.4±0.45 K) while 3-FTyr-azurin (T_m 359.2±0.22 K) has an enhanced thermal stability (2.8 K) in respect to the wt-protein.

Careful inspection of the melting curves indicates that unfolding of fluorinated annexin V and chlorinated azurin mutants are characterised by rather sharp co-operative transition between the native and the denatured state as known for the wt-proteins. Conversely, chlorinated annexin V (sample from Fig. 4B) and the fluorinated azurin variants have less steep transition profiles, indicating a reduced co-operativity in the unfolding process (Fig. 6).

Biological activity

Artificial liposomes containing the calcium-sensitive dye FURA-2 were used to monitor annexin V induced calcium influxes into liposomes as described elsewhere.[32,49] The average activity factors (arbitrary activity units determined according to Ref. 49) are 25.0 for wt-annexin V and 8.19 for 3-Tyr-annexin V. Preliminary results of the activity measurements show that different partially chlorinated annexin V forms are able to mediate calcium influx into liposomes like wild-type forms. On the other hand, electron transfer processes in azurin are not altered by the fluorination of its Tyr-residues (Professor Israel Pecht, personal communication).

Discussion

The concept of 'atomic mutations' in the protein folding and stability studies

In attempts to identify the mechanisms responsible for proper folding and the stability parameters for the proteins, immense complexities are encountered. This stretch our contemporary understanding of this matter to the limits. Therefore, the desirable approach should be to reduce these complexities radically. The efficient substitution of conserved canonical amino acids with isosteric non-canonical counterparts by SPI methodology might be a way for such purpose. Indeed our recently proposed concept of 'atomic mutations' for protein folding and stability studies[32,37] is based on this assumption. Atomic mutations are supposed to simplify the interpretation of the experimental data since they are expected to produce measurable physicochemical properties that arise solely from exchanges of single atoms. Such isosteric replacements in the context of protein structure might provoke negligible local or even unexpected and significant global effects. For example, H→F atomic mutations at different positions of the single Trp187 indole ring of annexin V resulted in significant differences in stability, folding cooperativity and biological activity although complete isomorphism between the crystal structures of native and mutant proteins was preserved.[32] Closer inspection of the H→F atomic exchanges indicated that although isosteric, limited changes in bond lengths and van-der-Waals radii were existing, the resulting properties can additionally be affected by inverted polarity at the site of replacement. Computer-assisted modelling of these effects indicates that fluorination alters the indole ring electrostatic properties and consequently the dynamic behaviour in the local environment, that is globally propagated to the whole structure.[32] Future studies on other well characterised systems should address the question whether and to which extent such effects are general and predictable.

In spite of a large body of experimental data dealing with the protein biosynthetic labelling with fluorinated aromatic amino acids[26,50] only recently, a series of high resolution crystallographic studies with rat glutathione transferase M1-1 with global substitutions by fluorinated aromatic amino acids for their canonical counterparts was performed.[51,52] Regarding substitution of 14 tyrosine side chains in this protein by 3-FTyr, it was demonstrated that (a) local conformations of many of the fluorinated residues are changed; (b) many new hydrogen bonds and other interactions involving F atoms were observable, e.g. with water molecules, as well as (c) changes in crystal packing interactions. Despite of these findings, the overall folding pattern of the crystal structure of the fluorinated enzyme was the identical to the native one.

The same was expected for fully fluorinated and partially chlorinated annexins and azurins. Indeed, their structural properties are not changed when compared with their native counterparts. The proof for this is our finding that the evaluation of their secondary structures by circular dichroism (Fig. 5), and tertiary structure by fluorescence attests the structural integrity of these protein mutants in solution. Conversely, thermal parameters (T_m-values) derived from the melting curves presented in Fig. 6 clearly show differences in stability. Particularly instructive are the differences in stability introduced by fluorination: while the presence of 12 3-FTyr in annexin V reduce its stability by 3.3 K, only two fluorinated tyrosines of azurin increase the T_m value of this mutant by 2.8 K. Since the Tyr-residues in both proteins are not uniformly distributed, it is difficult to recognise the particular contribution of each of the Tyr-side chains. Thus, the atomic mutation approach in combination with site-directed mutagenesis might offer an attractive research aspect. In addition, the question arises as to whether the topological positions of the Tyr residues, in the context of two proteins with different structures (annexin: α-helix, azurin: β-sheet secondary structure elements) exert a general effect on experimental parameters. Detailed structural and functional analyses of the fluorinated and partially chlorinated proteins are in progress, and these might shed more light on this phenomena.

3-Chloro-tyrosines in proteins: forced protein building and folding under the efficient selective pressure in vivo

In the context of the amino acid repertoire determined by the

genetic code, fluorinated and chlorinated amino acids are 'xeno'-compounds and consequently fluorine and chlorine are 'xeno' elements. Although both are neighbours in the group of halogens in the periodic system of elements, they are markedly different in their electrochemical and sterical properties. As the most electronegative element, fluorine induces much stronger electronegativity (3.98 according to the Pauling scale) in its local surrounding than chlorine does (3.16 according to the Pauling scale). Both have a strong electron-withdrawing inductive effect (−I effect) and produce a moderate electron-donating resonance effect on the whole aromatic ring (+M effect). In addition they possess non-shared electron pairs, and carbon-bound fluorine is a weak hydrogen bond acceptor. Their dipole moments are opposite to that of a C–H bond (C–F: 1.41, C–Cl: 1.46). The most dramatic differences between these halogens lie in their steric properties. Chlorine is more bulky than fluorine (van der Waals radius of F is 1.35 Å and Cl 1.8 Å) and is much more polarizable. Replacement of the meta-hydrogen by fluorine in tyrosine produces a very minor steric perturbance. On the other hand the steric effects of fluorine replacement by chlorine are expected to be much more dramatic (C–H: 1.09 Å, C–F: 1.39 Å, C–Cl: 1.77 Å).[43]

In contrast to the biosynthetic labelling of proteins with fluorinated tyrosines, the co-translational introduction of bulky chlorinated, brominated or iodinated tyrosines into proteins in vivo is hardly imaginable. Since these atoms are too bulky, it is easier to have them at protein surface then to pack them efficiently into the protein interiors. From the protein chemists perspective, it should not be surprising that it is only possible context for such reactions to occur are post-translational modifications.[42] Nevertheless, the relaxed substrate specificity of the phenylalanine-tRNA synthetase was used for introduction of chlorinated phenylalanine into recombinant luciferase.[53] Although the introduction was successful and the full length protein translated, even the addition of chaperones did not help to prevent total loss of enzymatic activity.[53] On the other hand, our approach indicates that is should be possible to activate 3-ClTyr for in vivo protein biosynthesis without relaxing the editing function of the tyrosyl-tRNA synthetase. Although it was believed that the major deterrent for 3-ClTyr incorporation might be its steric bulkiness and subsequent difficult accommodation in the folded protein,[10] our mass analysis indicates that at least small amounts of fully substituted (and folded) protein exists (Fig. 4C). However it should be noted that even under the stringent fermentation conditions (i) the full labelling with 3-ClTyr of both proteins was never possible, (ii) protein yields were always extremely low in comparison with the fluorinated and wild-type variants. Obviously, during such translation of tyrosine codons into of chlorotyrosines, under the in vivo selective pressure conditions, many other still unknown factors, intervene at the level of ribosomal synthesis.

Future studies of these phenomena should answer the challenging questions such as: (i) to which extent the proteins can be loaded with bulky amino acids like 3-ClTyr and still retain their structure and function; (ii) which other factors beside sterical bulkiness can be regarded as major stumbling blocks for a successful translational integration of various non-canonical amino acids and finally (iii) what can be learned about protein folding and the possibilities for rational design with such substances.

Engineering enzyme activity and catalysis

The substitution of canonical side chain with non-canonical ones in the proteins does not produce any significant effect for the activity only if mutated residues do not play functionally important roles. Fluorination of all annexin V Tyr-residues makes this protein 3 times less active than the parent protein whilst it produces no effects on azurin activity. Obviously, Tyr-residues are somehow important for annexin V activity (mediation of Ca^{2+} influx into liposomes) but not for electron transfer processes mediated by azurin. In contrast, fluorination of a single Trp-side chain in azurin significantly affects electron transfer, since this residue is placed at a 'strategically important' site in the intramolecular electron transfer pathway of azurin (I. Pecht, personal communication). Until now, Tyr residues in the structure of annexin V are not expected to play any significant role for mediating calcium influx through the liposome membranes. Thus, careful dissection of the observed effects in future studies might shed more light on annexin V activity mechanisms.

Recombinant human annexin V as well as azurin are only to certain extent labelled with 3-ClTyr (see Fig. 4). Since there is no information about possible topologically preferred sites in the protein structure (surfaces, minicores) that might be more permissive for such labelling than other sites, any attempt for explanations about the role of chlorine atoms in protein activity would be too speculative. Detailed studies of 3-ClTyr incorporations in combination with site-directed mutagenesis might add our understanding of these processes.

There are well-studied examples of model proteins with tyrosine as the crucial catalytic residue. 3-FTyr was used as substitution for tyrosine to probe whether it is involved in the catalytic activity of β-galactosidase.[36,54] More recently, altered kinetic properties of enzymes upon 3-FTyr introduction were reported: ketosteroid isomerase from Pseudomonas testosteroni[55] and rat glutathione transferase M1-1.[51,52] The rationale behind these experiments is to use differences in phenolic pK_a values of 3-FTyr and Tyr to see to what extent the catalytic properties are affected (pK_a value for 3-FTyr is 8.3, and 10.1 for Tyr).[44]

Histidine residues are especially attractive for such purposes since they are often found in the active sites of different enzymes, functioning as catalysts in acid-base and nucleophilic processes. For example, H→F substitution that results in 2-FHis and 4-FHis reduces the pK_a of the imidazole ring by about 5 pH units.[44] Such a dramatic increase in the acidity of the imidazole ring should alter dramatically the activity of the fluoro-His-containing proteins.[56,57] More recently, it was demonstrated that substitution of His by its non-canonical counterpart triazole-3-alanine yields an enzyme with high activity at acidic pH.[29]

With the exception of the serine→cysteine conversion, any change in the frame of the genetic code of the residues that

take part in catalysis involves relatively large modifications of charge and size. In this context, isosteric, non-canonical amino acid analogues introduced into proteins by SPI-method should be useful to dissect, probe and manipulate enzyme catalysis and functionality.

Pharmaceutical and biomedical potentials of fluorinated and chlorinated tyrosines

Since long time it is known that halogenated amino acids are capable of producing a variety of biological effects. They may act as specific mechanism-based irreversible enzyme inhibitors, interact with various catabolic enzymes, intervene in the process of amino acid biosynthesis, turnover, transport and storage, and finally they can be substrates for incorporation into proteins.[44] Therefore, the strong *anti*-microbial, *anti*-mycotic, and *anti*-tumour activities of such substances are plausible and it is surprising how little attention was paid to these interesting pharmacological properties.

One of the best characterised biological effect of 3-FTyr is the mechanism of its extreme metabolic toxicity. This toxicity stems from 3-FTyr enzymatic conversions in the cellular milieu via the dominant tyrosine pathway to fluorinated derivatives of tricarboxylic acid cycle intermediates (fluoroacetate, fluorocitrate) which in turn inhibit the citric acid metabolism.[58] As a consequence, its systemic administration in experimental animals produces acute convulsions and lethal effects.[59] Thus, it is not surprising that the fluorinated tyrosines are never reported to be abundantly present in the cellular cytosol nor co-translationally or post-translationally introduced into proteins. 3-FTyr was also reported as tissue tracer. For example the brain uptake of 3-^{18}FTyr exhibits a greater increase with time than of the native tyrosine and the label was incorporated into cerebral proteins without significant catabolism. This property might be promising to measure protein synthesis rates in different tissues, e.g. tumour tissues and normal ones.[60]

Literature sources dealing with the presence of chlorinated tyrosines in the metabolism of the cellular cytosol or proteins are scarce. The presence of 3-ClTyr is described in the insect cuticle, where this amino acid is considered as an unavoidable by-product of the sclerotization. It was therefore assumed that it does not have any specific function in proteins.[61] More interestingly, chlorinated aromatic amino acids play an important role in behavioural brain chemistry.[62] 3-ClTyr is a metabolite of *para*-chlorophenyl alanine and both substances act as drugs that alter the brain concentrations of the neurohormone serotonine, and subsequently cause specific behavioural effects.[63] It was recently reported that 3-ClTyr appears in the host defence (e.g. microbial killing) and inflammation reactions as a results of the hypochlorous acid presence. Thus, it is a potential marker for the production of hypochlorous acid, a strong biological oxidant produced by neutrophile and monocyte immune cells.[64]

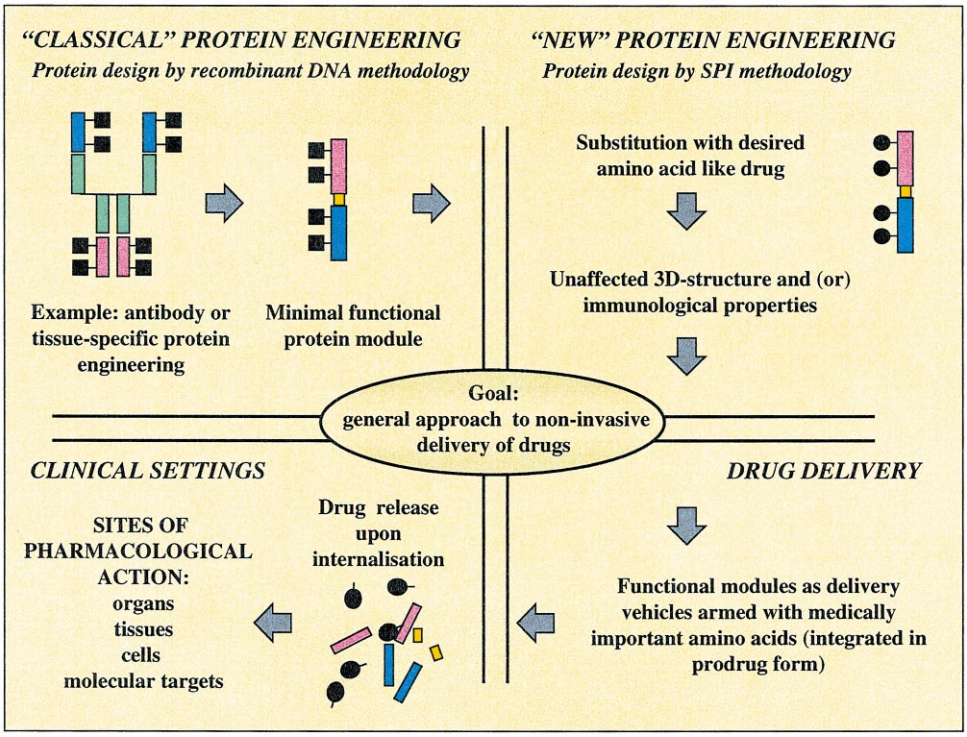

Figure 7. Schematic representation of the concept of 'protein shuttles' for general non-invasive drug delivery. A possible supplementation of the classical protein engineering with new protein engineering is presented here by the production of minimal functional protein modules (e.g. single chain antibodies, catalytic domains). Such specific proteins designed by classical engineering methods may serve as delivery vehicles for various markers or cytotoxic agents (radioisotopes, drugs, toxins) to different target cells either for imaging or therapy. Prior to their possible use in clinical settings, they can be further redesigned by substitution of their particular canonical amino acid building blocks (full rectangles) with non-canonical amino acid-like drugs (full circles) by means of SPI methodology. In this way, protein can be 'armed' with amino acid-like cytotoxic drugs (e.g. 3-FTyr) or other pharmacologically active substances (e.g. 3-ClTyr) which are expected to be released in their active form upon internalisation in the specific target sites. Such proteins due to their specificity of targeting, and efficiency of delivery, may represent 'magic bullets' for rational treatment of a variety of diseases at a molecular level.

Attributes and perspectives of proteins as shuttles for pharmacologically active amino acids

In drug design for chemotherapy it is imperative to achieve specificity of action and selectivity of delivery. The potential power of proteins as therapeutic vehicles resides in their intrinsic compatibility with living cells. Recombinant proteins substituted with drug-like amino acids by in vivo biosynthesis as candidates for specific carriers ('shuttles') in targeted drug delivery were recently proposed.[31] The rationale behind this proposal is as follows. Substitution of putative shuttle proteins with drug-like amino acids should introduce as few as possible perturbations in its structural, functional and immunogenic properties, as demonstrated by recent studies.[31,32] This is indeed, plausible since most of the non-canonical amino acids introduced into recombinant proteins by SPI-method resemble their canonical counterparts by shape, size and chemical properties.[10] Combined with the choice of suitable 'shuttle' candidates like antibodies, cytokines or other tissue-specific proteins, their substitution offers the opportunity to intervene in a pathological process with a high degree of specificity and minimal perturbation of normal physiological processes as outlined in Fig. 7. Similarly, peptides were proposed as vehicles for drug uptake.[44] A variety of synthetically substituted small peptides prepared under different names like 'smugglins' or 'portable transporters' were used as carriers for some antibacterial amino acids.[65]

In the frame of our shuttle concept, it is conceivable that e.g. lethal intracellular synthesis of fluorocitrate from fluoroacetate can be induced by a suitable protein shuttle substituted with 3-FTyr via specific route of administration (skin, lungs, mucosus membranes, intestine, systemic circulation) to the local target sites of action like cancer cells while sparing normal cells. The extreme toxicity of 3-FTyr during delivery can be circumvented by its conversion into inactive prodrug form upon covalent integration into polypeptides. Once the local target is reached, the prodrug is expected to be converted into the active drug by its release from the polypeptide chain upon internalisation. Reincorporating (recycling) of the drug into other proteins is less probably, since such substitution works well only under the experimentally produced selective pressure. Even if it would happens, such local reincorporating can produce particularly profound effects on the biological behaviour of some proteins, especially if replacement occurs at residues important for catalytic activity or compromise structural integrity.

However, we should exercise caution here. Namely, it is possible to anticipate many technical difficulties in attempts for practical realisation of these ideas. For example, molecular size, electrical charge, sensitivity to pH and proteolytic activity and even relative hydrophilic nature of most protein surfaces (which diminishes membrane transport) of such hypothetical delivery shuttles represents a serious limitation to their possible practical use. On the other hand, the number of substances that can be used for such substitutions is still very low, at this level of SPI-methodology development. Even then, this study shows how difficult is to introduce such non-canonical amino acids like 3-ClTyr. It would be actually impossible without further design and optimisation of the protein-shuttles structures using different approaches.

In general, although information available about the pathology of the process we wish to intervene in might often be sufficient, our knowledge for the preparation and suitable design of protein shuttles as tools for such intervention is still grossly inadequate. Obviously, much more work must be done in this area of rational drug design.

Experimental

Chemicals, bacterial strains, and media for the growth

The non-canonical amino acids 3-DL-FTyr and 3-DL-ClTyr were from Sigma. All other chemicals were purchased from Sigma or Aldrich unless stated otherwise. For all bioincorporation experiments we used the tyrosine deficient *E. coli* strain AT2471 (λ^-, *tyrA4*, *relA1*, *spoT1*, thi^{-1}) which was a generous gift from Professor Barbara Bachmann from Yale University, New Haven. All growth experiments were performed in New Minimal Medium (NMM).[27,31,32,47]

Growth, culture conditions, expression and purification of labelled proteins

Growth curves of *E. coli* AT2471 transformed with two plasmids in NMM with different concentration of tyrosine and its fluorinated and chlorinated analogues are presented in Fig. 3. They were determined by following the optical density at 600 nm of two independent cultures over a 14 h time period at 30°C. The protein expression host *E. coli* AT2471 was routinely co-transformed with two plasmids: ampicillin resistant pRSET-PP4 harbouring the annexin V gene sequence under the control of T7 promoter, and kanamycin resistant pGP1-2 containing a gene for T7 polymerase.[67] For recombinant azurin expressions two plasmids were also used: pRSET-azurin and pGP1-2.

The incorporation experiments were performed using cultures grown in NMM in the presence of 100 mg L^{-1} ampicillin and 70 mg L^{-1} kanamycin and 0.03 mM tyrosine as optimal limiting concentration of the native substrate at 30°C. This medium provides enough 'healthy' cells before depletion of tyrosine as a native substrate, in the mid-logarithmic phase of growth (OD_{600} about 0.5–0.6)—an optimal point where the non-canonical substrate is added and target protein expression switched on by a temperature jump to 42°C for 20 minutes. The culture fermentation after the induction of protein synthesis was 4 h or overnight at 30°C.

The purification procedures for the labelled forms for both proteins were identical to those of the wild-type forms. The purity of the proteins was analysed by SDS/PAGE (silver staining), HPLC and electrospray mass analysis. While the expression of fluoro-tyrosine mutants gives similar yields to those of the wt-protein form for both proteins (10 mg per litre of culture), it was not a surprise that the yields of 'chloro-proteins' were always considerably reduced (about 0.2–0.4 mg/1 L medium). Both proteins are purified in the soluble form. The presence of their inclusion body forms in

cellular pellets due to the chloro-tyrosine incorporations was not possible to detect.

Modifications of the fermentation protocol

To enhance the pressure on the *E. coli* AT2471 strain for higher levels of 3-ClTyr incorporations, the fermentation protocol is modified as follows. Since the presence of 0.3 mM 3-ClTyr from the beginning of the fermentation enhance labelling, 1000 mL of such culture (NMM with 0.3 mM 3-ClTyr, but without tyrosine) was inoculated with transformed AT2471 cells grown in LB medium, to a final dilution 1:50 and allowed to grow overnight. The cellular growth was supported until an OD_{600} of about 0.25, whereby the source of tyrosine was diluted LB-medium from inoculum. After growth stabilisation, the cells were collected by centrifugation (3000 g) and resuspended into 100 mL NMM medium containing 1 mM 3-ClTyr. The induction of protein synthesis was performed under the conditions described above. In recombinant azurin the higher extent of substitution at two sites was achieved, while in the case of annexin V higher an additional number of tyrosines were substituted. It should be noted, however, that this procedure does not increase fermentation yields of labelled proteins significantly, when compared with standard labelling protocols.

Analytical and spectroscopic methods

Mass spectrometric analysis. The quantitative replacement of the native tyrosine residues (12 in annexin V, 2 in azurin) by the fluorinated non-canonical analogues was routinely confirmed by electrospray mass spectrometric analyses (ESI-MS) as described elsewhere.[27]

Amino acid analysis. Protein probes of approximately 10 nM were hydrolysed in 6 M HCl containing 2.5% (v/v) thioglycolic acid for 48 h. The hydrolysates were analysed with a Biotronic LC 6001 amino acid analyser. The fluorinated tyrosine counterparts were subjected to hydrolysis under identical conditions to determine both retention times and recoveries. 3-FTyr exhibits an identical retention time to that of leucine and cannot be resolved from that signal. Therefore, the increased intensity of the leucine signal in the composition chromatogram of the annexin V sample indicates the presence of 3-FTyr in the protein sequence. In the case of azurin containing 3-FTyr a slight shoulder of the leucine peak is even visible in chromatogram. Thus, the extent of 3-FTyr or 3-ClTyr presence was calculated from the absence of the Tyr peak from the composition chromatogram.

Circular dichroism (CD)

Far UV CD experiments were carried out at 20°C on a JASCO J-715 spectrometer in a configuration described by JASCO hardware manual P/N:0302-0265A (1995). For secondary structure determination spectra were taken in the 200–250 nm wavelength range and expressed as mean residue ellipticity ($[\Theta]_R$) in degrees $cm^2\ dmol^{-1}$. Protein concentrations of 0.08 mg mL^{-1} in PBS (phosphate buffered saline) containing 10% glycerol were used, and the spectra were measured in quartz Hellma 110-QS cells with 0.1 cm optical path length. Raw data are processed using the 'processing method' in the JASCO software package (Software manual P/N:0302-0266A, 1995). A sufficient signal to noise ratio is achieved by recording four accumulations for the far UV CD spectra. The chosen spectral regions cover all important spectral characteristics.

Thermal denaturing experiments

For thermal unfolding measurement experiments the JASCO spectrometer equipped with a Peltier type FDCD attachment, model PFD-350S/350L was applied and probes were pipetted in rectangular 110-QS Hellma quartz cells with optical path of 0.1 cm. The melting curves of wild-type as well as substituted proteins were measured in the range 10–100°C (heating rate was 30°C h^{-1}) by monitoring the changes in dichroic intensity at 222 nm (annexin V) and at 218 nm (azurin) as function of temperature change. More than 1000 data points for each melting curve were collected and normalised, whereby T_m value was estimated.

Biological activity

Annexin V moves calcium across membranes and calcium influx into artificially prepared liposomes was monitored by using the calcium-sensitive dye *FURA2*. Liposome vesicle preparations and calcium influx assay of native and substituted proteins was performed following the protocol described elsewhere.[49] Electron transfer measurements on substituted azurins were performed as described elsewhere[66] in the laboratory of Professor Israel Pecht (Weizman Institute, Rehovot, Israel)

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References

1. Böck, A.; Forchhammer, K.; Heider, J.; Leinfelder, W.; Sawers, G.; Veprek, B.; Zinoni, F. *Mol. Microbiol.* **1991**, *5*, 515–520.
2. Budisa, N.; Moroder, L.; Huber, R. *Cell. Mol. Life Sci.* **1999**, *55*, 1626–1635.
3. Offord, R. E. *Protein Engng* **1987**, *1*, 151–157.
4. Merrifield, R. B.; Stewart, J. M. *Nature* **1963**, *207*, 522–523.
5. Moroder, L.; Göhring, W.; Nyfeler, R.; Scharf, R.; Thamm, P.; Wendlberger, G. *Hoppe Seyler's Z. Physiol. Chem.* **1983**, *364*, 157–171.
6. Wilken, J.; Kent, S. B. H. *Curr. Opin. Biotechnol.* **1998**, *9*, 412–426.
7. Offord, R. E.; DiBello, C. *Semisynthetic Peptides and Proteins*; Academic: London, 1978.
8. Cotton, G. J.; Ayers, B.; Xu, R.; Muir, T. W. *J. Am. Chem. Soc.* **1999**, *121*, 1100–1101.

9. Hutchison, C. A.; Phillips, S.; Edgell, M. H.; Gillam, S.; Janke, P.; Smith, M. *J. Biol. Chem.* **1978**, *253*, 6551–6560.
10. Budisa, N.; Minks, C.; Alefelder, S.; Wenger, W.; Dong, F.; Moroder, L.; Huber, R. *FASEB J.* **1999**, *13*, 41–51.
11. Richmond, M. H. *Bacteriol. Rev.* **1962**, *26*, 398–420.
12. Hortin, G.; Boime, I. *Methods Enzymol.* **1983**, *96*, 777–784.
13. Wilson, M. J.; Hartfield, D. L. *Biochim. Biophys. Acta* **1984**, *781*, 205–215.
14. Garen, A. *Science* **1968**, *160*, 149–159.
15. Engelhardt, D. L.; Webster, R.; Wilhelm, R.; Zinder, N. *Proc. Natl. Acad. Sci. USA* **1965**, *54*, 1791–1797.
16. Chapeville, F.; Lipmann, F.; von Ehrenstein, G.; Weisblum, B.; Ray, W. J.; Benzer, S. *Proc. Natl. Acad. Sci. USA* **1962**, *48*, 1086–1092.
17. Crick, F. H. C. *Symp. Soc. Exp. Biol.* **1958**, *12*, 138–163.
18. Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517–4520.
19. Spirin, A. S.; Baranov, V. I.; Ryabova, L. A.; Ovodov, S. J.; Alakhov, Y. B. *Science* **1989**, *242*, 1162–1164.
20. Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182–188.
21. Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, R. A.; Diala, R. S. *J. Am. Chem. Soc.* **1989**, *111*, 8013–8014.
22. Sisido, M. *Prog. Polym. Sci.* **1992**, *17*, 699–764.
23. Steward, L. E.; Collins, C. S.; Gilmore, M. A.; Carlson, J. E.; Ross, J. B.; Chamberlin, R. A. *J. Am. Chem. Soc.* **1997**, *119*, 6–11.
24. Thorson, J. S.; Cornish, V. W.; Barret, J. E.; Cload, S. T.; Yano, T.; Schultz, P. G. *Protein Synthesis: Methods and Protocols*; Martin, R. Ed.; Humana: Totowa and New Jersy, 1998, pp 43–75.
25. Hohsaka, T.; Kajihara, D.; Ashizuka, Y.; Murakami, H.; Sisido, M. *J. Am. Chem. Soc.* **1999**, *121*, 34–40.
26. Sykes, B. D.; Wiener, J. H. *Magnetic Resonance in Biology*; Choen, J., Ed.; Wiley Interscience: New York, 1980; pp 171–196.
27. Budisa, N.; Steipe, B.; Demange, P.; Eckerskorn, C.; Kellerman, J.; Huber, R.; Eur *J. Biochem.* **1995**, *320*, 788–796.
28. Soumillion, P.; Jespers, L.; Vervoort, J.; Fastrez, J. *Protein Engng* **1995**, *8*, 451–456.
29. Beiboer, S.; Van den Berg, B.; Dekker, N.; Cox, R.; Verheif, H. *Protein Engng* **1996**, *260*, 628–643.
30. Ross, J. B.; Szabo, A. G.; Houge, C. V. *Methods Enzymol.* **1997**, *278*, 151–190.
31. Budisa, N.; Minks, C.; Medrano, F. J.; Lutz, J.; Huber, R.; Moroder, L. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 455–459.
32. Minks, C.; Huber, R.; Moroder, L.; Budisa, N. *Biochemistry* **1999**, *38*, 10649–10659.
33. Pratt, E.; Ho, C. *Biochemistry* **1975**, *13*, 3035–3040.
34. Hendrickson, W.; Horton, J.; LeMaster, D. *EMBO J.* **1990**, *9*, 1665–1672.
35. Boles, J.; Lewinski, K.; Kunkle, M.; Odom, J. D.; Dunlap, B. R.; Lebioda, L.; Hatada, M. *Nature Struct. Biol.* **1994**, *1*, 283–284.
36. Ring, M.; Armitage, I. M.; Huber, R. E. *Biochem. Biophys. Res. Commun.* **1985**, *131*, 675–680.
37. Budisa, N.; Huber, R.; Golbik, R.; Minks, C.; Weyher, E.; Moroder, L.; Eur *J. Biochem.* **1998**, *253*, 1–9.
38. Berdy, J. *Handbook of Antibiotic Compounds*. Part IV, CRC: Boca Raton, Florida, 1980.
39. Fink, K.; Fink, R. M. *Science* **1948**, *108*, 358–359.
40. Hunt, S. *Methods Enzymol.* **1984**, *107*, 413–438.
41. Jimenez, E. C.; Craig, A. G.; Watkins, M.; Hillyard, D. R.; Gray, W. R.; Gulyas, J.; Rivier, J. E.; Cruz, L. J.; Olivera, B. M. *Biochemistry* **1997**, *36*, 984–989.
42. Craig, A. G.; Jimenez, E. C.; Dykert, J.; Nielsen, D. B.; Gulyas, J.; Abogradie, F. C.; Porter, J.; Rivier, J. E.; Cruz, L. J.; Olivera, B. M.; McIntosh, J. M. *J. Biol. Chem.* **1997**, *272*, 4689–4698.
43. Walsh, C. *Adv. Enzymol.* **1983**, *55*, 197–289.
44. Kirk, K. L. In *Biochemistry of Halogenated Organic Compounds*; Kirk, K. L., Ed.; Plenum: New York, 1991; pp 253–317.
45. McCord, T.; Smith, D.; Winters, D. W.; Grimes, J.; Hulme, K. L.; Robinson, L. Q.; Gage, L. D.; Davis, A. L. *J. Med. Chem.* **1975**, *18*, 26–29.
46. Hohster, R. M.; Quastel, J. H. *Metabolic Inhibitors*; Academic: New York, 1963; Vol. 1.
47. Budisa, N.; Karnbrock, W.; Steinbacher, S.; Humm, A.; Prade, L.; Moroder, L.; Huber, R. *J. Mol. Biol.* **1997**, *270*, 616–623.
48. Mann, M.; Wilm, M. *Trend Biochem. Sci.* **1995**, *20*, 219–223.
49. Hofmann, A.; Benz, J.; Liemann, S.; Huber, R. *Biochim. Biophys. Acta* **1997**, *1330*, 254–264.
50. Sykes, B. D.; Weingarten, H. I.; Schlesinger, M. *J. Proc. Natl. Acad. Sci. USA* **1974**, *71*, 469–473.
51. Xiao, G.; Parsons, J.; Tesh, K.; Armstrong, R. N.; Gilliand, G. *J. Mol. Biol.* **1998**, *281*, 323–339.
52. Xiao, G.; Parsons, J.; Armstrong, R. N.; Gilliand, G. L. *J. Am. Chem. Soc.* **1997**, *119*, 9325–9326.
53. Ibba, M.; Hennecke, H. *FEBS Lett.* **1995**, *364*, 272–275.
54. Ring, M.; Huber, R. E. *Biochem. Cell. Biol.* **1993**, *71*, 127–132.
55. Brooks, B.; Phillips, R. S.; Benisek, W. F. *Biochemistry* **1998**, *37*, 9738–9742.
56. Kirk, K. L.; Cohen, L. A. Biochemistry and pharmacology of ring-substituted imidazoles. In *Biochemistry Involving Carbon–Fluorine Bonds*; ACS Symposium Series, Filler, R., Ed.; ACS: Washington, DC, 1976; No. 28, pp 23–36.
57. Klein, D. C.; Weller, J. L.; Kirk, K. L.; Hartley, R. W. *Mol. Pharmacol.* **1977**, *13*, 1105–1110.
58. Wiesmann, A.; Koe, B. K. *J. Pharmacol. Exp. Ther.* **1966**, *155*, 135–144.
59. Baumel, I.; Carlson, G. P.; Lal, H. *Brain Res.* **1971**, *28*, 357–360.
60. Ishiwata, K.; Kubota, K.; Murakami, M.; Kubota, R.; Sasaki, T.; Ishii, S.; Senda, M. *J. Nuclear Med.* **1993**, *34*, 1936–1943.
61. Andersen, S. O. *Acta Chem. Scand.* **1972**, *26*, 3097–3100.
62. Sheard, M. H. *Brain. Res.* **1969**, *15*, 524–528.
63. Stark, P.; Fuller, W. *Neuropharmacology* **1972**, *11*, 261–272.
64. Kettle, A. J. *FEBS Lett.* **1996**, *397*, 103–106.
65. Payne, J. W. *Drugs Exp. Clin. Res.* **1986**, *12*, 585–594.
66. Karlsson, B.; Pascher, T.; Nordling, M.; Arvidsson, R.; Lundberg, L. *FEBS Lett.* **1989**, *246*, 211–217.
67. Tabor, S.; Richardson, C. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 1074–1078.