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Replacement of Tyr62 by Trp in the designer protein Milk Bundle-1 results in significant improvement of conformational stability

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Abstract Protein design is currently used for the creation of new proteins with desirable traits. In our lab, we focus on the synthesis of proteins with high essential amino acid content, having potential application in animal nutrition. One of the limitations we face in this endeavor is the achievement of stable proteins in spite of a highly biased amino acid content. We report here the synthesis and characterization of MB-1Trp, a protein with a tailored content in selected essential amino acids. The protein is a Tyr62-Trp mutant of the parent molecule MB-1 described earlier. The new protein is largely helical as per design, is well folded, and has a melting temperature of 55°C. Its resistance to proteolytic degradation compares to that of cytochrome *c*, a protein of similar size. Design strategy used for MB-1Trp is discussed with regards to its applicability toward the creation of efficient nutritional proteins.

Key words: Protein design; Mutagenesis; Conformational stability; Tryptophan; Protein fluorescence; Hydrophobicity

1. Introduction

De novo design of artificial proteins has been an emerging area of research that tests our understanding of protein structure and allows the creation of macromolecules with desirable and specific structures and properties. The property that we (and other groups) have chosen is the nutritional quality of protein, in view of its importance for animal production and human health [1–5].

In our lab, we have been interested in nutritional value, focusing on proteins with an essential amino acids (EAA) profile tailored for dairy animal production. Our first de novo designed protein, Milk Bundle-1 (MB-1), has been reported earlier [1]. Characterization of MB-1 indicated that the design process used resulted in the stable expression of a new, largely helical protein enriched in the selected EAA (60% in M, T, K and L). After a first round of design, the protein MB-1 was found to have a folded core and low affinity for 8-anilino-1-naphthalenesulfonic acid (ANSA) [4]. Its behavior and expression levels in vivo were found to be far superior to earlier attempts in the area of high EAA polypeptide design [6].

Investigation of MB-1's properties did reveal some flaws, however. The proteins appear to associate into dimers that could dissociate into monomers in the presence of a high salt concentration [4]. Its melting temperature was found to be very low (39°C), and its resistance to proteases at a physiological temperature was also found to be limited, a possible consequence of partial unfolding [6]. As a consequence of these weaknesses, efforts in growing crystals were unsuccessful [7].

In order to improve MB-1's folding and resistance to degradation, strategies for a new cycle of design were considered. One possible modification involved the sole Tyr in position 62, predicted to be buried in the hydrophobic core. This spectral probe has been useful on two counts: it permitted the confirmation of protein purity using fluorescence, and it has been used as a conformational probe, as it was indeed buried as planned [6]. Burial of a hydroxyl group in the core is a potential source of destabilization, however, and may have contributed to MB-1's poor behavior in terms of stability [6]. Replacement of Tyr62 by Trp should provide increased hydrophobicity to the protein core, and would maintain, if not expand, the useful spectroscopic properties of the side chain in position 62. In this paper, we report the substitution of Tyr62 by Trp and the impact of this mutation on a number of structural features, including thermostability.

2. Materials and methods

2.1. Preparation of MB-1Trp mutant

Substitution of the Tyr in position 62 by a Trp was performed using the oligo-directed mutagenesis kit 'Altered Sites® II' (Promega). The mutational oligonucleotide Tyr62-Trp (shown below with the corresponding MB-1 sequences) was purchased from Gibco BRL/Life Technologies, purified using denaturing polyacrylamide gel electrophoresis (PAGE) and phosphorylated.

MB-1: 5′-ATG GCC ACT ACG **TAC** TTC AAA ACG ATG-3′
Tyr62-Trp: 5′-ATG GCC ACT ACG **TGG** TTC AAA ACG ATG-3′

The mutation was then confirmed by dideoxynucleotide sequencing using T7 Sequenase kit (Amersham Life Science). The mutated MB-1 gene was cloned back in the pCMG20 4-X expression vector [1] and positive clones were checked again by DNA sequencing.

2.2. Protein expression and purification

Bacteria carrying the mutant vectors were grown at 37°C, 300 rpm

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Abbreviations: ANSA, 8-anilino-1-naphthalenesulfonic acid; BCA, bicinchoninic acid; CD, circular dichroism; EAA, essential amino acids; EDTA, ethylenediaminetetraacetate; MB-1, Milk Bundle-1; MBP, maltose binding protein

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in 1 l of LB Miller medium (Difco) to an optical density (OD) of 0.4. Transcription was induced using 1 mM isopropylthio-β-D-galactoside for 3 h. The cells were then harvested by centrifugation at 3000×*g*. The purification procedure was essentially as described in [1] with minor modifications. Precipitated cells were resuspended in ice-cold column buffer (10 mM Tris, 200 mM NaCl, 10 mM ethylenediaminetetraacetate (EDTA), 1 mM sodium azide (NaN_3), pH 7.4). Phenylmethylsulfonylfluoride, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetate, benzamidine-hydrochloride and benzamide were added to a final concentration of 0.1, 10, 2 and 2 mM, respectively. Cells were then lysed by 10 30 s sonication pulses using a Branson Sonifier 250 at 60% output control. The sonicate was centrifuged at 11 500×*g* for 30 min at 4°C.

The supernatant was then loaded onto a 15 ml amylose column. The maltose binding protein (MBP) fused with MB-1Trp (MBP-MB-1Trp) was eluted by washing the column with column buffer containing 10 mM maltose (elution buffer). Pooled peak fractions were placed in dialysis tubing (Spectra/Por; molecular weight cut off 3500 Da) with 50 μl factor Xa per 10 ml fusion protein. The bag was placed in 20 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$ (cleavage buffer) overnight at 4°C. The following morning, the bag was transferred to 10 mM Tris–1 mM EDTA (TE) buffer, pH 8.0. After a 2 h dialysis, the sample was applied to DEAE-Sepharose equilibrated in TE buffer, pH 8.0 (Fast Flow; Pharmacia) and washed with the same buffer. MB-1Trp was collected as the flow-through. The different fractions were analyzed for protein content by the bicinchoninic acid (BCA) assay and the positive fractions were pooled and concentrated using BIOMAX-5K concentrators (Millipore). Protein samples were prepared in a borate-phosphate buffer (55 mM NaH_2PO_4, 35 mM $Na_2B_4O_7$, pH 6.8) and dialyzed overnight against this buffer prior to measurements. Protein concentration was adjusted to 0.4 mg/ml unless specified otherwise.

2.3. Protein quantification and electrophoresis

Protein concentration was determined by the BCA assay (Sigma), using bovine serum albumin as the standard. The protein was visualized by sodium dodecyl sulfate (SDS)–PAGE using 12% polyacrylamide–tricine gels, followed by silver nitrate staining. SDS–PAGE experiments were conducted prior to measurements to confirm protein purity.

2.4. Conformational investigation by circular dichroism (CD)

Protein samples were degassed for 20 min at 20°C before measurements. Spectra were measured with a Jasco J-720 spectropolarimeter, which was routinely calibrated with a 0.06% (w/v) ammonium (+)-10-camphorsulfonate solution. For measurements in the far-UV region, a quartz cell with a path length of 0.01 cm was used. Ten scans were accumulated at a scan speed of 20 nm per minute, with data being collected at every nm from 180 to 260 nm. Sample temperature was maintained at 20°C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvettes. Spectra were corrected for buffer signal and conversion to $\Delta\varepsilon$ (on the basis of amide bond concentration) was performed with the Jasco Standard Analysis software. Secondary structure calculations were performed using the CDsstr program developed [8], using default settings.

2.5. Thermal denaturation

Samples were prepared as described in the preceeding Section 2.4. In order to measure thermostability, temperature was increased from 15 to 85°C at a rate of 30°C per hour using a Neslab RTE-11 controlled by the Jasco spectropolarimeter software. CD spectra were collected at every 5°C, from 200 to 260 nm, at a scan speed of 20 nm/min. In order to assess reversibility of thermal denaturation, the protein solutions were cooled down at a rate of 30°C per hour, and spectra were measured at 70, 50 and 20°C.

Thermal stability was calculated assuming a unimolecular, two-state process as previously described [9]. The $\Delta\varepsilon_{MRW}$ at 222 nm measured at various temperatures was used as the property (y) indicative of the extent of unfolding. In the folded state, the parameter $y = y_f$ and the fraction of folded protein f_f is equal to 1. When the protein is unfolded, the parameter $y = y_u$, and the fraction of unfolded protein f_u is equal to 1. For intermediate states, y is given by $y_f f_f + y_u f_u$. Thus, by measuring y, we can calculate the fraction of protein unfolded: $f_u = (y_f - y)/(y_f - y_u)$. The equilibrium constant for the unfolding process is $K_u = f_u/(1-f_u)$ and melting temperatures (T_m) are obtained at $K_u = 1$ [9].

2.6. ANSA fluorescence enhancement

Protein concentration was adjusted to 0.1 mg/ml in B/P pH 6.8 and equilibrated at room temperature (RT) for 1 h. Then ANSA was added to a final concentration of 10 μM and equilibrated 5 min prior to measurements. Spectra were recorded using an LS50-B Perkin-Elmer fluorometer with an excitation wavelength of 380 nm. Spectra were collected from 410 to 550 nm. Correction for buffer signal on ANSA was keyed in when applicable.

2.7. Intrinsic fluorescence measurements

Protein concentration of samples was adjusted to 0.1 mg/ml and equilibrated at RT for 1 h. For chemical denaturation, urea (Sigma U-5378) was added to a final concentration of 8 M and equilibrated at RT 5 min before measurement. For thermal denaturation, temperature was increased to 85°C at a rate of 15°C per hour using Neslab RTE-11. Spectra were recorded using an L550-B Perkin-Elmer fluorometer with an excitation wavelength of 280 nm. Spectra were then corrected for buffer signal before analysis.

2.8. Size-exclusion chromatography/estimation of molecular size

Protein samples were adjusted to a concentration of 0.6 mg/ml and loaded onto a Beckman Ultraspherogel SEC-2000 column hooked to a Waters 510 HPLC. The column was standardized with the Pharmacia Low Molecular Weight gel filtration calibration standards prepared in the same buffer.

2.9. Proteolytic degradation measurements

Experiments were carried out as described by Krisnamoorty et al. [10]; with few modifications as described in Morrison et al. [11]. Reactions were stopped by the addition of 2% SDS buffer followed by heating for 3 min at 100°C. Samples were then analyzed on SDS–PAGE followed by silver nitrate staining. The amount of protein remaining after incubation with proteases was determined by measuring the OD of each band using the image analysis system Imaging Research MCID. Cytochrome *c* from horse heart (Sigma C-7752) was used as a reference protein for all degradation runs in order to correct for possible variations in crude proteolytic activity.

3. Results

Position 62 in MB-1 was chosen for the emplacement of a spectroscopic probe at the moment of initial design [1]. As shown on the model in Fig. 1, this position is part of the hydrophobic core, and a niche made of five Ala was built

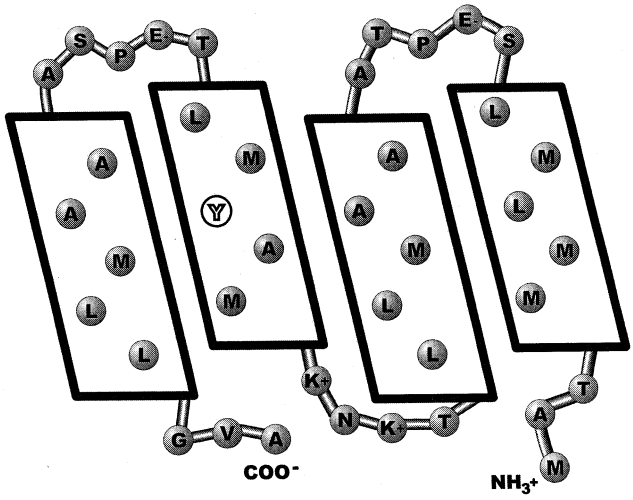

Fig. 1. MB-1's hydrophobic core as predicted by design. The intended structure is a four-helix bundle, with residue polarity distributed along the canonical heptad pattern. The pictogram shows only the interior of the bundle after 'opening' and 'flattening', each box representing the non-polar face of a helix. Note that the large Tyr in position 62 is surrounded by five small alanine residues.

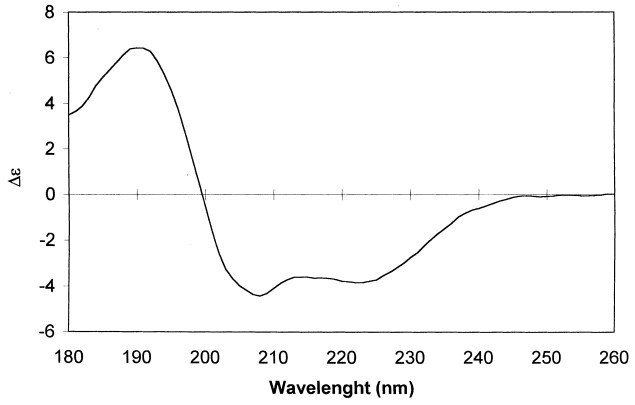

Fig. 2. CD spectra of MB-1Trp. A 50% helical content was predicted from such spectra as described in Section 2.

around it in order to accommodate a larger side chain in this region of the core. Substitution of Tyr62 for Trp was performed on MB-1 by site-directed mutagenesis and confirmed by DNA sequencing and fluorospectroscopy (not shown); then the mutant, named MB-1Trp, was characterized. First, CD measurements were performed and spectra typical of helical proteins were obtained (Fig. 2). Using the CDsstr algorithm [8], 50% of amide groups were predicted to be in a helical environment. Thus, the secondary structures of the mutant MB-1Trp were found to be similar to the parent molecule MB-1.

The formation of a tertiary structure in the mutant was confirmed by fluorescence measurements: the folded protein fluorescence peaked at 333 nm, and its emission shifted to approximately 345 nm when unfolded using heat or urea (Fig. 3). The Trp side chain appears to be protected in the folded protein, and exposed to solvent upon unfolding, as predicted by design. Similar shifts in fluorescence λ_{max} were observed for another designer protein (α4) after insertion of a Trp side chain [12].

Results from fluorescence measurements using the probe ANSA are shown in Fig. 4. The enhancement of ANSA emission by the protein was limited to a factor of 3, which is comparable to the parent molecule [4], and lower than the

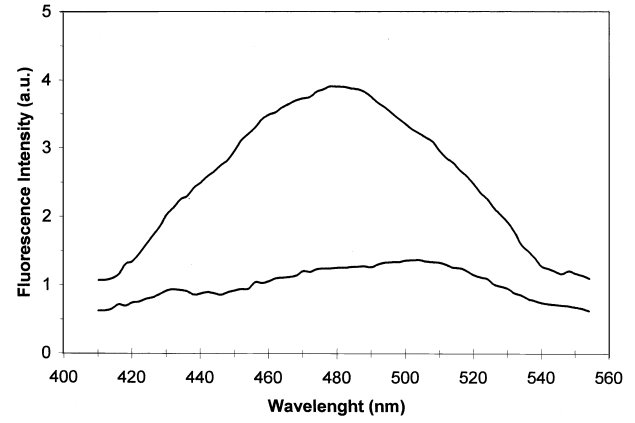

Fig. 4. Enhancement of ANSA fluorescence by MB-1Trp. Spectra collected with 10 μM ANSA alone (bottom curve) and ANSA with MB-1Trp (top curve). When exposed to the mutant protein, ANSA fluorescence shifts from 510 to 480 nm, and its fluorescence intensity at 480 nm increases by a factor of 3, as a result of binding to hydrophobic clusters in folded MB-1Trp.

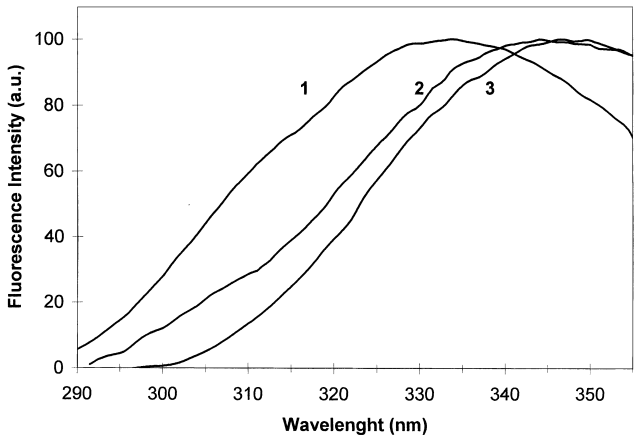

Fig. 3. Unfolding of MB-1Trp as monitored by fluorospectroscopy. Spectra were obtained for MB-1Trp at 20°C (curve 1), after thermal denaturation at 85°C (curve 2) and after urea denaturation (curve 3).

values expected for poorly folded proteins [13]. These results suggest that MB-1Trp is rather well folded, and not in a molten globule. Size-exclusion chromatography analysis revealed that MB-1Trp migrated as a 12 kDa protein, closed to the expected size of an MB-1Trp monomer. Substitution of Trp in position 62 appears to correct one weakness of the initial design of MB-1, which had a size approaching that of a dimer [4].

The impact of the substitution in position 62 on conformational stability was verified using two different approaches. First, the CD signal at 222 nm was recorded at various temperatures in order to monitor unfolding of MB-1Trp helices. The denaturation of MB-1Trp (Fig. 5) indicated a melting temperature of 55°C, a significant improvement over MB-1 (melting temperature of 39°C) and other mutants characterized so far ([6], Hefford and Beauregard, in preparation). The thermal denaturation was found to be fully revers-

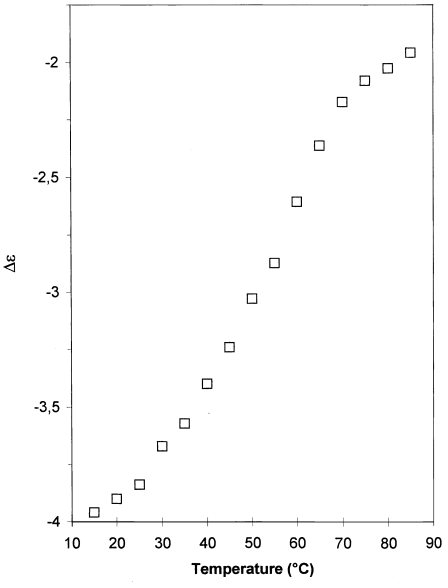

Fig. 5. Thermal denaturation curve for MB-1Trp. $\Delta\varepsilon$ was measured at 222 nm as described in Section 2.

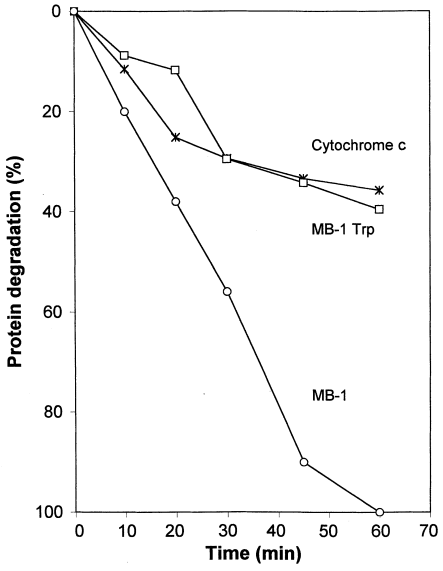

Fig. 6. Degradation of MB-1 and MB-1Trp mutant using pronase E. MB-1 resistance to proteolytic degradation (open circles) is increased by the insertion of a Trp instead of the Tyr at position 62 (open square). MB-1Trp's resistance to proteolytic degradation compared to that of cytochrome *c*, a natural protein of similar size that is used as an internal standard for such measurements.

ible, another improvement over MB-1, but the transition was spread over a wide temperature range (30–65°C). However, this apparent lack of cooperativity in MB-1Trp thermal unfolding also characterized MB-1 and other small designer proteins [6,12].

Stabilization of MB-1 fold by the mutation was also confirmed by proteolytic degradation experiments. Degradation curves shown in Fig. 6 clearly demonstrate a gain in resistance to proteolytic attack. Under our standard conditions, MB-1Trp was twice as resistant as MB-1, and behaved like a natural protein of similar size (cytochrome *c*).

4. Discussion

Trp and Tyr residues are comparable on various counts: they have large aromatic side chains, they undergo a limited but similar loss of conformational entropy upon protein folding, and they have similar secondary propensities [14,15]. They differ in their hydrophobicities, however, due to the presence of a hydroxyl group in p position on the phenyl moiety. When such a polar group is involved in a hydrogen bond, the difference between Trp and Tyr hydrophobicity decreases [14], but in MB-1, no such H-bonding partner was properly positioned while designing the protein. Most results shown here indicate that the increase in hydrophobicity in position 62, due to the removal of tyrosine's hydroxyl group from the core, led to an important improvement of MB-1 fold stability. The important impact of a Tyr-Trp mutation observed here is not totally unexpected: Honda et al. [16] observed a significant drop in T_m values for the chitosanase from *Streptomyces* sp. N174 upon removal of Trp residues. This may be explained by tryptophan's ability to make extensive van der Waals contacts with neighboring residues due to its large size, in addition to its contribution to the hydrophobic effect. The improvement of MB-1 properties also included the specification of a monomeric organization. A full understanding of such a correction to this design weakness awaits further investigations though.

Trp and Tyr both belong to the class of large side chains, but Trp is significantly larger than Tyr, with an additional 34 Å^3 [16,17]. The original design strategy allowed for a Tyr in position 62, but not for Trp, which would result in a layer volume that would be above average for natural bundles. The results shown here all indicate that MB-1 secondary and tertiary structures were not disturbed by the substitution Tyr62-Trp. The protein is helical and well folded and it appears that the niche around position 62 readily accommodates the larger side chain of Trp. The mutation resulted in an important improvement in the protein stability. In a comparable designer protein where the intended location of a Trp was not engineered in a way as to accommodate an increase in bulk, the mutation actually destabilized the protein [12].

Our results suggest that while Trp stabilized MB-1 fold, it may not be as buried as Tyr was in MB-1: its fluorescence λ_{max} is at 333 nm, and not near 325 nm as observed for fully protected Trp. Similar results obtained by Handel et al. [12] on a similar designer protein may indicate that Trp cannot be buried completely in a core as small as the one involved here.

The gain in conformational stability afforded by the Tyr62-Trp mutation is of paramount importance for future advances in the development of MB-1 family of proteins. What we achieved here by a single point mutation is the conversion of a low stability protein into a protein that is now folded at physiological temperature. Its high resistance to proteases could permit the production of this high quality protein by transgenic crops to be used in animal production. A comparison of previously reported analyses of plant protein degradability indicates that MB-1Trp compares to sunflower 2S seed albumin 8 protein, a protein with a high methionine content intended for production of transgenic crops with enhanced nutritional quality [18]. Note that the EAA profile of SFA8 is not optimized for lactating cows needs, while MB-1Trp is, due to its balanced content of methionine, lysine and threonine. This comparison indicates that although few cycles of design may be required, our design approach produced a high quality protein that competes with natural proteins at the level of stability.

A number of MB-1 mutants have been designed and characterized so far. The design strategy was based on the assumption that MB-1 folded as an α-helical bundle, as shown in Fig. 1. Most mutants, including MB-1Trp, behaved as helical and folded protein ([7,19], Williams et al., in preparation). These results (and results from investigations of MB-1 structure using a number of low resolution techniques) all indicate that the designer protein MB-1 is approximately folded as planned. Nevertheless, no detailed structure (nuclear magnetic resonance or X-ray) is available for the designer protein MB-1 as of yet, since our efforts have been hampered by lack of solubility and stability in solution [7]. The mutant presented here clearly outperforms the parent molecule on several counts, including the ability to stay monomeric under conditions used here, and to resist degradation in solution. Crystallization experiments are under way and it is hoped that solving the MB-1Trp structure will permit a critical evaluation of the design strategy used for MB-1.

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