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Engineering Nutritious Proteins: Improvement of Stability in the Designer Protein MB-1 via Introduction of Disulfide Bridges

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Protein design is currently used for the creation of new proteins with desirable traits. In this laboratory the focus has been on the synthesis of proteins with high essential amino acid content having potential applications in animal nutrition. One of the limitations faced in this endeavor is achieving stable proteins despite a highly biased amino acid content. Reported here are the synthesis and characterization of two disulfide-bridged mutants derived from the MB-1 designer protein. Both mutants outperformed their parent protein MB-1 with their bridge formed, as shown by circular dichroism, size exclusion chromatography, thermal denaturation, and proteolytic degradation experiments. When the disulfide bridges were cleaved, the mutants' behavior changed: the mutants significantly unfolded, suggesting that the introduction of Cys residues was deleterious to MB-1-folding. In an attempt to compensate for the mutations used, a Tyr62-Trp mutation was performed, leading to an increase in bulk and hydrophobicity in the core. The Trp-containing disulfide-bridged mutants did not behave as well as the original MB-1Trp, suggesting that position 62 might not be adequate for a compensatory mutation.

KEYWORDS: Amino acid; mutations; protein; synthesis; agrobiotechnology

INTRODUCTION

The expense associated with feed additives used for animal production has led to the study of intracellular production of high-quality protein by transgenic crops and other organisms, as a means of obtaining efficient and less costly sources of essential amino acids (EAA) (*1*−*3*). Three main approaches for improving protein quality in a given organism are being explored (*4*, *5*). The first approach involves transferring a gene coding for a high-quality protein from one organism to another that is more suitable for farming practices (heterologous expression). Recently, soybean and sunflower albumins have been chosen for their high methionine contents for the development of transgenic crops by major agro-biotechnology companies (*6*, *7*). In this approach, the amino acid composition of a natural protein is predetermined and may not conform to the desired EAA ratio. The second approach involves modifying the genes of an organism so that more selected EAAs are inserted in the proteins. Attempts to change amino acid content, however, often destabilize the protein and/or prevent it from folding, which may jeopardize its recovery (*4*, *8*, *9*). Recently, some success has been reported with this approach by Chui et al. (*10*) and Hey et al. (*11*). The third approach, chosen here, involves creating a new protein with a biased composition of selected EAAs (*12*−*14*). Theoretically, this strategy allows for full control of the amino acid composition of the protein and is, thus, an advantage over the previously mentioned options.

Previous efforts to express synthetic proteins with high EAA contents in vivo in bacteria (*14*−*16*) have not yielded high quantities of protein, which is possibly attributable to the lack of structural stability of the final peptide. To improve on the properties of such new proteins, we have tried to identify dominant folding principles in selected natural proteins and attempted to encode them into the new amino acid sequence. The underlying assumption of this strategy is that by encoding information that confers structure, compactness, and stability, a protein normally stable in vivo could be designed, regardless of its biased composition. The ideal protein we hope to design would (1) have a strong biased composition, with methionine, lysine, and other EAAs being dominant (the limiting amino acids for milk protein production were targeted) and (2) be expressed in vivo as a soluble, stable, full-length protein.

At the time of its design (*13*), creation of this ideal protein was a challenge yet to be met; most reported advances in protein design had not involved expressed and soluble in vivo full-length proteins (*17*). Nevertheless, the investigation of folding principles in α-helical bundle proteins was relatively advanced (*18*), and because this fold could harbor most of the residues desired, we selected the bundle as our ideal protein. The basic

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assumptions for the design of the new protein called milk bundle 1 (MB-1) were as follows: (1) the range of possible residues in each recurring position in the helices of the bundle protein would be determined by the physiochemical properties of the amino acid residues and the topological constraints due to their particular location in the tertiary structure; (2) the driving force for bundle formation would be a "hydrophobic collapse", where hydrophobic side chains are energetically favorably buried in the interior of the bundle; (3) as a result, the loops would be relatively unimportant. The design process was an iterative one in which criteria such as secondary structure propensities, charged residues distribution, and other global factors found to be determinant for folding (*19*−*22*), were subsequently used to refine the initial design (*13*).

Characterization of MB-1 indicated that the design process used resulted in the stable expression of a new, largely helical protein enriched in the desired amino acids (60% in M, T, K, and L). After a first round of design, the MB-1 protein was found to have a folded core and a low affinity for 8-anilino-1-naphthalenesulfonic acid (ANSA) (*23*). Its behavior and expression levels in vivo were found to be far superior to those obtained from earlier attempts of high EAA polypeptide design (*24*).

Investigation of MB-1 properties did, however, reveal some flaws. Its melting temperature was found to be very low (39 °C), and protease resistance at physiological temperatures was limited, possibly due to partial unfolding (*24*). Such results suggests that MB-1 would not accumulate in transgenic organisms, where it would be exposed to proteases. Proteolytic degradation experiments have predicted that MB-1 lifetime in rumen conditions will be shorter than that of several natural proteins (*25*). Thus, feeding MB-1 to ruminants may also be inefficient.

One way of improving the properties of MB-1 is to increase its conformational stability, which often correlates with resistance to proteolytic degradation (*26*−*28*). To improve MB-1's stability, additional design cycle are under study. In the absence of an X-ray-resolved structure, extensive core redesign and elaborated fold-specifying devices had to be ruled out. In view of its apparent simplicity, we chose the insertion of disulfide bridges. This strategy was chosen not only for its simplicity but for other reasons as well: (1) an intramolecular disulfide bridge would bring stability to the protein by reducing the entropy of the unfolded state (*29*, *30*); (2) the disulfide bridge is a well-known stabilizer against proteolysis, probably due to its impact on target access by proteases (*31*−*33*); (3) its engineering involves only a small modification of the amino acid composition, which is critical in order for MB-1 to remain nutritionally efficient.

This study also involves MB-1Trp, a mutant that was obtained recently (*34*). This mutant is derived from MB-1 and was obtained by the replacement of a core Tyr by Trp, a larger, less polar amino acid. Several protein properties were positively modified by this mutation, including thermostability and resistance to proteolytic degradation, which were largely increased (*34*). Thus, in this paper, we will attempt to build stabilizing disulfide bridges into MB-1 and MB-1Trp.

EXPERIMENTAL PROCEDURES

Preparation of the New Mutants. Substitution of Cys in positions 13 and 87 of MB-1 (MB-1LH) and positions 10 and 91 of MB-1 (MB-1RH) was performed using the oligo-directed mutagenesis kit "Altered Sites II" (Promega). The mutational oligonucleotides (**1** for position 13, **2** for position 87, **3** for position 10, and **4** for position 91, shown below with the corresponding MB-1 sequences) were purchased from GibcoBRL/Life Technologies, purified using denaturing polyacrylamide gel electrophoresis (PAGE), and phosphorylated.

MB-1: 5′-ATG ATG ACC ACC **CTG** TTT AAA ACT ATG-3′
Oligo 1: L13C: 5′-ATG ATG ACC ACC **TGC** TTT AAA ACT ATG-3′

MB-1: 5′-ACG GCT ACA ACC **ATG** AAA AAT CAT CTG-3′
Oligo 2: M87C: 5′-ACG GCT ACA ACC **TGC** AAA AAT CAT CTG-3′

MB-1: 5′-ATG ACC GAC ATG **ATG** ACC ACC CTG TTT-3′
Oligo 3: M10C: 5′-ATG ACC GAC ATG **TGT** ACC ACC CTG TTT-3′

MB-1: 5′-ATG AAA AAT CAT **CTG** CAG AAC TTG ATG-3′
Oligo 4: L91C: 5′-ATG AAA AAT CAT **TGC** CAG AAC TTG ATG-3′

MB-1: 5′-ATG GCC ACT ACG **TAC** TTC AAA ACG-3′
Oligo 5: Y62W: 5′-ATG GCC ACT ACG **TGG** TTC AAA ACG-3′

The Tyr62-Trp mutation led to MB-1Trp and was achieved directly in the expression vector pMal-c2. The mutational oligonucleotide **5** was used and treated as described above. The mutations were then confirmed by dideoxynucleotide sequencing using a T7 Sequenase kit (Amersham Life Science). The mutated MB-1 genes were cloned back in pCMG20 4-X, the expression vector. All positive clones were checked again by DNA sequencing after cloning.

Protein Expression and Purification. All mutated proteins were prepared as described in Gagnon et al. (*34*). After purification, all proteins were checked for purity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (protein purity >95%). Unexpectedly, during purification, the mutant MB-1TrpRH formed oligomers of various sizes. For this mutant, an additional purification step was used to isolate the monomeric proteins, as described in Doucet and Beauregard (*35*).

Protein Quantification and Electrophoresis. Protein concentration was determined by using the bicinchoninic acid (BCA) assay (Sigma), with bovine serum albumin as standard. The protein was visualized by SDS-PAGE using 12% polyacrylamide−tricine gels, followed by silver nitrate staining. SDS-PAGE experiments were conducted prior to measurements to confirm protein purity.

Quantification of Cys Residues and Assessment of Bridge Formation. Chemical modification of the Cys residues by iodoacetamide and iodoacetate was performed as described in Hollecker and Larcher (*36*). Comparison of the electrophoretic mobility of the reduced and nonreduced form of the MB-1 mutant was done by SDS-PAGE (*35*). The Cys residues were also quantitated by reacting with *p*-hydroxymercurybenzoate (*p*-HMB) and measuring absorbance of *p*-HMB at 260 nm with a UV−vis spectrophotometer (Novaspec, Pharmacia).

Conformational Investigation by Circular Dichroism (CD). Unless specified otherwise, protein samples were prepared at a concentration of 0.6 mg/mL with a phosphate buffer (128 mM NaH_2PO_4, pH 6.8). The reductive agent dithiothreitol (DTT) was added to a final concentration of 10 mM where specified. The samples were then degassed and equilibrated 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/min, with data being collected at every nanometer 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 cuvette. Spectra were corrected for buffer signal, and conversion to $\Delta\epsilon_{MRW}$ (mean residue weight) was performed with the Jasco Standard Analysis software. Secondary structure calculations were performed using the CDsstr program developed by Johnson (*37*), using default settings.

Thermal Denaturation. Samples were prepared as described in the preceding section. To measure thermostability, temperature was increased from 10 to 95 °C at a rate of 30 °C/h using a Neslab

RTE-11 controlled by the Jasco spectropolarimeter software. CD spectra were collected every 5 °C, from 200 to 260 nm, at a scan speed of 20 nm/min, and CD signals at 222 nm were collected at 1 °C intervals. To assess the reversibility of thermal denaturation, the protein solutions were cooled at a rate of 30 °C/h, and spectra were measured at 70, 50, and 20 °C.

Thermal stability was calculated by assuming a unimolecular, two-state process as previously described (*38*). The CD signal at 222 nm 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 qjto 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 $K_u = 1$ (*38*).

ANSA Fluorescence Enhancement. Protein concentration was adjusted to 0.2 mg/mL and equilibrated at room temperature for 1 h. Then ANSA was added to a final concentration of 10 μM and equilibrated for 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 and for the effect of DTT on ANSA was keyed in when applicable.

Fluorescence Quenching Measurements. The protein concentration of samples was adjusted to 0.2 mg/mL and equilibrated at room temperature for 1 h. Where specified, DTT was added to a final concentration of 10 mM and equilibrated at room temperature for 15 min before the quencher citrate (final concentration = 0.25 M) was added. Control samples were exposed to 0.75 M NaCl to keep the same ionic strength as in the samples exposed to 0.25 M citrate. Mutants containing Tyr were excited at 280 nm, and fluorescence spectra were recorded from 290 to 330 nm. For the Trp mutants, fluorescence was collected from 310 to 400 nm. Quenching of the Trp mutants was performed with 0.3 M potassium iodate, and NaCl was used in controls. Spectra were then corrected for buffer signal before analysis. The effect of DTT on fluorescence was corrected when needed.

Proteolytic Degradation Measurements. Proteins were dialyzed against 2000 volumes of borate−phosphate buffer (pH 6.8) at 4 °C overnight. Experiments were then carried out as described by Krisnamoorty et al. (*39*) with few modifications as described in Morrison et al. (*25*). 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 optical density of each band using the image analysis system Imaging Research MCID. Cytochrome *c* (horse heart, Sigma catalog no. C-7752) was used as a reference protein for all degradation runs to correct for possible variations in crude proteolytic activity.

Size Exclusion Chromatography (SEC). Proteins were applied to a Beckman Ultraspherogel SEC-200 column at a concentration of 0.6 mg/mL. A constant flow of 0.8 mL/min was maintained using a Waters HPLC pump. Pharmacia low molecular weight gel filtration calibration standards were used for column calibration.

RESULTS

Design Strategy. The putative modifications to MB-1 structure are illustrated in Figure 1. The design strategy used here focused on two aspects: (1) the restrictive effect of a covalent bond between remote residues on the protein as a whole and (2) the precise location of Cys, which permits disulfide bridge formation. By choosing positions as far apart as possible, one can reduce the entropy gain upon unfolding for most of the protein (*29*, *40*). Thus, we attempted to insert a bridge between helices I and IV. Another consideration for using helix I is that this MB-1 segment is sensitive to proteolytic degradation (Hefford and Beauregard, unpublished observations). The restriction of helix I by a bridge could help prevent such a phenomenon.

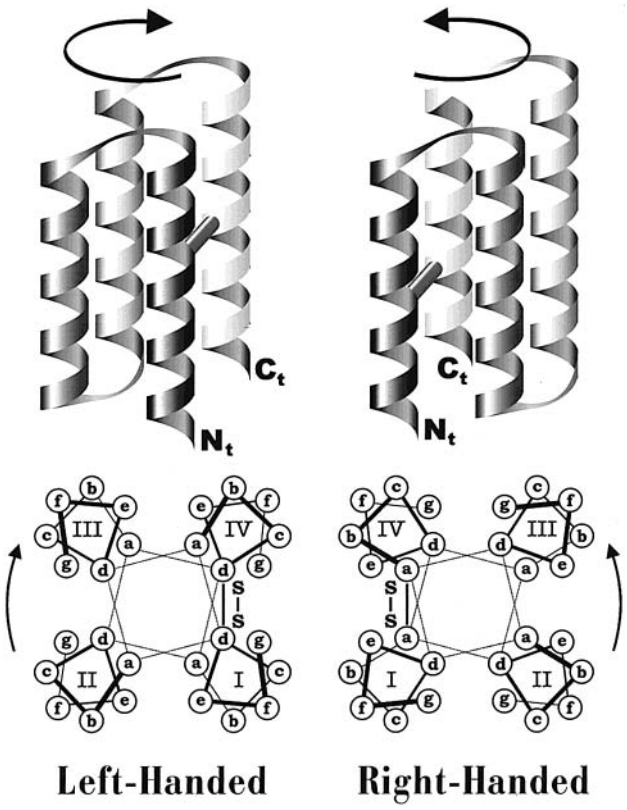

Figure 1. Schematic representation of MB-1LH and MB-1RH: (top left) ribbon model of an α-helical bundle with a left-hand connectivity; (bottom left) end-on view showing the canonical heptad pattern used to design MB-1. The two ribbon models show the disulfide bridge (tube connecting helices) to be formed between helices I and IV. Positions d are chosen for mutations to Cys for MB-1LH. Pictograms on the right side of the figure apply to MB-1RH (with a right-hand connectivity) where positions a are selected for the mutations.

The position of Cys in helices I and IV must allow sulfhydryl groups to be properly aligned in order to minimize strain induced by bridge formation. On the basis of geometric models built for similar proteins (*41*), it appeared that position d of the heptad pattern used for MB-1 design would offer the best geometry for bridge formation. Therefore, L13 and M87 residues were selected for mutation to Cys. Figure 1 depicts the expected location of the bridge in the mutant (hereafter referred to as MB-1LH, assuming it folds as per design). Note that for proper alignment of position d in helices I and IV, a left-hand connectivity of the helices had to be assumed (i.e., the bundles are positioned such that when helix I is at the forefront, with its N terminus pointing down, then helix II is placed to the left of helix I). A second scenario was considered, in which a right-hand connectivity could be specified. Examination of the second model in Figure 1 suggests mutations at positions a in helices I and IV, because positions d would be too far apart. By choosing M10 and L91 residues for mutation to Cys, we attempted to generate a mutant (named MB-1RH) that would resemble MB-1LH as much as possible, except for reversing its connectivity.

Disulfide bridges will also be inserted into MB-1Trp. This protein is a derivative of MB-1 in which Tyr62 was replaced by Trp. Position 62 in MB-1 was chosen for the emplacement of a spectroscopic probe at the moment of initial design (*13*).

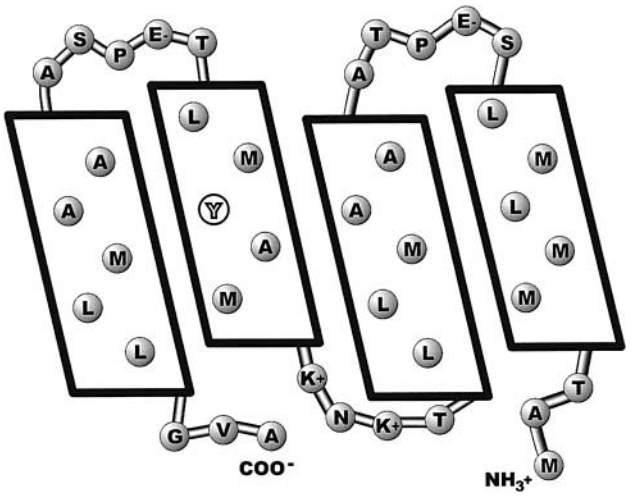

Figure 2. MB-1's hydrophobic core as predicted by design. The intended structure is a four-helix bundle, with residue polarity distributed along the canonical heptad pattern. The pictogram shows only the interior of the bundle after "opening" and "flattening", each box representing the nonpolar face of a helix. Note that the large Tyr in position 62 is surrounded by five small alanine residues. This Tyr62 is changed to Trp in MB-1Trp.

As shown in the model in Figure 2, position 62 is part of the hydrophobic core, and a niche made of five Ala was built around it to accommodate a larger side chain in this region of the core. The replacement of Tyr by Trp was thought to improve on stability and, indeed, characterization of MB-1Trp confirmed the strategy. MB-1Trp has a melting temperature of 55 °C and is more resistant to protease action than MB-1. Here we are going to use MB-1Trp because of the increase in bulk offered by Trp in the core, in a way to compensate for the loss of volume consequent to the mutations used for bridge insertion.

Initial Characterization of Mutant Structure. The presence of two Cys in all mutants was confirmed by Cys derivatization with mixtures of iodoacetamide and iodoacetate and by reacting Cys with *p*-HMB, in agreement with the DNA sequences of the expression vectors (data not shown). Formation of the bridges was confirmed by a comparison of the protein migration after and before treatment with DTT, a reductive agent. Reduction of the protein changed its migration speed, and the impact of reduction appeared to modify the mobility of all proteins treated (not shown). Replacement of Tyr by Trp was confirmed by spectrofluorometry.

Secondary Structures Analysis. Secondary structure analysis of the mutant was performed using CD (Figure 3). The spectra measured for all mutants with their bridge closed (no DTT) were typical of helical proteins, and calculations of helical amide contents were in the range expected for these proteins (Table 1). MB-1LH, MB-1RH, and MB-1TrpLH appeared to have secondary structures similar to those of their parent molecules MB-1 and MB-1Trp (which contain ~50% helical amides) (*23*, *34*). The protein MB-1 appeared to be tolerant to bridge insertion, regardless of the connectivity imposed by this bridge (either handedness).

Secondary structures in MB-1TrpRH differed significantly from the other proteins' secondary structures, with only 42% helical content (Table 1). The right-hand connectivity in MB-1TrpRH imposes a different conformation, with less helical content.

The mutants were then analyzed in the presence of DTT (Figure 4), to separately assess the effect of the mutations (insertion of Cys) from the impact of bridge formation. All

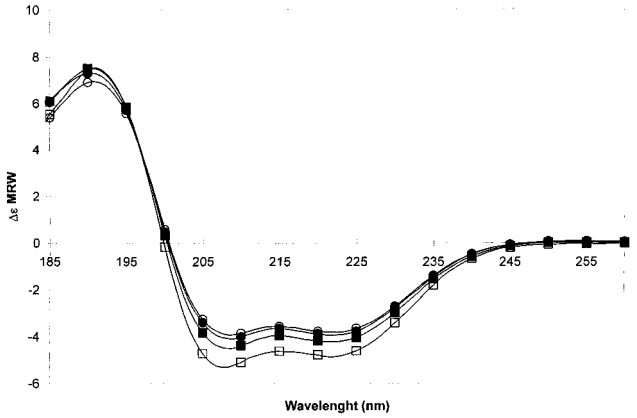

Figure 3. CD spectra of the four mutants: (□) MB-1RH; (○) MB-1LH; (■) MB-1RHTrp; (●) MB-1LHTrp. CD signal is expressed as change in extinction coefficient per residue. Note that after a cycle of reduction–oxidation or after renaturation, the CD spectra measured superimposed on the ones shown here (see Materials and Methods for details).

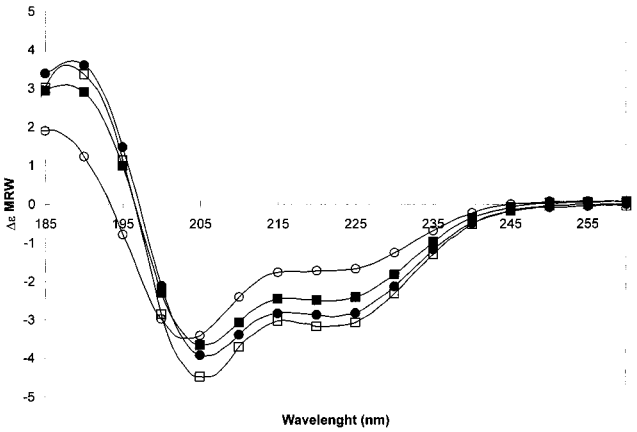

Figure 4. CD spectra measured in the presence of DTT. Symbols are the same as those in Figure 3.

Table 1. Comparison of Physical Properties Measured for the Four Mutants

mutant	α-helix[a] ± 3%	monomer population SEC[b] ± 5%	monomer population SDS-PAGE[c] ± 5%	T_m[d] ± 1 °C	protein undegraded[e] ± 8%
RH	50 (23)[f]	100	100	50	35
LH	48 (18)	100	100	48	43
RHTrp	42 (25)	90	95	49	40
LHTrp	55 (34)	100	100	42	17

[a] Helix percentage calculated with CD spectra. [b] Oligomerization population estimated from exclusion chromatography. [c] Oligomerization population estimated from SDS-PAGE gel analysis. [d] Melting temperature derived from thermal denaturation. [e] Percentage of protein undegraded after 60 min of incubation with Pronase E. [f] Numbers in parentheses are for proteins with opened disulfide bridge (treated with DTT).

mutants' helical content dropped when exposed to DTT as indicated by a loss of signal intensity at 190 and 225 nm, with MB-1LH being nearly completely unfolded (Table 1). This suggests that in the absence of the bridge, mutations used here are destabilizing. Among the four mutants presented, MB-1LH had the largest drop in helical content after bridge opening (down to 18%, see Table 1). This means that the mutations M13-Cys and L87-Cys promote unfolding of the protein and that formation of the bridge compensates for this deleterious effect.

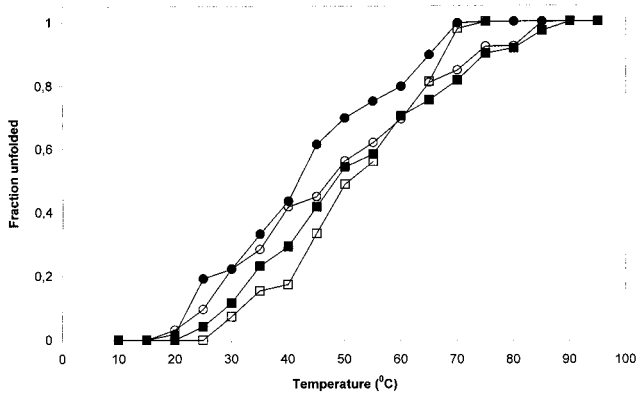

Figure 5. Thermal denaturation curves. The proteins are folded (fraction unfolded = 0) at low temperature and unfolded at high temperature (fraction unfolded = 1). Representative data are shown. For symbols see Figure 1.

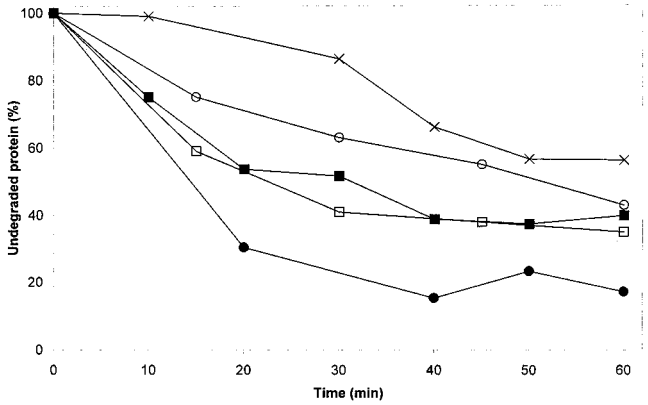

Figure 6. Proteolytic degradation of the proteins. Percentages are based on total protein (MB-1 or mutant) detected prior to incubation. Representative data are shown. Symbols are the same as those in previous figures, and results obtained for cytochrome *c* are shown by "×".

When the same treatment was performed on MB-1TrpLH, the loss of helical content was less severe (down to 34%) (Table 1). The fact that MB-1TrpLH stayed somewhat better folded (34%) than MB-1LH in the presence of DTT may suggest that Trp compensate for the loss of bulk consequent to Cys insertion in the core with a left-hand connectivity. Partial unfolding after DTT treatment was confirmed by fluorescence quenching and ANSA binding for all mutants (not shown).

Reversibility of DTT-induced unfolding was verified by CD measurements. After DTT removal, CD spectra were similar to the native spectra, suggesting that all four proteins refolded after bridge repair (Figure 3).

Quaternary Structure. The ability to control protein association and aggregation has been (and still is) an important stumbling block in de novo protein design. Thus, we monitored protein association for our mutants under two sets of conditions. First, quaternary organization was monitored by SEC under benign buffer conditions in a way to observe the impact of both covalent and non-covalent bonds. Then, the proteins were denatured and migrated on an SDS-PAGE gel to detect any intermolecular covalent bond. Results in Table 1 indicate that all mutants, except MB-1TrpRH, were monomeric, regardless of the treatments used here. This confirms that intramolecular bridges are formed and suggests that the three proteins fold as planned. At variance, MB-1Trp RH was found to contain 5−10% dimer that resisted SDS treatment, which indicates that these dimers are formed via an intermolecular bridge. Other oligomerization states have been observed for this protein under specific experimental conditions (*35*). An increase in surface hydrophobicity was detected using ANSA binding measurements (ratio I_{480}/I_{510} for MB-1TrpRH = 12, compared to a ratio of 7−8 for the other three mutants), which indicates that the MB-1TrpRH core is more fluid, allowing for intermolecular bridges.

Effect of the Mutations on Conformational Stability. Conformational stability of both mutants was measured using CD as described under Materials and Methods. Figure 5 shows the four denaturation curves, and calculated T_m values are listed in Table 1. Both MB-1 mutants clearly outperform MB-1 [T_m = 39 °C (*24*)], by ∼10 °C in thermostability. Approximately 100% of the helical content was recovered after renaturing the protein by cooling to room temperature as described under Materials and Methods, whereas the MB-1 parent molecule could not be refolded under similar experimental conditions (*24*).

Both mutants derived from MB-1Trp were less thermostable than their ancestor molecule [T_m for MB-1Trp = 55 °C, Gagnon et al. (*34*)], with MB-1TrpLH being the least stable (T_m = 42 °C). The mutants are also less stable than MB-1LH and MB-1RH, suggesting that the compensatory mutation Tyr62-Trp is in fact not compatible with the conformations specified by the bridges.

Effect of the Mutations on Proteolytic Degradation. Proteolytic degradation experiments were carried out as described in Figure 6. Both mutants derived from MB-1 outperformed MB-1, with ∼40% intact protein left after a 1 h treatment with proteases [MB-1 cannot be detected after the same treatment (see ref *25*)]. MB-1LH is more resistant than MB-1RH in the first 45 min, but the difference decreases after 60 min. The mutants derived from MB-1Trp were less stable than their parent molecule [60% intact MB-1Trp left under the same conditions (see ref *34*)]. MB-1TrpRH behaved like MB-1RH and the other MB-1 mutants, whereas MB-1TrpLH has the lowest resistance, with 17% proteins left after treatment (Table 1). Once again, the insertion of Trp near the bridge did not lead to any improvement on stability. Degradations were performed in the presence of DTT, and higher degradation rates were measured. However, difficulties were encountered due to the inhibitory effect of DTT on the proteolytic activity and the need to prevent reoxidation of the disulfide bridge, thus strongly limiting the reliability of these data (data not shown).

DISCUSSION

The insertion of disulfide bridges in MB-1 was found to have an important impact on protein behavior. Both mutant proteins were more resistant and stable than MB-1 (*25*). Their unfolding (be it after bridge reduction or due to high temperature) was found to be reversible, and their apparent size in solution indicates they are monomeric. Both strategies led to serious behavior improvement when compared to the MB-1 parent molecule, in accordance to the known impact of disulfides on protein resistance (*31*−*33*). This phenomenon cannot be ascribed to Cys mutations per se (which are destabilizing) but is a consequence of specifying connectivity and limiting protein skeleton freedom through disulfide bond formation.

Predicting (and understanding) the impact of Cys mutations separately from the effect of bridge formation has been difficult in the past. As a result, engineering disulfides in natural proteins has sometimes led to loss of stability (*29*, *42*, *43*). Here we attempted to separately monitor the effect of mutation from that of bridge formation. Results obtained in the presence of DTT

indicate that Cys mutations are destabilizing for both proteins. Looking back at the design strategy, it appears that the choices made for the Cys mutations may be questioned on several accounts: Cys does not have a property profile (size, hydrophobicity) that perfectly matches the consensus profile for positions 10 and 91 (*44*, *45*), where leucines are the preferred residues; Cys is a poor helix former (*46*); and finally, the positions chosen are part of the core, where a protein is most sensitive to mutations.

Closing the bridge led to an increase in stability, which suggests that possible strain induced by cross-linking helices I and IV is rather unimportant. Thus, the geometry and the position of the Cys residues appear to be adequate for bridge formation as expected per design.

The disulfide-bridged constructions harboring Trp 62 analyzed here were not better behaved than the ones based on MB-1. Furthermore, comparing MB-1TrpRH or MB-1TrpLH to MB-1Trp revealed that inserting the bridges was destabilizing. MB-1TrpRH appeared to form concatemers linked by intermolecular bridges, indicating a high conformational flexibility. MB-1TrpLH had the lowest resistance to proteases, suggesting some undetected flaw in folding. Therefore, the insertion of Trp in position 62 of bridged MB-1 protein is not appropriate, and one might infer that this position is too close to the bridges, promoting steric hindrance. Also, loss of backbone flexibility by insertion of the bridge can lead to a misalignment of the Trp residue in its "niche" formed by the five surrounding alanines. More definitive answers to this question will be available when we get access to more refined structural data.

Because we did not explicitly specify connectivity in MB-1 design, it would have been tempting to elucidate this topological feature on the basis of a comparison of MB-1LH and MB-1RH (the two connectivities are shown in Figure 1). Unfortunately, with their bridge closed, the proteins behave in a similar manner. The slight differences which would indicate that MB-1RH is better folded may be readily explained by the impact of Cys mutations on MB-1LH. The opposing effect of the bridge and the mutations compromises any attempt at elucidating connectivity. Thus, our results do not clearly indicate any intrinsic preference for a right- or left-hand connectivity in the MB-1 parent molecule. The original MB-1 design was based on a statistical survey of helices in four helical bundles (*13*). Little attention was initially paid to many topological details, so the set of proteins used for analysis contained proteins of both connectivities. We have undertaken a larger analysis of helical bundles in which we now consider different connectivities separately. Preliminary results indicate that amphiphilic positions such as heptad positions e and g may have a determining role for this topological feature.

The mutations used here allowed increased MB-1 stability and resistance to proteases, which should improve MB-1 efficiency as a food additive. In fact, a comparison of previous analyses on plant protein degradability indicates that some of our mutants compare to sunflower 2S seed albumin 8 protein, a protein with a high methionine content intended for production by transgenic crops with enhanced nutritional quality (*32*). Note that the EAA profile of SFA8 is not optimized for the needs of lactating cows, whereas MB-1 is, due to its balanced content of methionine, lysine, and threonine. The comparison between SFA8 and our mutants indicates that although few cycles of design may be required, our design approach produced high-quality proteins that compete with natural proteins at the level of stability.

ABBREVIATIONS USED

ANSA, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; DTT, dithiothreitol; EAA, essential amino acid; *p*-HMB, *p*-hydroxymercurybenzoate; MB-1, milk bundle 1 protein; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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