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Oxidative folding of synthetic polypeptides *S*-protected as *tert*-butylthio derivatives

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Abstract: A new method for oxidative folding of synthetic polypeptides assembled by stepwise solid phase synthesis is introduced. Folding is obtained in excellent yields by reacting *S*-*tert*-butylthiolated polypeptides with a 100-fold molar excess of cysteine at 37 °C in a slightly alkaline buffer containing chaotropic salts, and in the presence of air-oxygen. This novel protocol has been applied to the folding of *S*-*tert*-butylthiolated human thymus and activation-regulated chemokine (hu-TARC) derivatives as well as to larger segments of *Plasmodium falciparum* and *Plasmodium berghei* circumsporozoite proteins. Folded *P. falciparum* polypeptides have been used as substrates of endoproteinase Glu-*C* (Glu-*C*) and endoproteinase Asp-*N* (Asp-*N*) in an attempt to identify their disulfide connectivities. Particular practical advantages of the present method are (i) easy purification and storage of the *S*-protected peptide derivatives, (ii) elimination of the risk of cysteine alkylation during the acidolytic cleavage deprotection and resin cleavage steps, (iii) possibility to precisely evaluate the extent of folding and disulfide bond formation by mass spectrometry, and (iv) facile recovery of the final folded product. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide synthesis; oxidative folding; polypeptides; vaccine; malaria; chemokine

INTRODUCTION

During the search for subunit vaccine candidates for malaria, we were confronted, at the very beginning, with the problem of assembling and folding of a large segment *Pf*CSP- (282–383) (102 amino acid residues) of the *Plasmodium falciparum* circumsporozoite protein, flanking on the *C*-terminal side by the immunodominant domain consisting of forthy -Asn-Ala-Asn-Pro- repeats. The 102-mer polypeptide with four cysteine residues of unknown connectivity and containing important elements for $CD4^+$ and $CD8^+$ T-cell responses was, at that time, one of the leading malaria vaccine candidates [1].

Problems in obtaining satisfactory yields of the target polypeptide did occur during the chain assembly on the solid support mainly because of the difficult coupling between Pro341 and Cys(Trt)342. Modified coupling protocols such as longer coupling/deblocking times or double/triple couplings were found to be ineffective. Furthermore, oxidative folding of the polythiol precursor gave invariably high amounts of dimeric soluble species (unpublished).

In an attempt to circumvent these problems, we replaced Fmoc-Cys(Trt)-OH with Fmoc-Cys(S*t*Bu)-OH and prepared the intermediate Cys(S*t*Bu)^{342,346}-*Pf*CSP-(282–383) with only two protected cysteines per chain to minimize solubility problems, if any. Coupling between Pro341 and Cys(S*t*Bu)342 did actually proceed in almost quantitative yield as the subsequent steps in the assembly of the target sequence. Folding of the doubly *S*-protected derivative occurred spontaneously at room temperature in PBS at pH 7.2 in the presence of air-oxygen. Indeed, after about 24 h a substantial conversion to the target product was reached [2]. In contrast to other malaria-related polypeptides [1], the folded *Pf*CSP- (282–383) segment was the first example in which a malaria vaccine candidate could simultaneously activate specific $CD4^+$, $CD8^+$ T and B cells [3,4].

These results as well as the regeneration of native proteins from their mixed disulfide derivatives with low molecular weight thiols (protein-S-S-R) reported in the literature [5–11] suggested to us to attempt a different but still related approach for further optimization of

Abbreviations: Asp-*N*, endoproteinase Asp-*N*; Boc, *tert*-butoxycarbonyl; des-Arg74-C5a, complement 5a protein desarginated by serum carboxypeptidase N; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; ED_{50}, the efficacy dose at 50% maximal activity; Fmoc, 9-fluorenylmethoxycarbonyl; GdnHCl, guanidinium hydrochloride; Glu-*C*, endoproteinase Glu-*C*; GSH-GSSG, glutathione; HOBt, *N*-hydroxybenzotriazole; hu-CCL1 24-96/I-309, T-lymphocyte secreted protein I-309; hu-TARC, human thymus and activation-regulated chemokine; MALDI-TOF, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; NEM, *N*-ethylmaleimide; NMP, *N*-methylpyrrolidinone; *Pb*CSP, *Plasmodium berghei* circumsporozoite protein; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PBS, phosphate buffer saline; *Pf*CSP, *Plasmodium falciparum* circumsporozoite protein; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid phase peptide synthesis; *t*Bu, *tert*-butyl; S*t*Bu, *tert*-butylthio; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl.

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the synthesis. We thought that a suitable amount of thiol added to dilute and slightly basic solutions of fully *S-tert*-butylthiolated polypeptides could generate the native disulfide framework by irreversibly displacing the highly volatile *tert*-butylmercaptan.

Rather than using large segments of complex malaria proteins of unknown structure and undetermined disulfide connectivities, we envisaged to experimentally test the validity of the approach by comparing the oxidative folding of the tetrathiol precursor of hu-TARC, a small protein with a known and stable structure, with that of *S-tert*-butylthio derivatives of hu-TARC promoted by low molecular weight thiols. In hu-TARC two disulfide bonds connecting Cys10 to Cys34 and Cys11 to Cys50 (1-3/2-4 connectivities) stabilize the structure of the 71 amino acid residue sequence [12]. It is known that polythiol chemokine precursors fold efficiently *in vitro* by the addition of glutathione (GSH-GSSG) or cysteine/cystine redox buffers to dilute slightly basic solutions under air-oxygen to yield the fully functional protein molecules [13,14]. From oxidative folding experiments of fully *S-tert*-butylthiolated hu-TARC we expected the information needed for an application of this novel procedure to the folding of fully *S-tert*-butylthio protected large fragments of malaria proteins of unknown structure. Moreover, the knowledge acquired by folding of partially *S*-protected hu-TARC derivatives would also allow an accurate reexamination of the spontaneous oxidative folding of Cys (S*t*Bu)^{342,346}-*Pf*CSP- (282–383).

In the present study, we report the results of oxidative folding of the hu-TARC polythiol precursor, Cys(S*t*Bu)^{10,11,34,50}-, Cys(S*t*Bu)^{10,11}-, Cys(S*t*Bu)^{10,34,50}-, and Cys(S*t*Bu)^{11,34,50}-hu-TARC derivatives. Additionally, the results of oxidative folding of Cys(S*t*Bu)^{10,11,26}-hu-CCL1 24-96/I-309, a member of the CC chemokine family that plays a functional role in leukocyte trafficking and inflammatory processes, are reported together with those of two fully *S-tert*-butylthiolated large fragments of *P. falciparum*, and a doubly S-protected *P. berghei* circumsporozoite malaria protein. The chemokines and the malaria protein derivatives were prepared by basically following Sheppard's Fmoc/tBu protocol [15]. Enzymatic digestion of *Pf*CSP-(319–383) and *Pf*CSP- (337–383) with Glu-*C* and Asp-*N* endoproteinases and related mass spectrometric analysis were carried out to possibly determine their disulfide connectivities.

MATERIALS AND METHODS

Solid Phase Chain Assembly

The peptides were assembled stepwise on an Fmoc-Ser(*t*Bu)-p-alkoxybenzylalcohol resin (0.6 mmol g^{-1}) using both 431A and 433A Peptide Synthesizers (Applied Biosystems, Framingham, MA) fitted with a UV monitoring system for Fmoc deprotection. Side-chain protection of Fmoc-amino acids was: Asp(O*t*Bu), Arg(Pmc), Arg(Pbf), Asn(Trt), Cys(Trt), Cys(S*t*Bu), Glu(O*t*Bu), Gln(Trt), His(Boc), Lys(Boc), Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu), and Trp(Boc). The peptide chain assemblies were performed using a fivefold molar excess of Fmoc-amino acid and DIPCDI/HOBt activating agents for each 60-min coupling step. Unreacted *N*-terminal amino groups were regularly capped with 10% acetic anhydride/5% DIPEA in NMP. The polypeptidyl resins were

Table 1 *S-tert*-butylthio derivatives of hu-TARC, hu-CCL1 24-96/I-309 and *P. falciparum* and *P. berghei* circumsporozoite protein fragments

tert-Butylthio derivatives	Sequence
hu-TARC (polythiol form)	ARGTNVGRE**CC**LEYFKGAIPLRKLKTWYQTSED**C**SRDAIVFVTVQGRAI**C**SDPNNKRVKNAVKYLQSLERS
Cys(S*t*Bu)^{10,11,34,50}-hu-TARC	ARGTNVGRE**C(StBu)C(StBu)**LEYFKGAIPLRKLKTWYQTSED**C(StBu)**SRDAIVFVTVQGRAI**C(StBuS)-**SDPNNKRVKNAVKYLQSLERS
Cys(S*t*Bu)^{10,11} -hu-TARC	ARGTNVGRE**C(StBu)C(StBu)**LEYFKGAIPLRKLKTWYQTSED**C**SRDAIVFVTVQGRAI**C-**SDPNNKRVKNAVKYLQSLERS
Cys(S*t*Bu)^{10,34,50}-hu-TARC	ARGTNVGRE**C(StBu)C**LEYFKGAIPLRKLKTWYQTSED**C(StBu)**SRDAIVFVTVQGRAI**C(StBu)-**SDPNNKRVKNAVKYLQSLERS
Cys(S*t*Bu)^{11,34,50}-hu-TARC	ARGTNVGRE**CC(StBu)**LEYFKGAIPLRKLKTWYQTSED**C(StBu)**SRDAIVFVTVQGRAI**C(StBu)**SD-PNNKRVKNAVKYLQSLERS
Cys(S*t*Bu)^{10,11,26}-hu-CCL1 24-96/I-309	KSMKVPFSR**C(StBu)C(StBu)**FSFAEQEIPLRAIL**C(StBu)**YRNTSSI**C**SNEGLIFKLKRGKEA-**C**ALDTVGWVQRHRKMLRH**C**PSKRK
*Pf*CSP- (337–383) (fully *S*-protected)	TEWSP**C(StBu)**SVT**C(StBu)**GNGIQVRIKPGSANKPKDELDYANDIEKKI**C(StBu)**KMEK**C(StBu)**S
*Pf*CSP- (319–383) (fully *S*-protected)	PSDKHIKEYLNKIQNSLSTEWSP**C(StBu)**SVT**C(StBu)**GNGIQV RIKPGSANKPKDELDYANDIEKK-I**C(StBu)**KMEK**C(StBu)**S
Cys(S*t*Bu)^{342,346}-*Pf*CSP-(282–383)	KNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSP**C(StBu)**S-VT**C(StBu)**GNGIQVRIKPGSANKPKDELDYANDIEKKI**C**KMEK**C**S
Cys(S*t*Bu)^{271,275}-*Pb*CSP-(242–310)	NDDSYIPSAEKILEFVKQIRDSITEEWSQ**C(StBu)**NVT**C(StBu)**GSGIRVRKRKGSNKKAEDLTLED-IDTEI**C**KMDK**C**S

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cleaved (1 h) with a mixture of 78% TFA, 12% TIS, 5% phenol, and 5% water to recover crude polypeptides. The MALDI-TOF mass spectrometric analysis of the crude materials always detected major signals of the target products together with peaks and shoulders of polypeptide impurities.

The assembled polypeptides and their sequences are reported in Table 1.

Peptide Purification and Analysis

Crude products, recovered by lyophilization, were dissolved in 50% acetic acid and run on Sephadex G-50 column (70 cm × 2.5 cm) using 50% acetic acid as mobile phase. Selected fractions containing the desired product were pooled and lyophilized. After lyophilization, the different pools were dissolved in 50% acetic acid, and loaded on a 250 mm × 10 mm semipreparative Vydac C4 column (The Separations Group, Hesperia, CA). Samples were eluted with a linear gradient up to 80% buffer B (0.1% TFA in acetonitrile) over 1 h at a flow rate of 3 ml min^{-1}. Buffer A was 0.1% TFA in water. The purity of each fraction was checked by analytical RP-HPLC on Vydac C18 and MALDI-TOF spectrometry.

MALDI-TOF mass analysis was performed using a TOF Voyager-DE spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser ($\lambda = 337$ nm) to desorb and ionize the sample. The accelerating voltage used was 25 kV. One microliter of a sample solution in water or 50% acetic acid ($c = 1$ mg ml^{-1}) was placed into a well of the plate, dried under vacuum, treated with 1 µl of a saturated solution of the matrix *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 1 : 2 acetonitrile/1% TFA in water and left to crystallize at room temperature.

Only homogenous fractions containing the target peptide were pooled, lyophilized, and used for folding and disulfide formation experiments.

Oxidative Folding

The oxidative folding of tetrathiol hu-TARC was carried out under air-oxygen, preferably, in the presence of the glutathione redox buffer. The purified tetrathiol was first dissolved in 6 M GdnHCl, 0.1 M Na_2HPO_4, 10 mM Tris at a concentration of 10 mg ml^{-1} (1.24 mM) at pH 8. After about 20 min, the solution was diluted 10 times to obtain folding buffer (i) consisting of 0.6 M GdnHCl, 10 mM Na_2HPO_4, 1 mM Tris for the spontaneous oxidation by air-oxygen, or folding buffer (ii) upon addition of 1 mM oxidized glutathione and 0.1 mM reduced glutathione; final pH 7.2. The progress of the process was monitored by analytical C4 RP-HPLC. The target product was checked for the presence, if any, of free-thiol groups by adding an excess of NEM to 1 mg ml^{-1} peptide solution and incubation for 1 h at 4 °C. A mass signal at +125 Da would detect a residual, free-SH group.

RP-HPLC-purified fully *S*-protected polypeptides were folded by dissolving 10 mg of purified product in 1 ml of a buffer comprising 6 M GdnHCl, 0.1 M Na_2HPO_4, and 10 mM Tris at pH 8.0. The resulting solution, kept at room temperature for about 20 min, was first 10-fold diluted with water to pH 7.2 and then treated with a 100-fold molar excess of cysteine over the concentration of the polypeptide precursor, final pH 7.2. The temperature was gradually increased to 37 °C and then maintained constant for about 24 h to allow disulfide formation to occur. The folding process was monitored both by analytical HPLC and MALDI-TOF mass spectrometry of 25 µl samples from the folding solution upon acid-quenching with TFA at 4 °C. Because of the loss of 178 Da per disulfide bond as compared to the loss of only 2 Da per disulfide bond of the polythiol precursor, the extent of disulfide bond formation of *S*-*tert*-butylthio derivatives was quickly and precisely evaluated. RP-HPLC analysis of the folding reaction was carried out by elution with a 20–60% acetonitrile gradient in 0.1% TFA/water in 40 min at a flow rate of 1.0 ml min^{-1}. The absorbance at 214 nm was used to calculate the amount of folded product as the percentage of the total protein present. After 24 h, an equilibrium condition was reached for all foldings, rendering useless further monitoring of the reaction. The presence, if any, of free-thiol groups in the final product was verified by reacting with NEM, as reported above.

RP-HPLC-purified partially *S*-protected polypeptides were folded in high yield by dissolving the derivatives in 6 M GdnHCl, 0.1 M Na_2HPO_4, and 10 mM Tris at a concentration of 10 mg ml^{-1}, pH 8.0. After about 5 min, the initial solutions were diluted 10 times and left to spontaneously fold at 37 °C for 24 h. After RP-HPLC and mass spectrometric analysis, a 100-fold molar excess of cysteine was added under stirring. The solutions were finally kept at 37 °C under air-oxygen, at a final pH of 7.2, to increase the extent of folding via further thiol–disulfide exchanges.

The observed conversions of all *S*-tert-butylthio precursors at 37 °C to final folded products were comparable or better than the 77% conversion reached in the folding of polythiol hu-TARC at the same temperature at 24 h. Standard error of folding, when reported, was determined on three independent folding experiments.

Chemokine Biological Assay

The biological activity of synthetic human chemokines was measured by their ability to chemoattract mouse thymoma cells BW 5147 [16]. Cell migration was determined using a lactate dehydrogenase-based calorimetric assay (OD 450 nm; 16).

Enzymatic Digestion Essay

Glu-*C* (Pierce, Rockford, IL) and Asp-*N* (Boehringer, Mannheim, Germany) were used for cleavage of *P. falciparum* CSP polypeptides according to Wilkinson and Maier and colleagues, respectively [17,18].

RESULTS AND DISCUSSION

Oxidative Folding of the hu-TARC Tetrathiol Precursor

Oxidative folding of the fully *S*-deprotected hu-TARC (tetrathiol form) gave within 24 h the target product with a conversion yield of about 77% at 37 °C. Much lower yields were observed to occur at room temperature (Figure 1(A) and (B)). Precipitation or aggregation during the folding reaction did not occur, indicating that the polypeptide was soluble in both the unfolded and folded form. As expected, the folded

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material eluted about 8 min earlier than the starting material. The measured mass was identical to the calculated mass (Figure 1(C)).

The results of hu-TARC polythiol oxidative folding suggest that the proximity, reactivity, and accessibility of the thiol groups as well as the stable three-dimensional structure of the chemokine chain were sufficiently specific to strongly favor the native disulfide bonds over the non-native ones [19].

Folding of Cys(S*t*Bu)^{10,11,34,50}-hu-TARC

We have found that the regeneration of native hu-TARC from the fully *S*-protected hu-TARC is best promoted and favored by a 100-fold molar excess of cysteine, while with lower and higher Cys concentration the yields are reduced. At pH 7.2, the cysteine thiolate form is able to slowly and gradually convert the derivative to the target product within 24 h, at 37 °C, and in the presence of air-oxygen in 80 ± 3% yield (Figure 2(A) and (B)). Glutathione appeared to exhibit the identical concentration dependence when used to fold the TARC derivative.

The slow oxidative folding rates are somewhat surprising if compared to the extremely fast (about 5 min) folding of cysteinylated lysozyme at pH 7.5 and 37 °C and the trace amounts of cysteine required to catalyze the reaction [11]. Being identical the initiating mercaptan and pH and also the quite close temperature of the folding reaction, the significantly different kinetics can be explained by a concentration effect (optimal rate for reactivation of cysteinylated lysozyme occurs at 0.111 mg ml^{-1}, 10-fold less than the concentration used for our experiments). However, the slow kinetics most probably derive from the very slow rate of reduction of the Cys(S*t*Bu) residues by cysteine. From comparative analysis of reduction of *S*-*tert*-butylthio-, *S*-methylthio-, *S*-isopropylthio-, and *S*-benzylthio-cysteine by GSH at pH 7.0 reaction rates of 0.008, 0.06, 0.019, and 0.05 M s^{-1}, respectively, were derived [20]. The more difficult thiol/disulfide exchange reaction and reduction of the Cys(S*t*Bu) disulfide has recently been subject of theoretical considerations

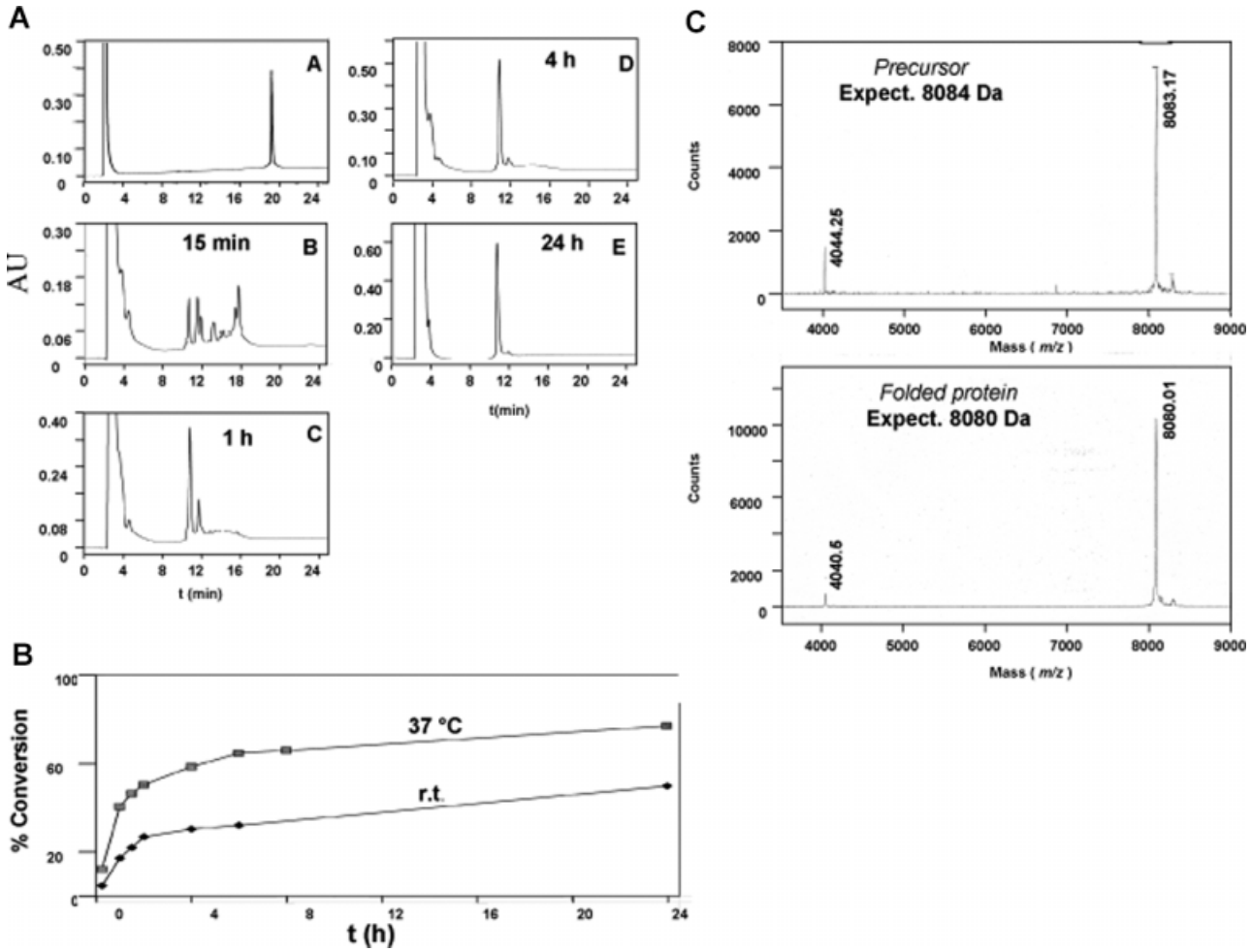

Figure 1 (A) Oxidative folding of hu-TARC tetrathiol precursor at 37 °C, pH 7.2, with the glutathione redox buffer in the presence of air-oxygen; (B) The effect of temperature on the rate of folding; (C) MALDI-TOF mass analysis of hu-TARC tetrathiol precursor and of folded hu-TARC.

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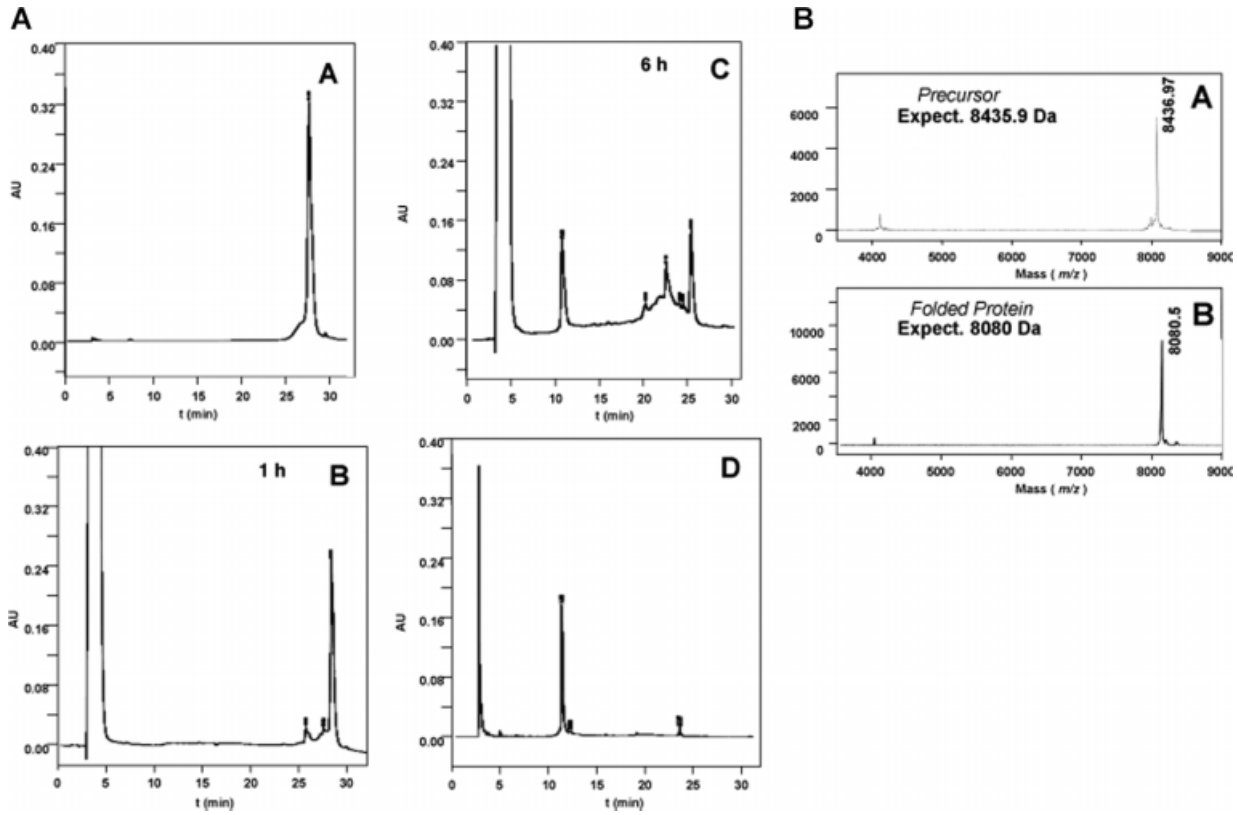

Figure 2 (A) Folding of Cys(S*t*Bu)10,11,34,50-hu-TARC induced by a 100-fold molar excess of cysteine at 37 °C, pH 7.2, in the presence of air-oxygen; (B) MALDI-TOF mass analysis of Cys(S*t*Bu)10,11,34,50-hu-TARC and folded hu-TARC.

[21]. Moreover, the cysteine thiolate attack occurs at the cysteine sulfur of the unsymmetrical disulfide displacing *tert*-butylmercaptan, which itself cannot be oxidized by the air-oxygen to the disulfide [22] and thus can react again with the cysteinylated peptide yielding the starting derivative, unless subtracted from the equilibrium because of its high volatility. The S-cysteinylated peptide has to then react with a second cysteine thiol to produce the thiol-peptide and thus to initiate the intramolecular thiol/disulfide exchange reactions via attack at a second Cys(S*t*Bu) residue. These reactions can be favored by conformation-induced local proximities, but may well be hampered by intermediate burying of the Cys(S*t*Bu) groups in hydrophobically collapsed structures and thus of difficult access to the hydrophilic charged cysteine residues.

In fact, after 8 h of reaction, the mass (m/z) signals at 8178, 8209, 8267, and 8269 in the MALDI-TOF spectrum clearly reveal the presence of many transient intermediates with one or two Cys(S*t*Bu) residues ($m/z = 8178$ and 8267, respectively), one Cys/Na^+ residue ($m/z = 8209$), and one Cys(S*t*Bu) + one Cys/Na^+ ($m/z = 8296$) (Figure 3(B)). The RP-HPLC chromatogram shows a main narrow peak of the desired product (MW = 8080.9 Da) in addition to a series of shoulders and unresolved peaks emerging from a broad rounded lump of folding intermediates (Figure 3(A)).

Most surprising is the low efficiency of oxidative folding of Cys(S*t*Bu)^{10,11,34,50}-hu-TARC in the presence of a 1000-fold molar excess of cysteine at 37 °C (Figure 4). The intramolecular disulfide formation occurs very slowly (about 3% in 6 h) with only 20% conversion to the desired product within 24 h (Figure 4(A)). During folding, the major peak appearing in the RP-HPLC chromatogram remains that of the starting derivative. The narrow, sharp peak of the target product is accompanied by several additional small peaks and shoulders (Figure 4(B)). For some of them, the MALDI-TOF spectrum registered after about 6 h of folding reveals m/z values of 8175, 8264, and 8353 corresponding to intermediates with one, two, and three residual *tert*-butylthio groups per chain, respectively (Figure 4(C)).

A possible explanation for the observed very slow and inefficient folding in the presence of a 1000-fold excess could be the fast reduction of formed disulfides that stabilize thermodynamically favored folded intermediates and by this way increase the efficient concentration (C_{eff}) of peptide thiolates for their attack on Cys(S*t*Bu) residues. This conformational effect that could well represent the bypass to the *per se* very slow thiol/disulfide exchange reaction at Cys(S*t*Bu) disulfides is lost in the presence of too high excesses of cysteine.

This explanation would also agree with the observation that the native hu-TARC species increases slowly

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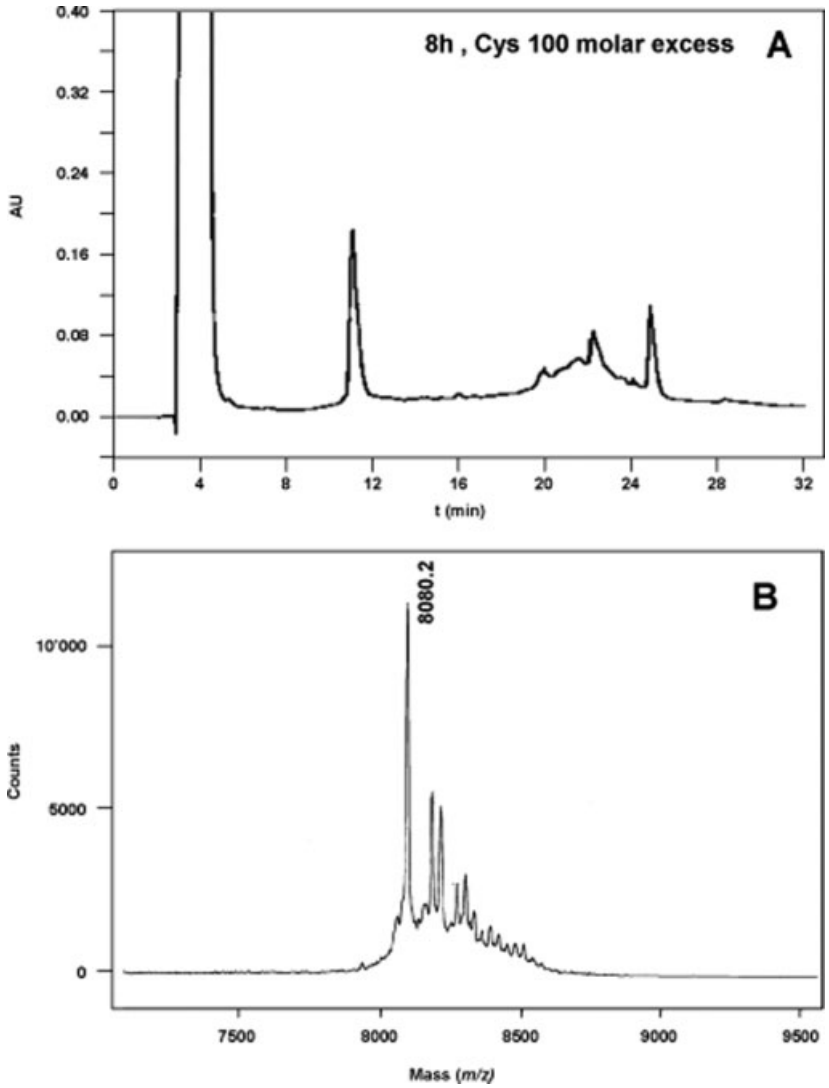

Figure 3 (A) Intermediates during Cys(S*t*Bu)10,11,34,50-hu-TARC folding; (B) MALDI-TOF mass analysis of folding intermediates.

and that native disulfide bonds, once formed, do not rearrange or reshuffle even in the presence of a large excess of cysteine thiolate. The formation of a stable tertiary structure in the folding intermediates as well as in the final folded form locks in the native disulfide bonds, preventing back-reactions and thereby inducing such species to accumulate.

No oligomers and cross-linked species have been found in the crude product, nor free thiols, upon reaction of the final folded product with NEM.

Oxidative Folding of the Partially *S*-protected Cys(S*t*Bu)^{10,11}-hu-TARC Derivative

The presence of two unprotected cysteine residues in the Cys(S*t*Bu)^{10,11} precursor of hu-TARC was sufficient to promote spontaneous, although incomplete, intramolecular disulfide bond formation at room temperature as well as at 37 °C, and pH 7.2 under air-oxygen (Figure 5). The peptide thiolate/heterodisulfide exchange reactions appear to be strongly affected by temperature during the initial stages (about 3 and 30% conversion after 1 h of reaction at room temperature and 37 °C, respectively). These results suggest that initially the hu-TARC derivative may require temperature-induced local unfolding near its reactive chain thiolate and heterodisulfide groups to bring them into juxtaposition and make them reciprocally accessible for thiol–disulfide exchanges and native disulfide bond formation.

After about 8 h, the folding becomes much less sensitive to temperature. A conversion of 45% is slowly and gradually attained during additional 16 h of reaction time. Taken together, the data suggest that the native forms accumulated during the initial steps coexist in solution with long-lived metastable, slowly folding species. A conversion to about 75% was slowly reached in 24 h by adding a 100-fold molar excess of cysteine to the reaction mixture initially

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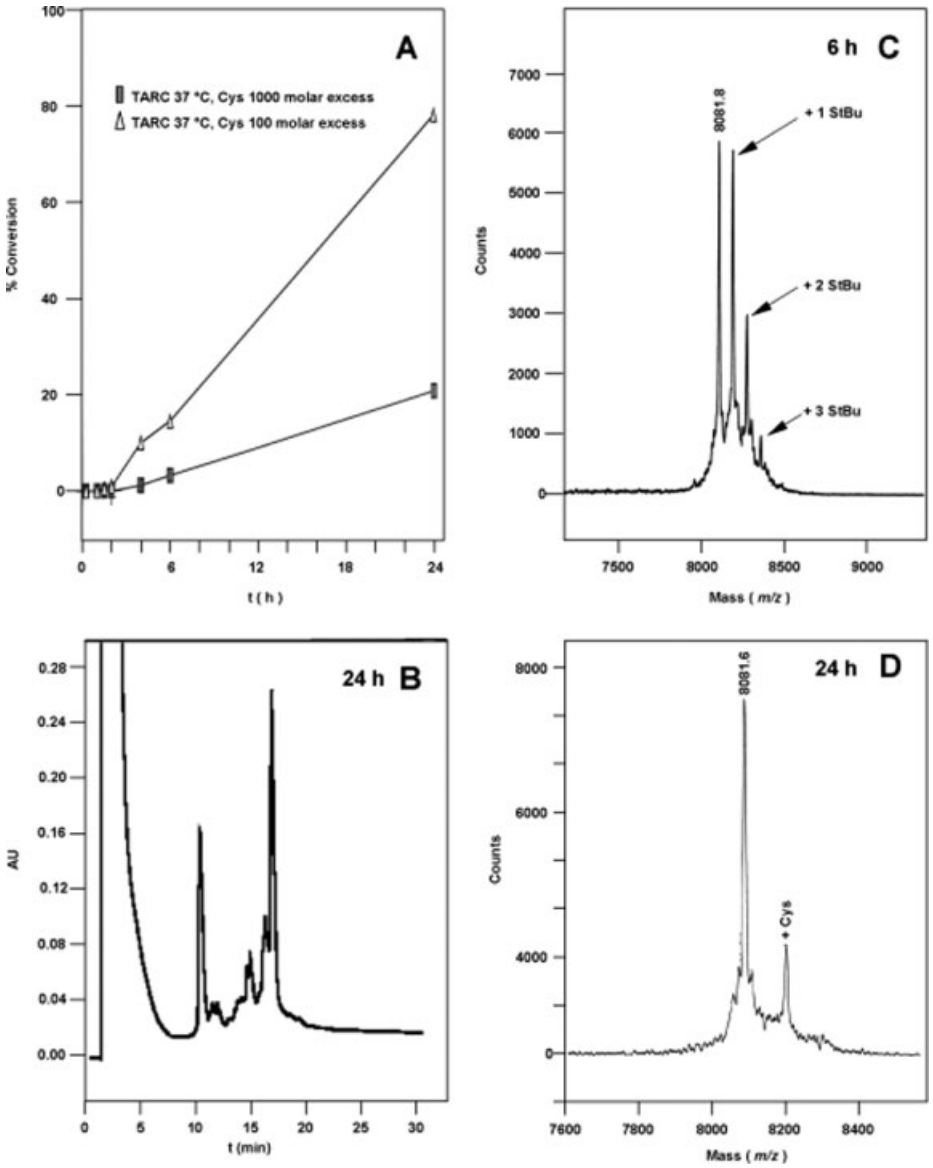

Figure 4 (A) The effect of cysteine concentration on the rate of folding of Cys(S*t*Bu)10,11,34,50-hu-TARC at 37 °C, pH 7.2, in the presence of air-oxygen; (B) Intermediates after 24 h of folding at 37 °C promoted by a 1000-fold molar excess of cysteine; (C) MALDI-TOF mass analysis of intermediates after 6 h and (D) after 24 h of folding.

kept to fold spontaneously at 37 °C for 4 h. Again, the intramolecular disulfide bonds of native species, more rapidly formed during the spontaneous folding, are not reshuffled back by the excess of added cysteine thiolate.

The procedure reported for the partially *S*-protected peptide is of general practical importance for folding of precursors that, when fully *S*-protected as *tert*-butylthio derivatives, are insoluble in aqueous buffers at 37 °C. In fact, a derivative of *des*Arg74-C5a with six Cys(S*t*Bu) out of seven cysteines was found to gradually gelify upon 1 : 10 dilution of the initially clear solution in 6 M GdnHCl, thus preventing oxidative folding. Folding was easily achieved in 24 h upon addition of a 100-fold molar excess of cysteine at pH 7.2 to a solution of the precursor containing three Cys(S*t*Bu) residues and the remaining four Cys residues unprotected.

Oxidative Folding of Cys(S*t*Bu)^{10,34,50}- and Cys(S*t*Bu)^{11,34,50}-hu-TARC Derivatives

In contrast to the hu-TARC with two cysteine residues protected as *tert*-butylthio derivatives, the peptide with three Cys(S*t*Bu) residues and a single unprotected Cys residue practically does not fold to the desired product. After one week at 37 °C, both the RP-HPLC profiles and MALDI-TOF mass spectra showed small signals corresponding to the target molecule and to derivatives with one and three Cys(S*t*Bu) derivatives. The major signals seen in the mass spectra correspond to the mass of derivatives with two Cys(S*t*Bu) and a single intramolecular disulfide bridge. About 80% conversion to the target molecules was again obtained

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for both Cys(S*t*Bu)^{10,34,50}- and Cys(S*t*Bu)^{11,34,50}-hu-TARC derivatives by adding a 100-fold or even a 50-fold molar excess of cysteine at 37 °C.

Oxidative Folding of the Partially *S*-protected Cys(S*t*Bu)^{10,11,26}-hu-CCL1 24-96/I-309 Derivative

The method described above for the partially *S*-protected hu-TARC derivatives has been successfully applied (about 80% conversion at 24 h) to the folding of tris-*tert*-butylthio derivative of segment 24–96 of human CCL1 chemokine, with 73 amino acid residues and three disulfide bridges (Figure 6).

The biological activity of the folded, purified sample is similar to that of the natural product (10–60 ng ml^{-1}; 16).

BIOACTIVITY OF THE SYNTHETIC hu-TARC

The biological activities of the synthetic hu-TARC obtained both from the fully and partially *S*-protected forms were compared to those of natural hu-TARC and of the product obtained from the polythiol precursor by measuring their ability to chemoattract mouse thymoma cells BW5147 [16]. The ED_{50} (the dose inducing half-maximal migration response) found for hu-TARC synthetic samples was similar to that of the natural molecule (0.1–0.6 ng ml^{-1}), further supporting the notion that the thermodynamically most stable molecules obtained by oxidative foldings of the different precursors at 37 °C were most probably coincident with the correctly folded one, i.e. the native conformation.

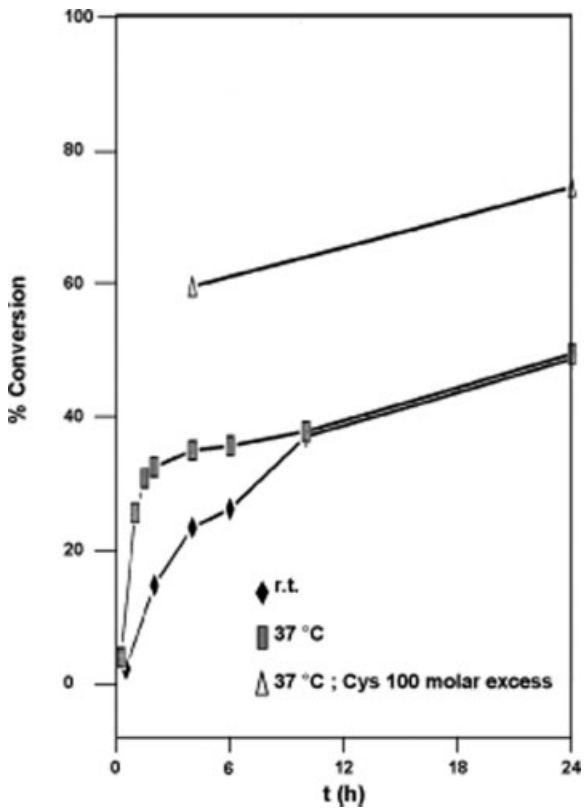

Figure 5 Spontaneous folding of Cys(S*t*Bu)10,11-hu-TARC at room temperature and at 37 °C and further folding upon addition at 37 °C of a 100-fold molar excess of cysteine.

Oxidative Folding of the *P. falciparum* and *P. berghei* Circumsporozoite Protein Fragments *Pf*CSP- (282–383) and *Pb*CSP- (242–310)

The positive outcome of the experiments with the partially *S*-protected Cys(S*t*Bu)^{10,11}-hu-TARC induced us to reexamine the oxidative folding of the malaria 102-mer *Pf*CSP- (282–383) for possibly increasing the yields. A 100-fold molar excess of cysteine was added at room temperature to the solution of the Cys(S*t*Bu)^{342,346}-precursor upon exposure to air-oxygen for spontaneous formation of the intramolecular disulfide bridges. After cysteine addition, the solution was kept at room temperature for about two days and the reaction was monitored by MALDI-TOF mass spectrometry. The fraction of incompletely and/or incorrectly folded products disappeared gradually with time, leading to a substantial increase of the target monomeric product (80 ± 4% conversion after 48 h).

To further prove the efficacy of the method, we applied the novel oxidative folding procedure to the 71 amino acid fragment of *P. berghei* circumsporozoite protein *Pb*CSP- (242–310) containing two Cys(S*t*Bu)^{271,275} and two unprotected Cys^{304,309} residues. After 5 h of spontaneous folding, the target product is seen to elute 1–2 min earlier than a major intermediate, rapidly formed during the first hour (Figure 7(A)). The intermediate, still present after 36 h, is very slowly converted to the target product. Later-eluting peaks contain the starting material and a number of minor folding species. MALDI-TOF mass spectrometry (Figure 7(B)) and enzymatic cleavage with Glu-*C* demonstrated that the slowly folding major intermediate has a disulfide bridge connecting Cys^{304} and Cys^{309} and the two intact Cys(S*t*Bu) residues.

Likely, the metastable intermediate is a molecule with a native-like spatial structure where the two Cys(S*t*Bu) residues are mostly buried. Its presence even after 36 h of folding confirms once again that thiol–disulfide exchanges, either spontaneous or promoted by a 100-fold molar excess of cysteine, are generally slow if sterically impeded. Furthermore, the persistence in the folding mixture of the monomeric doubly *S*-*tert*-butylthiolated species indicates that covalent interpeptide cross-links do not form with time.

Disulfide Connectivities of *Pf*CSP- (282–383)

Although the *P. falciparum* circumsporozoite protein and related fragments have been studied to identify

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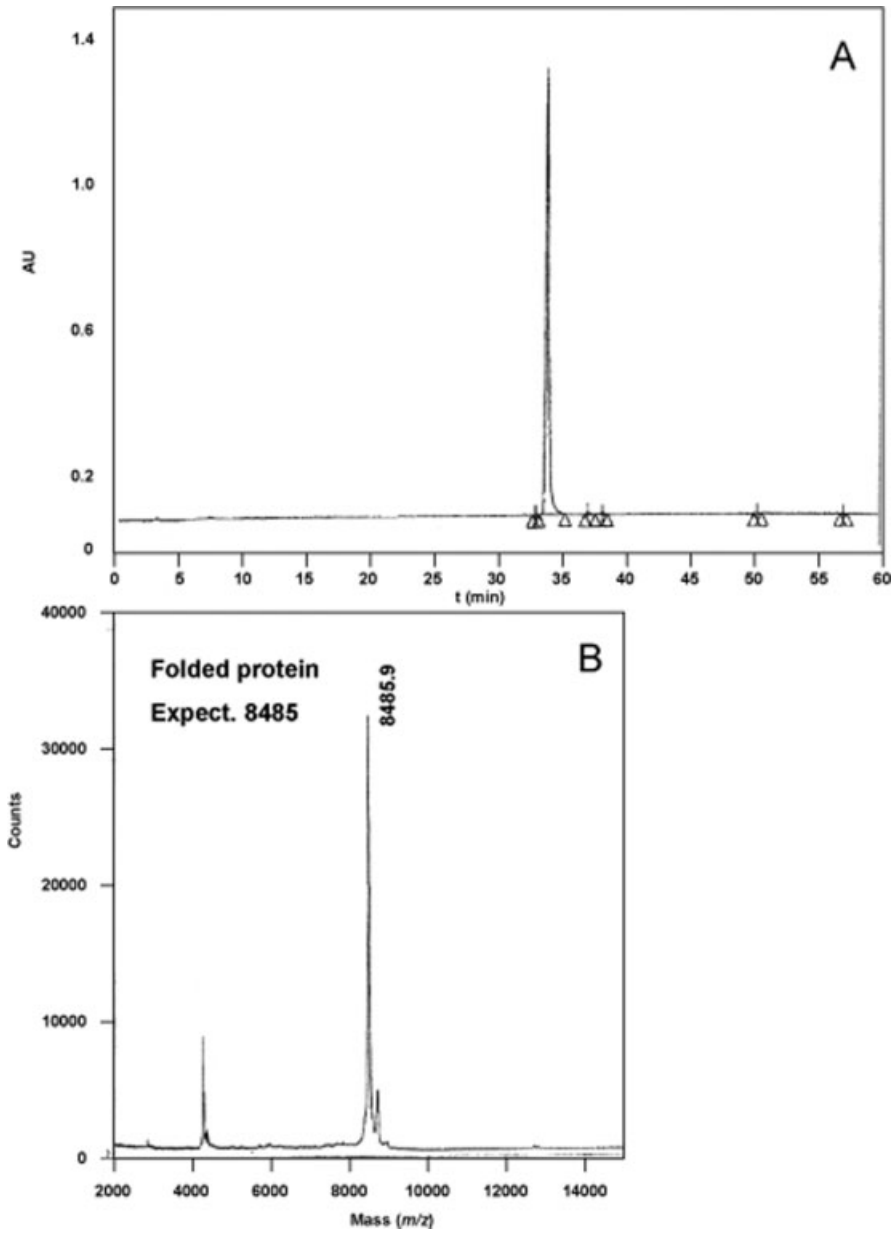

Figure 6 (A) RP-HPLC and (B) MALDI-TOF mass analysis of folded hu-CCL1 24-96/I-309.

vaccine candidates, the disulfide framework is still unknown. Such information is even more important, as two cytotoxic T-cell epitopes in the protein are nested in between the two cysteine-containing domains [23]. To obtain new information on the disulfide connectivities, the oxidatively folded malaria polypeptide fragments *Pf*CSP- (337–383) and *Pf*CSP- (319–383) were subjected to enzymatic digestion with the specific Glu-*C* and Asp-*N* endoproteinases.

For this purpose, the *S*-*tert*-butylthio derivatives of *Pf*CSP- (336–383) and *Pf*CSP- (319–383) were oxidatively folded analogously to *Pf*CSP- (282–383) by addition of a 100-fold excess of cysteine. Figure 8(A) and (B) shows the folding of *Pf*CSP- (336–383) and the MALDI-TOF mass analysis of the folded product, respectively. Three main isomeric products A, B, and C with MW = 5216 Da, and a number of peptide impurities, preeminent among them product D with two remaining *tert*-butylthio groups, were detected during folding.

Asp-*N* enzymatic hydrolysis at Lys^{363}-Asp^{364} and Leu^{366}-Asp^{367} of isomer A produced three peptide fragments corresponding to MALDI-TOF mass signals at $m/z = 3218$, 2860, and 2020 (Figure 8(C)). Each fragment contains a couple of Cys residues connected by a disulfide bond. The two longer fragments show both 342–346 disulfide connectivity, while the shorter fragment contains a 377–382 disulfide bond. Glu-*C* enzymatic hydrolysis at Glu^{365}-Leu^{366} gave two fragments with the Cys residues 342–346 and 377–382 connected by a disulfide bond, respectively (not shown).

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The combined results of Glu-*C* and Asp-*N* enzymatic hydrolysis of isomer A demonstrate that it contains two disulfide bridges with 1-2/3-4 Cys connectivity.

Digestion of major isomer B by Asp-*N* gave initially three peptide fragments corresponding to the signals appearing at $m/z = 4879$, 4773, and 4416 in the MALDI-TOF mass spectrum (Figure 8(D)). The signals at m/z 5216 (initial peptide) and $m/z = 4879$ disappeared after an additional 20 min digestion, while the signal at $m/z = 4416$ remained unchanged after 1 h. Glu-*C* hydrolysis of isomer B at Glu^{365}-Leu^{366} and Glu^{373}-Lys^{374} gave a polypeptide of 4300 Da with a single disulfide bridge connecting the *C*-terminal peptide KKICKMEKCS to the 3103 Da fragment TEWSPCSVTCGNGIQVRIKPGSANKPKDE. Therefore, the results of isomer B digestion by both enzymes exclude the 1-2/3-4 connectivity found for isomer A and rather indicate 1-3/2-4 or 1-4/2-3 connectivities.

The 1-2/3-4 cysteine pairings have not been found by Asp-*N* and Glu-*C* digestion of the longer 65 amino acid residue *Pf*CSP- (319–383) fragment. Enzymatic hydrolysis by Asp-*N* gave a fragment with MW = 6307 Da, while that by Glu-*C* produced a fragment with MW = 6377 Da both with cysteine 1-3/2-4 or 1-4/2-3 connectivities. The 1-2/3-4 cysteine pairings have not been found by Asp-*N* and Glu-*C* digestion of the longer 65 amino acid residue *Pf*CSP- (319–383) fragment. Enzymatic hydrolysis by Asp-*N* gave a fragment with MW = 6377 Da both with Cys 1-3/2-4 or 1-4/2-3 connectivities. Then the 1-2/3-4 connectivity seen only in the isomer A of the shorter 47 amino acid residue *Pf*CSP- (337–383) fragment can be reasonably excluded for the entire *P. falciparum* circumsporozoite protein, contrary to the hypothesis of Cerami and colleagues [24].

The unambiguous determination of the cystine framework of *Pf*CSP- (319–383) is unfortunately prevented, at present, by the lack of enzymes able to cleave at any of the bonds of the CSVTC sequence.

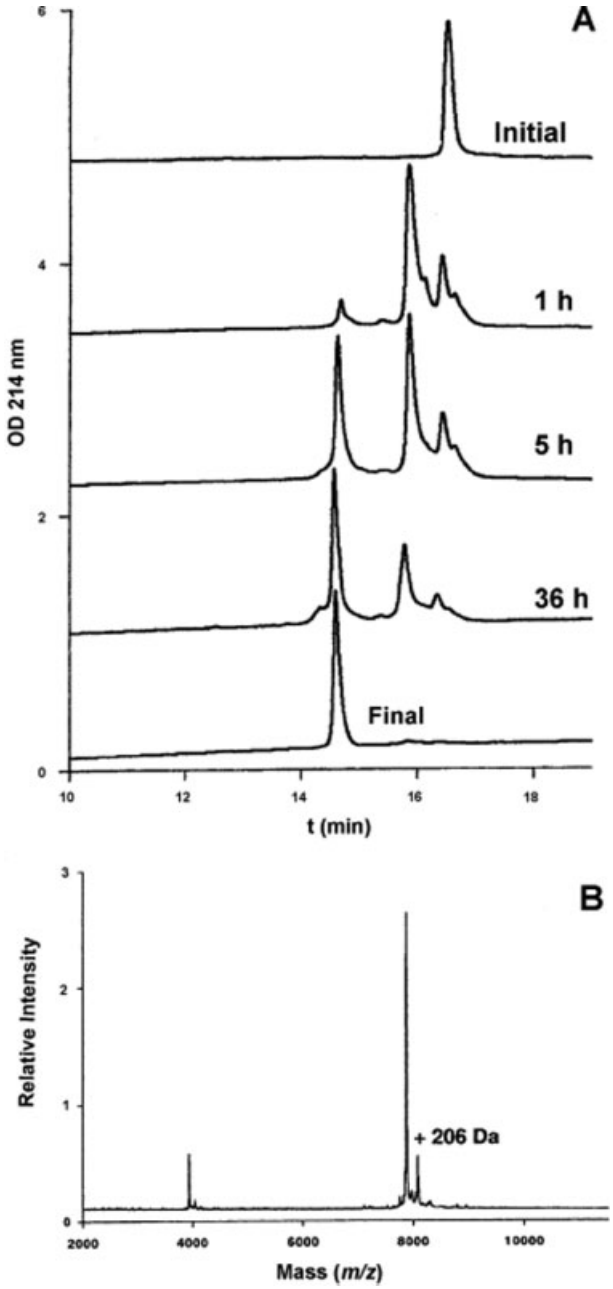

Figure 7 (A) RP-HPLC runs showing the folding of Cys(S*t*Bu) 271,275-PbCSP- (242–310) derivative; (B) MALDI-TOF mass analysis of the folded antigen. Mass: calculated 7870 Da, found 7871 Da. The small peak on the right of the main signal is a sinapinic acid adduct formed during acquisition.

CONCLUSIONS

To avoid the problems often encountered in the synthesis, isolation, and oxidative folding of polythiol peptides precursors, we have established a simple yet efficient folding procedure that does not require the use of the fully *S*-deprotected species. The precursors that we used successfully for oxidative folding are the *S*-*tert*-butylthio derivatives. Indeed, fully or partially *S*-protected peptide derivatives are efficiently folded by simple addition of a 100-fold molar excess of cysteine to a slightly alkaline buffer containing the derivatives and a chaotropic salt at 37°C and in the presence of air-oxygen as demonstrated for hu-TARC and hu-CCL1 24-96/I-309 chemokines and for *P. falciparum* and *P. berghei* circumsporozoite protein fragments. Correctly folded synthetic chemokines showing biological activities similar to those of the natural molecules are invariably obtained. The folding of the *S*-*tert*-butylthio precursors is straightforward and can provide at laboratory scale up to 100 mg of products, usually sufficient to carry out structure determination as well as *in vitro* and *in vivo* studies. We feel that the progress in folding of fully or partially *S*-protected polypeptides achieved in our laboratory coupled to the ease of their purification

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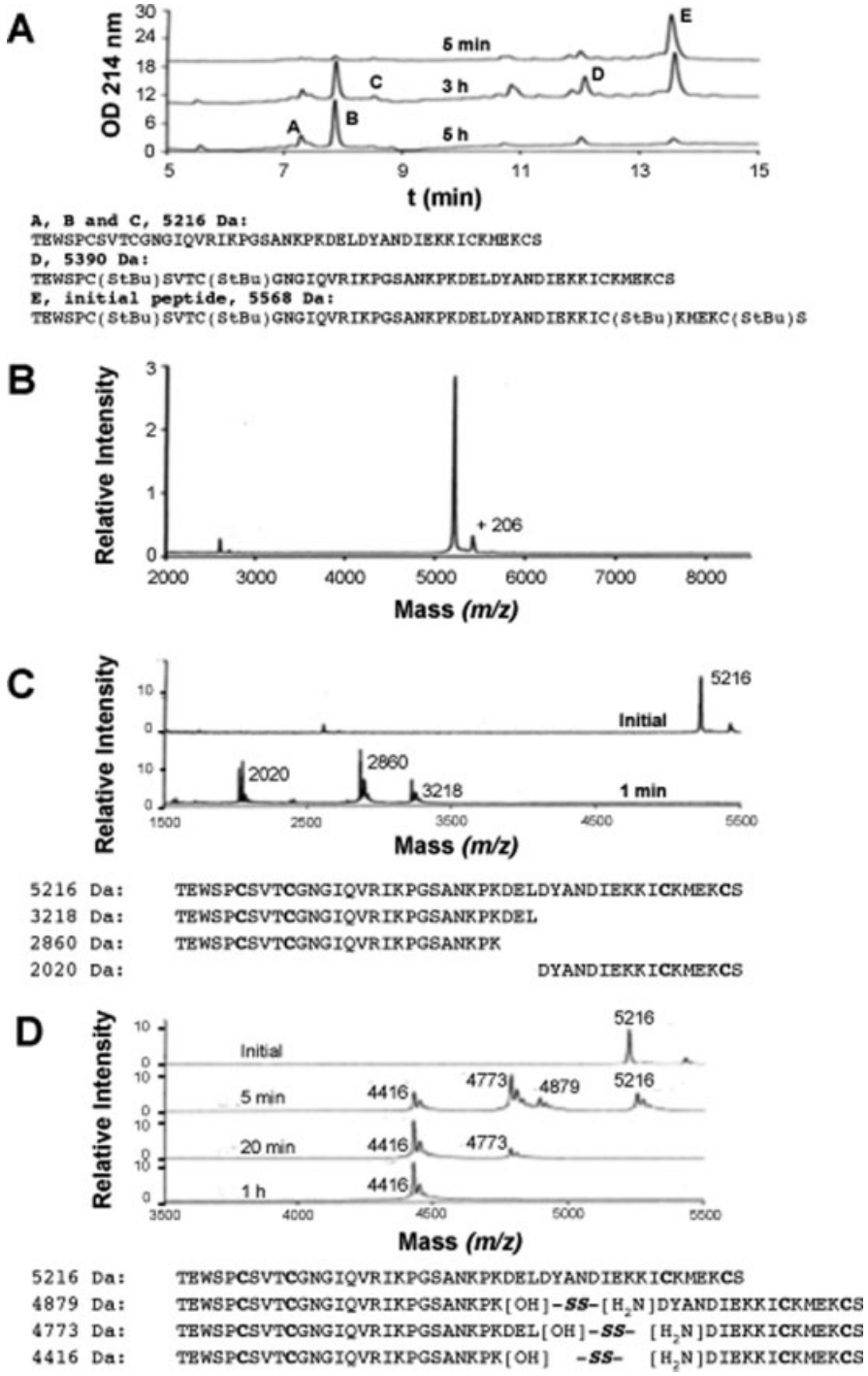

Figure 8 (A) RP-HPLC runs showing the folding of Cys(S*t*Bu)342, 346-*Pf*CSP-(337–383) derivative; (B) MALDI-TOF mass analysis of the folded antigen. Mass: calculated 5216 Da, found 5217 Da. The small peak on the right of the main signal is a sinapinic acid adduct formed during acquisition; (C) Asp-*N* endoproteinase hydrolysis at 37 °C of isomer A; (D) Asp-*N* endoproteinase hydrolysis at 37 °C of isomer B.

by routinely used RP-HPLC techniques will ultimately facilitate their synthesis on a multigram scale or more.

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