Amino Acids

Air oxidation method employed for the disulfide bond formation of natural and synthetic peptides

--Manuscript Draft--

Manuscript Number:	AMAC-D-15-00059R1
Full Title:	Air oxidation method employed for the disulfide bond formation of natural and synthetic peptides
Article Type:	Original Article
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Response to Reviewers:	Dear Editor, Thank you for giving us the possibility to revise our manuscript. We also kindly thank the Reviewer for his valuable suggestions. In particular we have rewrote the Abstract trying to address the Reviewer comments. We also added into the Introduction several new sentences, in order to better specify the synthetic advantages that are gained by using mild oxidation conditions. In the same section, we also clarified the reason of the choice of the studied peptides. Concerning with the oxidation methods employed to prepare the distinctin and the CXCR4 inhibitors, the authors of this work always employed the air oxidation protocol, which gives high yield of the final products. Actually, we were not involved into the article (Biopolymers, 2012, 98: 479-484) and we don't see the reason to perform the distinctin synthesis by employing such a long procedure (in this regard see the Discussion section). Moreover, we decided to add some specifications in Material and Methods section, in

	order to clarify the choice of the AMBER force field. Finally, we have performed all the minors corrections. With best regards Stefania De Luca

Air oxidation method employed for the disulfide bond formation of natural and synthetic peptides

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Keywords: disulfide bridges; peptide folding; oxidation methods; native-like oxidation conditions

Abstract

Among the available protocols, chemically-driven approaches to oxidize cysteine may not be required for molecules that, under the native-like conditions, naturally fold in conformations ensuring an effective pairing of the right disulfide bridge pattern. In this contest, we successfully prepared the distinctin, a natural heterodimeric peptide, and some synthetic cyclic peptides that are inhibitors of the CXCR4 receptor. In the first case the air oxidation reaction allowed to connect two peptide chains via disulfide bridge, while in the second case allowed the cyclization of rationally designed peptides by an intramolecular disulfide bridge. Computational approaches helped to either drive de-novo design, or suggest structural modifications and optimal oxidization protocols for disulfide-containing molecules. They are able to both predict and to rationalize the propensity of molecules to spontaneously fold in suitable conformations to achieve the right disulfide bridges

Introduction

Disulfide bridges represent natural conformational constraints which make peptides of great interest as potential therapeutics. They, in fact, increase the biological activity and the stability of the

molecules, inducing their natural folding and structural stabilization (Buchner et al. 2009; Wedemeyer et al. 2000; Annis et al. 1997; Thornton 1981; Creighton 1988; Pace et al. 1988; Matsumura et al. 1989).

Several methods can be employed for preparing intra and intermolecular disulfide bridged peptides, either in solution or on solid phase (Akaji et al. 2002, Kimura T. 2002, Postma et al. 2014). For peptides containing multiple disulfide bonds, orthogonally protected cysteine residues are generally employed in order to achieve selective pairing of bridges via progressive deprotection and oxidation reactions promoted by oxidants (Chan et al. 2000; Andreu et al. 1994), like iodine, thallium trifluoroacetate, potassium ferricyanide, dimethylsulphoxide (Kamber et al. 1980; Zhang et al. 2008; Engebretsen et al. 1997; Eritja et al. 1987; Tam et al. 1991). Highly constrained, non-natural structures can be forced to form by this approach. Single disulfide bridges can also be conveniently formed on unprotected polypeptides under very mild conditions in the presence of atmospheric oxygen, at high dilution and under slightly alkaline conditions (Reinwart et al. 2013; Steiner et al. 2011; Kudryavtsena et al 1998).

In this regard, there are two points to be discussed, in order to clarify advantages and disadvantages of promoting disulfide bond with oxidant agents with respect to the straightforward air oxidation method. The first procedure allows higher rates and yields of the disulfide linkage formation, while the second, due to the quite long duration for the complete reaction, can lead to accumulation of side products, like oxidized methionine. Moreover, strong oxidant reagents can affect also other sensitive amino acid residues, like tryptophan and tyrosine (Andreu et al.1994; Annis et al. 1997; Chan et al. 2000; Kamber et al. 1980). On the other hand, even in case of regioselective routes, in presence of oxidant agents a scramble cysteine pairing can occur, thus lowering the yield of the desired disulfide linkage. It is worth remembering that this kind of oxidation takes place in organic media, which eventually does not favor the naturally occurring peptide conformation, characterized by the proper cysteine pairing. Instead, the air oxidation method, performed in a buffered aqueous medium, can often successfully produces the molecular regioisomer with the more conformationally stabilized disulfide bridges.

Herein, we describe the synthetic strategy for preparing the natural dimeric distinctin (Dalla Serra et al. 2008; Cirioni et al. 2008; Verardi et al. 2011; Simonetti et al. 2012; Raimondo et al. 2005; Giacometti et al. 2007; Becucci et al. 2011) and rationally designed cyclic peptides, inhibitors of CXCR4 receptor (Portella et al. 2013). It consists in a chemical method able to speed up the synthesis and to increase the yields of the chosen disulfide-containing peptides.

The final aim has been to demonstrate that the mild conditions approach, in order to form disulfide bridges, is in general strongly dependent on the polypeptide structure, which can be, eventually,

induced to spontaneously refold under native-like conditions. (Calce et al. 2014; Ragone et al. 2000-2001). For our study we chose two different class of compounds, natural and synthetic, in order to prove that the folding-driven oxidation method can be successfully applied in both cases. Finally, a computational approach has been used to rationalize the results obtained on the different dimers of the chains of the natural peptide.

Materials and Methods

Chemical and Equipment

Fmoc-protected amino acids, Wang resin, Nhydroxybenzotriazole (HOBT), benzotriazol-1-yl-oxy-trispyrrolidino-phosphonium (PyBOP) were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland), piperidine and diisopropylethylamine (DIPEA) were purchased from Fluka (Milwaukee, WI), all other chemicals were purchased from Aldrich (St Louis, MI) or Fluka (Milwaukee, WI) and were used without further purification, unless otherwise stated.

Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus using a Phenomenex (Torrance, CA) C18 column, 4.6 • 250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C18 column, 22 • 250 mm with a flow rate of 20 mL min^{-1}. For all the RP-HPLC procedures the system solvent used was H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), with a linear gradient from 5% to 70% B in 30 min. Mass spectral analyses were carried out on Finnigan Surveyor MSQ single quadrupole electrospray ionisation mass spectrometer coupled with a Finnigan Surveyor HPLC (Finnigan/Thermo Electron Corporation San Jose, CA, USA).

Peptide synthesis

Wang resin was used for the synthesis of both distinctin peptide chains (hereafter named A and B, according to Raimondo et al. 2005) and CXCR4 antagonists (Portella et al. 2013).

The first amino acid is attached to Wang resin using as activating agent diisopropylcarbodiimide (DIC), a catalytic amount of 4-dimethylamino-pyridine (DMAP) and HOBt to reduce racemization (Sheppard et al. 1982). In more detail, the resin was suspended in 9:1 v/v CH_2Cl_2/DMF in a round bottom flask. In a separate flask, 2 equivalents (relative to the resin) of the Fmoc-amino acid and of HOBt were dissolved in a minimum amount of DMF, then the solution was added to the resin. In a separate flask 0.1 equivalent (relative to the resin) of DMAP was dissolved in a minimum amount of DMF and added to the resin mixture with 1.0 equivalent (relative to the amino acid) of DIC. The mixture was stirred for 3 hours at room temperature; then the resin was filtered and washed with

DMF, DCM and methanol. The loading was evaluated by Fmoc test (0.80 mmol/g calcd substitution; 0.100 g resin; 0.080 mmol scale).

The peptide synthesis was performed on an Applied Biosystems 433A synthesizer for distinctin peptide chains and manually for the CXCR4 antagonists, by solid phase method using the standard Fmoc procedures.

All couplings were performed twice for 30 min in DMF, by using an excess of 4 equiv for each amino acid coupling.

The peptide cleavage from the solid support and the simultaneous removal of all protecting groups from the amino acid residues were carried out by suspending the fully protected compound-resins in TFA/H_2O/EDT (94:4:2) for 3 h followed by filtration. The solution was then concentrated and the crude product isolated by precipitation into cold diethyl ether. The precipitate was collected by centrifugation and dried in vacuo. The crude products were characterized by RP-HPLC and mass spectrometry analysis.

Oxidation reaction

- *Distinctin*

Final heterodimeric AB product was obtained from 10 mg of each crude peptide chain via disulfide bond formation. This reaction was a 4 hr-long spontaneous process, which was performed in 6 mL of 0.1M NH_4HCO_3. This product was purified by preparative RP-HPLC and characterized as pure components by analytical RP-HPLC and LC-MS analysis.

Distinctin: t_R= 13.72 min; $[M+6H]^{6+}$= 913.7 (calcd= 913.2), $[M+5H]^{5+}$= 1095.8 (calcd= 1095.6), $[M+4H]^{4+}$= 1369.7 (calcd= 1369.3), $[M+3H]^{3+}$= 1825.7 (calcd= 1825.4)

- *CXCR4 antagonists*

The cyclization reaction, by disulfide bond formation between the two Cys residues, was performed by dissolving the crude peptide (final concentration 10^{-4} M) in 0.1M solution of NH_4HCO_3 in water to promote the oxidation reaction. After 4 h the reaction mixture was concentrated and the desired compound isolated by chromatographic purification.

RP-HPLC purification and mass spectrometry analysis confirmed the presence of the desired compounds.

Peptide R: t_R= 12.42 min; $[M+H]^+$= 899.8 (calcd= 899.4). Peptide R dimer: t_R= 10.67 min; $[M+H]^+$= 1798.1 (calcd= 1797.8); $[M+2H]^{2+}$= 900.1 (calcd= 899.4); $[M+3H]^{3+}$= 600.2 (calcd= 599.9). Peptide S: t_R= 10.79 min; $[M+H]^+$= 928.8 (calcd= 928.4). Peptide I: t_R= 8.57 min; $[M+H]^+$= 701.3 (calcd= 701.3). Peptide T: t_R= 11.15 min; $[M+H]^+$= 954.7 (calcd= 954.4).

Distinctin oxidation process analysis

The formation of homodimer AA and homodimer BB was performed dissolving 5 mg of each chain in acetic acid/water (4:1) to reach a final concentration of 10^{-3} M. To each solution 10 equiv of iodine were added and the reaction mixtures were stirred for 4 h. In order to quench the reaction, the solutions were placed in a 250 mL separatory funnel, the volumes were diluted five-fold with H_2O and the iodine was extracted with $CHCl_3$ (2-4 times, equal volume each time). The Elman's Test was performed on the aqueous layer in order to assay the formation of disulfide bonds (Ellman 1959). The final homodimers were collected after lyophilization process and characterized by LC-MS analysis. Afterwards, each obtained homodimer was dissolved in 3 ml of NH_4HCO_3 (0.1 M; pH 7-8) and 2 equiv of the complementary chain was added. The reaction mixtures were stirred at room temperature for 24 h and monitored by LC-MS analysis.

Molecular Dynamics and Modeling

Monomers A and B of distinctin were modeled from the NMR structure in solution of the non-covalent dimer of the distinctin covalent heterodimer AB (PDB entry 1XKM, Raimondo et al. 2005). The structures of the chains A and B of the representative conformation 1 of the PDB entry underwent mutation of the cystine into cystein residue, energy minimization (EM) *in vacuo*, solvation in a rectangular TIP3P water (Jorgensen et al. 1983), with a minimum distance between the peptide and the solvent box walls of 10 Å, and addition of Cl^- ions to neutrality, EM and 10 ns Molecular Dynamics (MD) in solution.

The representative structures of the last 2 ns of MD simulation for the two monomers were used to manually build the covalent homo- and heterodimers AA, BB and AB, which underwent the same protocol described above for monomers. Analyses were performed on the last 8 ns of the MD trajectories, unless differently stated.

The details for EM/MD programs and parameter and for the force field employed in the calculations are:

EM in vacuo: 100 steps of steepest-descent minimization followed by 2000 steps of conjugate gradient EM.

EM in solution: 100 steps of steepest-descent minimization followed by 2000 steps of conjugate gradient EM, with periodic boundary conditions, and both peptide and chloride ions positionally restricted by a harmonic potential with a force constant of 10 kcal mol^{-1}.

MD in solution: 150 ps of equilibration with both peptide and chloride ions positionally restricted by a harmonic potential with a force constant of 5 kcal mol^{-1}, using a timestep of 1.5 fs, followed by 10 ns of unrestrained production run, with a time step of 2 fs. All simulations were performed at constant temperature (300 K) by using a Langevin thermostat with a collision frequency of 5 ps^{-1}

(Zwanzig 1973), and pressure (1 atm) by employing a Berendsen "weak coupling" barostat with a pressure relaxation time of 4 ps (Berendsen et al. 1984).

Force field: AMBER ff12SB (Case et al. 2012), Particle Mesh Ewald treatment of long-range electrostatic interactions (Essmann et al. 1995) with a direct space sum limit of 10 Å, and a cutoff of 10 Å for nonbonded interactions. Bond including hydrogen atoms were restrained by applying the SHAKE algorithm (Ryckaert et al. 1977).

Programs: EM and MD were run with the AMBER 12 suite (Case et al. 2012), the AmberTools 13 suite (Case et al. 2012) was employed for system setup and numerical analysis, while model building, visual analysis and figure drawing were performed with the UCSF Chimera program (Pettersen et al. 2004).

The adopted version ff12SB of the AMBER force field allowed to describe with good accuracy the relative stability of helix vs. extended conformations, providing results comparable with CHARMM force field on the systems under examination. It is also natively and efficiently implemented into AMBER suite and can be easily parametrized for organic molecules and noncoded residues.

Results

Synthesis of Distinctin

The synthesis of the natural peptide distinctin, an heterodimeric molecule (hereafter named AB) consisting of two peptide chains, A and B, connected by a disulfide bridge (Dalla Serra et al. 2008; Cirioni et al. 2008; Verardi et al. 2011; Simonetti et al. 2012; Raimondo et al. 2005; Giacometti et al. 2007; Becucci et al. 2011) (Fig. 1), was performed in solid phase by using Fmoc chemistry standard protocols. The Wang resin was used as solid support on which the first amino acid was loaded in order to prepare the two peptide chains. The peptide cleavage from the solid support and the deprotection of all amino acid residues were obtained upon treatment with a high percentage of trifluoroacetic acid. The peptides were obtained in good yield and were fully characterized for their identity by mass spectrometry.

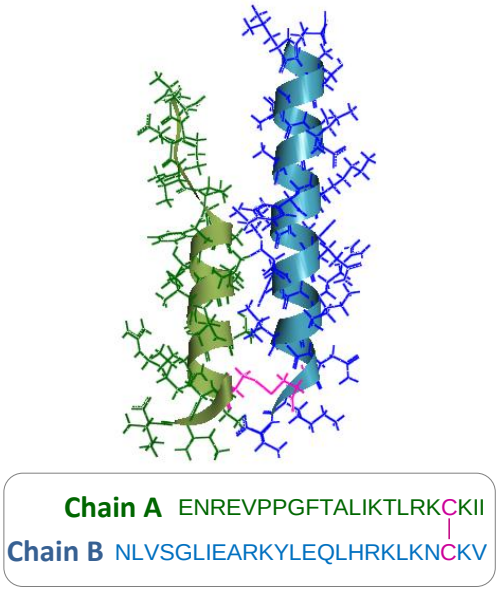

Fig. 1 Structure and amino acid sequence of Distinctin

The oxidation reaction was performed in solution on the free thiol form of the two different crude peptides (A and B), under air oxygen and basic conditions (0.1M NH_4HCO_3). Several concentration of two chains in the buffered solution were tried, in order to optimize the yield of the final heterodimer.

We firstly employed the recommended concentration of the thiolic moieties (0.01-0.1 mM) (Chan et al. 2000). By mass spectrometry analysis, only the wanted AB was found, the formation of homodimers AA and BB (hereafter named AA and BB) was not even detectable. Subsequently, we performed the reaction exploring lower concentration of the peptides, until reaching a concentration of around 0.5 mM for each chain. The desired AB was obtained in higher yield (Figure S1) after HPLC purification, due to the less diluted employed reaction conditions.

Oxidation process analysis

To further study the oxidation process, we firstly performed the oxidation of A and B utilizing iodine as oxidant. The formation of both homodimers occurred into 4 h (Fig. S2-S3) Afterwards, each purified homodimer was dissolved in ammonium bicarbonate and the single complementary chains were added, reaching the following ratios: B:AA, 2:1; A:BB, 2:1 (Fig. 2). The mixture was left under magnetic stirrer for 24 h. HPLC-MS analysis revealed that AA almost disappeared, being converted into AB, which was clearly detectable by mass spectrometry analysis (Fig. 2). On the other hand, BB was only partially (50%) incorporated into AB (Fig. 2). These results are also in agreement with previously published date that underlay the major stability of BB compared to AA (Evaristo et al. 2013).

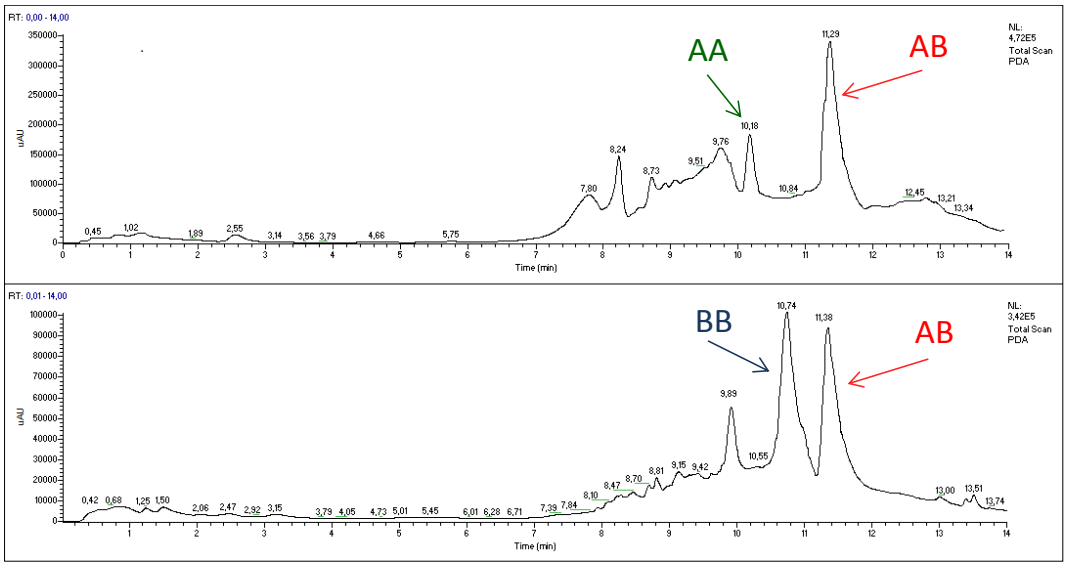

Fig. 2 HPLC profile of the oxidation reaction AA+B (upper panel) and BB+A (lower panel)

Molecular Modeling

A rationale for the experimental results heretofore described has been searched by employing molecular modeling and molecular dynamics (MD) simulations. In particular, starting from the solution structure of distinctin (Raimondo et al. 2005), the monomeric A and B chains, the homodimers AA and BB and the heterodimer AB have been modeled and simulated by MD (see Materials and Methods). This procedure was adopted since, rather than an exhaustive conformational analysis of monomers, we were looking for a comparison of the effect of covalent dimerization on the intrinsic properties of each chain and, in particular, of features potentially affecting the stability of the homodimers and their conversion to the heterodimer.

The computational results fully support the experimental findings by showing a much more stable homodimer for B than for A, the relative contribution of the disulfide bridge on the overall stability of the chain packing in the dimer being much less relevant in the former than in the latter. The two chains in BB exhibit a substantially larger average helicity (76.4±0.1% of residues classified in α helical conformation, Fig. 3A), very similar to that calculated for B (77.8±0.2%) and they form a coiled-coil-like structure stabilized by interchain interaction both hydrophobic, involving aliphatic and aromatic sidechains, and polar (H-bonds and salt-bridges) (Fig. 3B). The interchain interface involves many Leu residues, with very tight Leu-Leu interactions involving four out of the five Leu residues in the sequence of chain B, and a tight Leu-Leu interaction for the fifth Leu residue, occurring at the N-terminus of the chain. A salt-bridge is observed between Glu8 on a chain and Arg10 on the other chain, and a H-bond is formed between Tyr12 on a chain and His17 of the other chain. AA appears much less ordered, with a fraction of helix of 53.4±0.1% (Fig. 3A), even lower than that observed in A simulations (57.0±0.1%), differently from what observed in AB, where the

A chain exhibits on average a larger fraction of helical residues (67.1±0.1%) than in the monomer, and also than the value obtained by averaging the helical contents of AA and BB (65.7±0.1%). It is interesting to note that the relative helicity of homodimers and monomers (BB~B>A>AA) determined in MD simulations correspond to that experimentally observed in Raimondo et al. 2005. Only AB apparently exhibit a lower relative helicity than the experimental value, but this latter has been determined under conditions where the noncovalent homodimer of AB is present as representative species. Also the flexibility of AA (Fig. 3B, average rmsd value of 2.57±0.01 Å) is higher than that of BB (Fig. 3B, average rmsd value of 1.88±0.01 Å). The higher flexibility of AA corresponds to a reduced interchain interaction pattern, characterized by a lower number of interhelical contacts and by the lack of the interhelical salt-bridges detected for BB. In AA the very tight hydrophobic interactions are reduced to a cluster of four Leu residues (two for chain), while progressively looser contacts involve Pro, Val and Phe residues (Fig. 4). The two representative structures of AA and BB shown in Figure 4 exhibit a single-chain solvent-accessible surface area (SASA) burial after dimerization of 401.8 $Å^2$ and 655.7 $Å^2$, respectively, which thus accounts for the different stability and rigidity of the two homodimers.

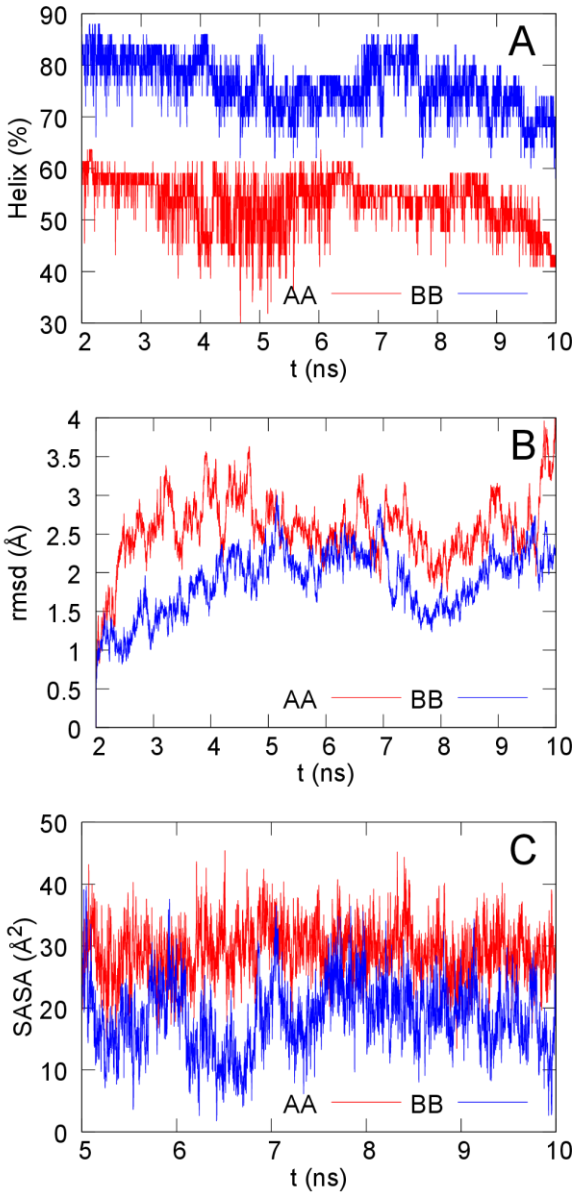

Fig. 3 Compared helicity, backbone atom rmsd and Cys sidechain accessibility from MD simulations of AA and BB models.(A) Percent of residues in α-helical conformation calculated for on the last 8ns of MD. (B) Backbone atom rmsd (in Å) after best fit on the last 8 ns of MD. (C) SASA (in Å2) of the Cys sidechains during the last 5 ns of MD. In all plots data for AA and BB are colored in red and blue, respectively.

An additional difference between the two homodimers that can be related to the relative ease of the AA→AB and BB→AB exchange reactions has been found in the accessibility of the disulfide bridges, estimated with the solvent accessible surface area (SASA) of the two CYS sidechains in Fig. 3C. The disulfide bridge is more shielded in BB, whose SASA (average value 20.0±0.1 Å^2) amounts to about two-third of the value calculated for AA (average value 29.2±0.1 Å^2).

The formation of the tetramer (i.e. the non-covalent dimer of a covalent dimer), previously predicted computationally and experimentally confirmed by NMR and ultracentrifugation for AB (Raimondo et al. 2005) and not observed for both homodimers, then provides the driving force to

shift the overall AA+2B→2AB and BB+2A→2AB equilibria toward the formation of AB, although the high stability and disulfide bond inaccessibility only allow a partial conversion in the case of BB.

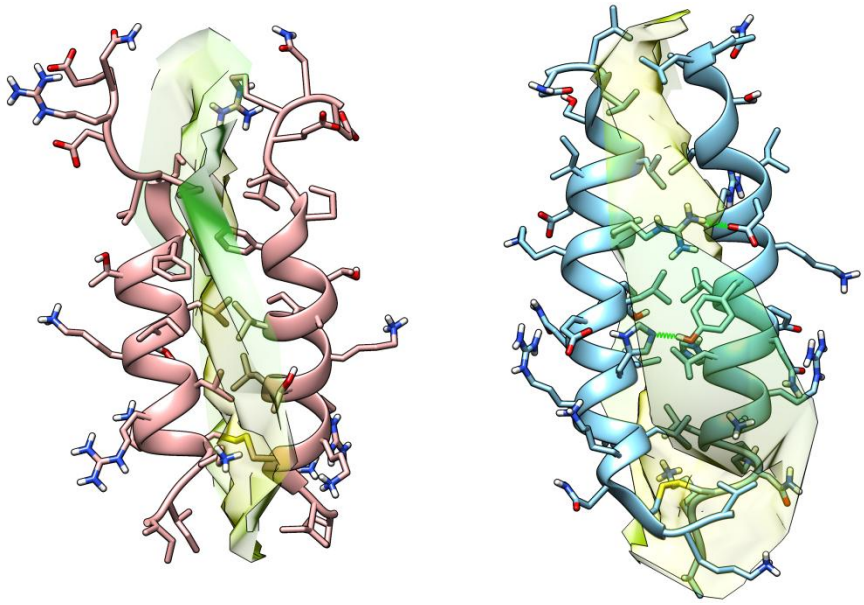

Fig. 4 Representative MD structures for AA and BB. A ribbon representation with sidechain heavy and polar hydrogen atoms sticks is shown for the representative structures of the most populated clusters in the last 5 ns of MD for AA (pink, A) and BB (light blue, B). Heteroatoms are painted according to the standard scheme (N: blue, O: red, S: sulfur, H: white), H-bonds are represented as green "springs". The transparent surfaces illustrate the interface between the two chains of each monomer and are painted with a colour gradient scheme ranging from yellow to green for progressively looser interactions.

Synthesis of CXCR4 antagonists

The rational designed CXCR4 inhibitors (Portella et al. 2013) (Fig. 5), consisting of different peptides cyclized via cysteine disulfide bond, were synthesized using the same solid phase Fmoc chemistry approach above mentioned. After cleavage from the resin and simultaneous deprotection of all amino acid side chains, the cyclization reaction was performed in solution by disulfide bridge formation. In particular the air oxidation reaction was performed on the crude products dissolved in 0.1M NH_4HCO_3.

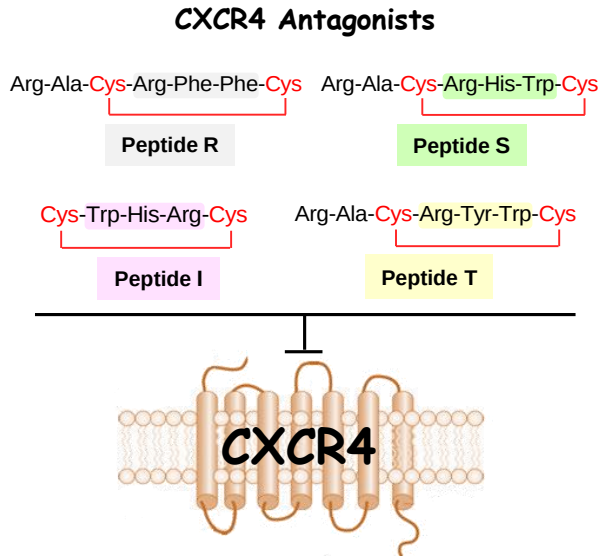

Fig. 5 Amino acid sequences of CXCR4 inhibitors

It is well established that high dilution is recommended in case of oxidation for intramolecular disulfide bridge, in order to avoid the formation of dimer, oligomer and in general polymer (Chan et al. 2000). We tried different concentrations of the linear peptide in a buffered solution and found that the best was around 0.1 mM (Fig. S4-S7 of SI).

In fact, any increase of this value allowed also the formation of the dimer, in which it is likely that the symmetric double oxidation took place between two peptide molecules. It was clearly detected by mass spectrometry analysis of the peptide R oxidation mixture, in fact we found that the product for 20-30% consisted of dimeric species (Fig. 6).

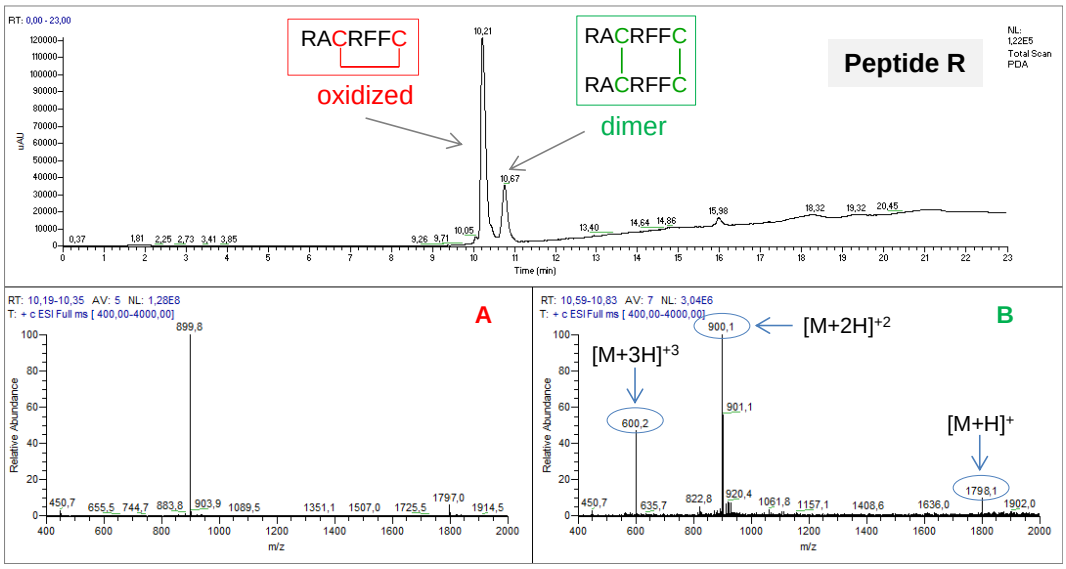

Fig. 6 LC-MS profile of the oxidized peptide R

Discussion

In our studies we demonstrated both for a naturally occurring peptide, distinctin, and for rationally designed peptides, like the CXCR4 inhibitors, that if the molecules spontaneously fold in a conformation which exhibit proper proximity and relative orientation of thiol groups, the formation of inter- or intramolecular disulfide bridges can occur in much milder, native-like, reaction conditions (air oxidation in a buffered aqueous medium) than those usually suggested in literature for peptides.

Instead of using the valuable protocols available in literature for the site-directed cysteine oxidation (Kamber et al. 1980; Zhang et al. 2008; Tam et al. 1991; Liu et al. 2014), we proved that the distinctin can be obtained in a very high yield (Cirioni et al. 2008; Verardi et al. 2011) by using a convenient method which allows a relevant conversion of the reactants. It is important to underlay that our method is not time-consuming, since it is performed with less synthetic and purification steps, and also straightforward requiring no chemical manipulation of the thiol functionalities (Mullen et al. 2012).

Concerning with the CXCR4 inhibitors, the mild protocol used to oxidize the two cysteine residues present on the same peptide chain provided a further validation of the performed rational designed. In fact, the peptides easily folded into a conformation with proper spatial arrangement of thiol groups, giving a very high yield of the oxidized peptide which represent a relevant result for a non-natural peptide sequence.

Computational approaches, both during design, and in the prediction or determination of structure-activity relationships, can provide considerable help in obtaining molecules providing higher yields of disulfide-bridged forms, and/or in allowing the use of milder oxidation conditions. They can also provide a rationalization of the variability of the oxidation yields in related peptides or in different aggregation states. These contributions, that generally improve the synthetic protocols of standard peptides, can be critical in special cases, such as peptides with low stability to drastic reaction conditions, or synthesized with low yields already before the oxidation stage, or in the case of different potential disulfide bridge patterns in the same system.

In conclusion, with our studies we intended to demonstrate that methods fully directed by chemical reactivity for the disulfide bond formation can be avoided for specific peptide molecules, that spontaneous oxidize in their natural conformation. This approach greatly simplified the synthetic chemistry and at the same time avoid to modify oxidation-sensitive residues, such as methionine, tryptophan, and tyrosine, that are often encountered in natural peptide sequences.

Conflict of interest

The authors declare that they have no conflict of interest.

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