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REGULAR ARTICLE

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Molecular dynamics simulations of proteins with chemically modified disulfide bonds

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Abstract Proteins that are used as therapeutic drugs act in the extracellular microenvironment. They usually have a small number of intramolecular disulfide bonds to help maintain their tertiary structure in the vascular circulation. In general, most cysteine residues are part of a disulfide bond with free sulfhydrals being uncommon. We have studied whether the site-specific chemical reduction of disulfides and the incorporation of a 3-carbon methylene bridge between the cysteines in interferon-α 2a would change the structure of this protein. Bridging of both of the disulfide bonds of interferon-α 2a was studied using two different molecular simulation protocols: (1) molecular dynamics, and (2) stochastic dynamics. We have shown that the disulfide bonds in interferon-α 2a can be reduced and chemically modified without significantly altering the tertiary structure of the protein. This offers the novel possibility of chemically modifying therapeutically important proteins without affecting their biological properties.

1 Introduction

Therapeutic proteins are the fastest growing class of new medicines that are being introduced into clinical practice [1]. Although there are many types of therapeutically active proteins, replacement cytokine and hormone based therapies currently constitute the core group of FDA approved medicines. Examples include interferon (IFN), colony stimulating factors (G-CSF), erythropoietin (EPO), interleukins (IL-4), and growth hormone (GH). Although some of these proteins are post-translationally glycosylated (e.g., EPO), they share several important structural and functional features [2,3]. The number of amino acid residues in these proteins ranges from 160 to 190 with a molecular weight of approximately 19,000 g/mol. They are usually folded in a 4α-helical bundle. Shorter helices and loops connect the four major helices. Functionally, some proteins (e.g., EPO, GH, and IFN) have two binding sites for cell surface receptors. Binding of the protein to each of its extracellular receptors leads to dimerisation of the complex formed and the initiation of the protein's biological activity.

Non-covalent interactions between residues in the primary amino acid sequence of a protein are crucial for defining and contributing to the maintenance of its overall tertiary structure and its biological activity. Therapeutically active proteins must exist in the extracellular microenvironment in order to exert their biological activity. They must also have covalent disulfide bonds to maintain their tertiary structure whilst circulating in the blood stream. In the case of four helical bundle proteins, they have 1–2 disulfides that are typically between the major helices. Consequently, some disulfides are derived from cysteines that span a large number of amino acid residues along the protein backbone. For example, EPO has a disulfide that spans cysteines 7–161 between the major helices A (residues 8–26) and D (residues 138–161), whilst interferon-α 2a has two disulfide bonds with one linking cysteines 1–98 and the other linking cysteines 29–138. Each disulfide defines a macromolecular substructure that is further constrained in its conformation by additional non-covalent interactions (e.g., electrostatic, hydrogen bonding, hydrophobic interactions). These span the amino acid backbone between the two cysteines of the disulfide. For example, in interferon-α 2a, structural integrity and biological activity are preserved when the disulfide bond between the cysteines 1–98 is reduced to liberate the free thiols for these two cysteines [4]. There are other examples where disulfides span a much smaller number of amino acids in the protein; for example, in EPO, the second of the two disulfides is

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between cysteines 29–33, and in GH, one of the two disulfides is between cysteines 181 and 189. Since interferon-α 2a has two disulfides that span a large number of amino acids, we hypothesized that any disulfide bridging perturbations that occurred would be compensated for by the structural conformational mobility and the non-covalent interactions that span the macrocycle substructure of the protein.

Typically in therapeutically relevant proteins, there are an even number of cysteines that are all paired in disulfide bonds. There are seldom-free naturally occurring sulfhydrals present. The notable exception is GCSF, which has five cysteines. There are two disulfides and one free sulfhydral in GCSF. Free sulfhydrals are often the cause of scrambling and mismatching of the disulfides during protein refolding when the protein is being manufactured. Free sulfhydrals also have a tendency to cause protein dimerisation and aggregation during manufacture, formulation, and storage. This results in the loss of biological activity and an increase in their immunogenicity.

Our interest in protein disulfide bonds relates to the improvement in clinical efficacy that is observed when proteins are covalently conjugated to poly (ethylene glycol) (PEG). The vast majority of PEG reagents form a covalent bond with different amine residues along the protein backbone; e.g., ε-amino moiety of lysine. This is because amine reactive reagents are not residue selective and this results in conjugation reactions at multiple residues along the protein. The result is positional isomers with PEG molecules positioned at different sites along the protein backbone. Such structural heterogeneity is not optimal for a therapeutically useful protein because each isomer displays a different physicochemical and biological profile.

Conjugation reagents that are selective for sulfydral groups over amine groups have been described [5–7]. Such reagents can be used in mild reaction conditions and they favor the preservation of the protein's stability. However, few clinically relevant proteins have free sulfhydrals, so these reagents can only be used in proteins that have been recombinantly engineered to have a free cysteine. This tedious process limits the use of this approach to the few proteins that have a free cysteine. For native proteins, it is imperative that the free cysteine is not required for biological activity, as is generally the case. Hence, reagents for single sulfhydrals have not found any utility for clinically relevant proteins.

In contrast, there are several sulfhydral specific, chemical functional groups that can undergo reaction with two thiol groups [8–10] in an interactive and thermodynamically driven manner. These latently *bis*-alkylating reagents can result in the incorporation of a 3-carbon methylene bridge between the two free sulfhydrals after partial reduction of a particular disulfide. Since disulfides are made up of two sulfurs, reduction of the disulfide will liberate two free thiols. To exploit the site-specific reactivity of the 2-thiols derived from a disulfide, we have computationally studied the effects of incorporating a 3-carbon methylene bridge between the two cysteines in each of the disulfide bonds of interferon-α 2a. We first compared the two different molecular simulation protocols: (1) molecular dynamics (solvated protein), and (2) stochastic dynamics (implicit solvent) in interferon-α 2a with a reduced disulfide bond. Subsequently, we followed the effects of disulfide bridging on the protein's tertiary structure by stochastic dynamics simulations. We have shown that the disulfide bonds in interferon-α 2a can be reduced and chemically modified by the insertion of a 3-carbon methylene bridge without significantly altering the tertiary structure of the protein.

2 Computational methods

The initial structure of interferon-α 2a used for the molecular modelling studies was based on the three dimensional NMR structure of interferon-α 2a (ExPDB code 1ITF) [11].

2.1 Molecular dynamics simulations of fully solvated proteins

The NMR structure of interferon-α 2a was fully solvated with a 10 Å radius thick layer of water molecules using Solvate 1.0 [12]. Two molecular structure files of the protein were generated in which each structure had one intact natural disulfide. The two sulfurs that comprised the other disulfide were left as free sulfhydrals; i.e., as they would be if the disulfide had been reduced. The first structure file (IFN I) had the intact disulfide between CYS29 and CSY138. Free sulfhydrals on CYS1 and CYS98 replaced the normal disulfide bond between these residues. Conversely, the second structure file (IFN II) had the intact disulfide at CYS1–CYS98 with free sulfhydrals at CYS29 and CYS138. Molecular dynamics simulations of explicitly solvated interferon-α 2a molecules were performed using CNS-SOLVE 1.1 [13] for 180 ps (IFN I with reduced disulfide bond between CYS1 and CYS98) and 200 ps (IFN II with reduced disulfide bond between CYS29 and CYS138) at 300 K using CHARMM 22 times force field and 0.5 fs timestep.

2.2 Stochastic dynamics simulations

These studies were conducted to determine the effects of the partial reduction of interferon (i.e., reduction of one of the two disulfides) followed by reannealing of the disulfide with a 3-carbon methylene bridge. Stochastic simulations were done with the AMBER forcefield in MacroModel v8.5 [14] using an aqueous generalized Born/surface area (GB/SA) continuum solvent model [15]. The modeling procedure consisted of the following steps:

(a) The stochastic dynamics simulation on partially reduced interferon-α 2a with two free sulfhydrals derived from one of the natural disulfides was performed with a 10 ps pre-equilibrium run. This was followed by further simulations at 300 K for 2,000 ps with 1.5 fs time steps and SHAKE constrained hydrogens.

(b) A 3-carbon methylene bridge between the free sulfur atoms on the two cysteines that comprise the reduced disulfide was constructed. The energy was minimized for these structures and then subjected to the same stochastic simulation protocol described in (a).

2.3 Analysis

All trajectory analyses, root mean square deviations (RMSD) calculations and graphic representations were conducted using VMD [16], gOpenMol [17], Vega ZZ [18, 19], and ViewerLite [20] software packages.

3 Results and discussion

Water plays an important role in the folding process and the folded state of proteins [21]. Implicit and indirect models of solvation are usually applied to reduce the computing time required for molecular simulation [22]. The folding simulations with explicit solvent molecules are becoming feasible and more practical to use [23]. Here, simulations with explicitly and implicitly defined solvent of the reduced IFN were conducted. Trajectories and RMSD values were compared to validate the use of stochastic simulations and the GB/SA model in the simulation studies of the chemically modified IFN.

3.1 Molecular dynamics simulation of fully solvated interferon-α 2a

The NMR structures of interferon-α 2a (ExPDB 1ITF) [11] were used as a starting point for molecular simulation calculations following the reduction of one disulfide bond to release two sulfhydrals. Two different models were built. The first was with the disulfide bond reduced between residues 1 and 98 (IFN I), and the second was with the disulfide bond reduced between residues 29 and 138 (IFN II). The simulations were conducted in the presence of solvent and the trajectories were analyzed by computing sets of geometrical properties (primarily RMSD), and the distance between the sulfur atoms of reduced disulfide bonds.

The RMSD plot obtained from the 180 ps simulation of IFN I for the backbone atoms of residues 1–158 is shown in Fig. 1a. The RMSD of IFN I was calculated against the native conformation of interferon-α 2a. After an initial RMSD increase to 2.5 Å, the structure stabilized and the RMSD was confined to 3–3.5 Å limits. The largest mobility was observed for the loop that connects helices E and F (loop E, F), where the greatest RMSD was observed in the NMR structures of native interferon-α 2a. The distance between the two free sulfur atoms of the CYS1 and CYS98 of the reduced disulfide bond is also shown in Fig. 1a. The distance increased by up to 9 Å for the first 60 ps and then fluctuated between 6 and 8.5 Å. As expected, the N-terminus of the polypeptide chain became more flexible with the free cysteines derived from the 1–98 disulfide bond (Supplementary material – http://www.aiki-dojo.f2s.com/eccc10c/itf-d-1.mpg, 10 Mb). Importantly, the lack of an intact disulfide bond between CYS1 and CYS98 did not cause unfolding of the protein or dramatic conformational changes in the helical structure of the protein. This indicated that the multiple, non-covalent interactions within the protein were making a significant contribution towards maintaining the tertiary structure of the protein.

Similar changes in protein conformation for IFN II were observed when the disulfide bond between CYS29 and CYS 138 was reduced (Fig. 1b). The RMSD for IFN II had a similar pattern to IFN I increasing up to 3.5 Å. The distance between the two sulfur atoms in IFN II did not increase as much because the CYS29 and CYS138 are part of a helix bundle and they are less mobile than the N-terminal CYS1 (Supplementary material – http://www.aiki-dojo.f2s.com/eccc10c/itf-d-2.mpg).

3.2 Stochastic simulation of reduced interferon-α 2a

The stochastic simulations were performed to mimic the presence of solvent. Calculations were conducted by randomizing the atom movements of interferon, as collisions with solvent molecules would occur. The simulation of IFN I was conducted for 2,000 ps and the RMSD plot is shown in Fig. 1c (Supplementary material – http://www.aiki-dojo.f2s.com/eccc10c/ifn-sd-1.mpg). The RMSD varied in a similar manner with the molecular dynamics simulation achieving a range of 3.5–4 Å. This observation was significant because the results between two different simulation protocols indicated a comparable trend. The RMSD values for IFN I varied between 2.5 and 3.5 Å for molecular dynamics of fully solvated protein (Fig. 1a) while RMSD was between 2.4 and 2.9 Å for stochastic simulation during the same time period (Fig. 1c). Also, the RMSD did not significantly increase during stochastic simulation and was ten times longer than the molecular dynamics simulation in explicit solvent (Fig. 1a). However, a distance of 20 Å between the two separated cysteines (CYS1 and CYS 98) was quite large. The N-terminal CYS1 was at the beginning of a protein chain and, as such, quite flexible. Interestingly, after the initial unfolding, the N-terminus folded back (distance going down to 15 Å) and therefore did not affect the overall tertiary structure of the protein to any significant degree. Future studies will use longer simulation times and will follow what happens with the N-terminus.

Similar characteristics were observed for the stochastic simulation of IFN II in which the disulfide between CYS29 and CYS138 was reduced (Fig. 1d) (Supplementary material – http://www.aiki-dojo.f2s.com/eccc10c/ifn-sd-2.mpg). The total RMSD was not greater than 4 Å, even after a 2,000 ps simulation, while the distance between the free sulfurs varied between 10 and 12 Å. This increased distance between the sulfhydrals, compared to 2.03 Å in the disulfide bond, did not have a significant impact on the tertiary structure of the protein. Therefore, a significant perturbation at the

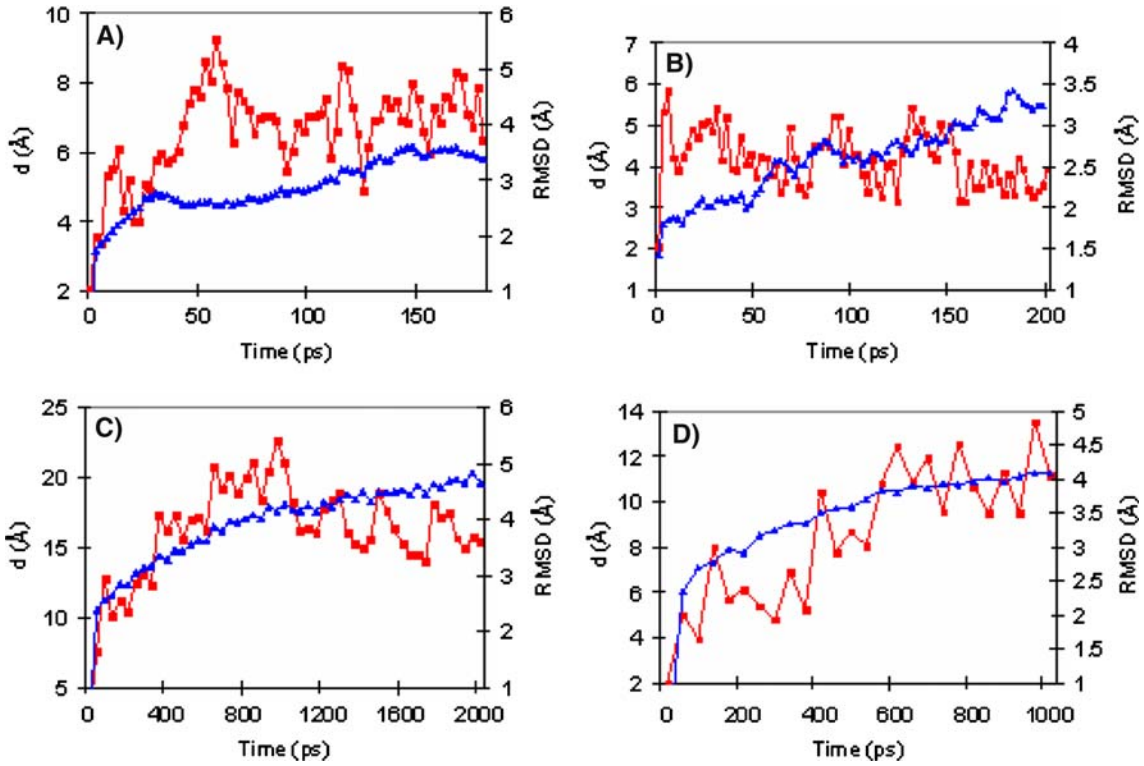

Fig. 1 The RMSD plot of backbone atoms of interferon-α 2a with a reduced disulfide bond between residues CYS1 and CYS98 (IFN I), or between residues CYS29 and CYS138 (IFN II). RMSD values were calculated against the native conformation of interferon-α 2a for residues 1–158 (*blue, triangle*). The time dependent distance between the two free sulfur atoms of a reduced disulfide bond are shown in *red, square*. (**a**) Simulation of IFN I (180 ps), (**b**) simulation of IFN II (200 ps), (**c**) stochastic simulation of IFN I (2,000 ps), (**d**) stochastic simulation of IFN II (1,000 ps)

site of the disulfide bond does not have a deleterious effect on the conformation of the protein.

As always true with these simulations (e.g. Fig. 1d), it is evident that further changes in the structures of the reduced proteins are possible. However we interpret these results as being broadly predictable that it should be possible experimentally to prevent such proteins from loss of tertiary structure. Our results suggest it is reasonable that reduced IFN will not rapidly unfold and denature within a reasonable time period in appropriate experimental conditions. This agrees with existing experimental data that suggests it is possible to reduce disulfide bonds in interferon-α 2a while preserving the tertiary structure of the protein [4]. Presumably the range of existing non-covalent interactions (e.g., electrostatic hydrogen bonding and hydrophobic interactions) within the protein are strong enough to maintain the tertiary structure of the reduced protein under appropriate experimental conditions.

Importantly, when the results of these two sets of simulations were compared to the RMSD values between the NMR structures of native interferon-α 2a (Fig. 2a), it was found that the RMSD values were very similar. The RMSD values of native interferon-α 2a were calculated for the set of 24 NMR based structures of interferon-α 2a (ExPDB 1ITF) [11] and the RMSD values for the backbone atoms were found to be in the range of 3–5 Å. This range reflected the dynamic properties of the protein in solution. An RMSD value of up to 4.5 Å was observed between native interferon-α 2a and interferon-α 2a with a reduced disulfide bond. This was comparable to the RMSD values observed for the native conformations of interferon-α 2a. It therefore appears that the simulated structures were located within the limits of the conformational space of the experimentally determined native structures.

Interestingly, it was also found that the structure of the native interferon-α 2a (with both of its natural disulfide bonds preserved) exhibited more flexibility than we expected during the stochastic simulation performed under the same conditions. The observed RMSD of the backbone atoms for residues 1–158 increased by up to 6 Å (Fig. 2b). This is considerably higher than the calculated RMSD for the conformations of the single disulfide structures, IFN I, and IFN II. It is possible that for some disulfides, conformational jumps can occur that lead to the structure displaying larger RMSD ranges than expected. Such conformational jumps may result in pathways that lead to protein destabilization. Certain disulfides may result in the proteins becoming so rigid that sufficient energy results in larger rather than multiple and smaller changes in its conformation. Therefore, conformational dependence is most likely to be a function of the protein's secondary structure and the number and type of amino acids that span the distance between the two cysteines. For example, it has been reported for copper plastocyanin that there was a greater degree and range of conformational variation when a disulfide bond was engineered onto the protein surface. Long-term dynamics simulation of this protein

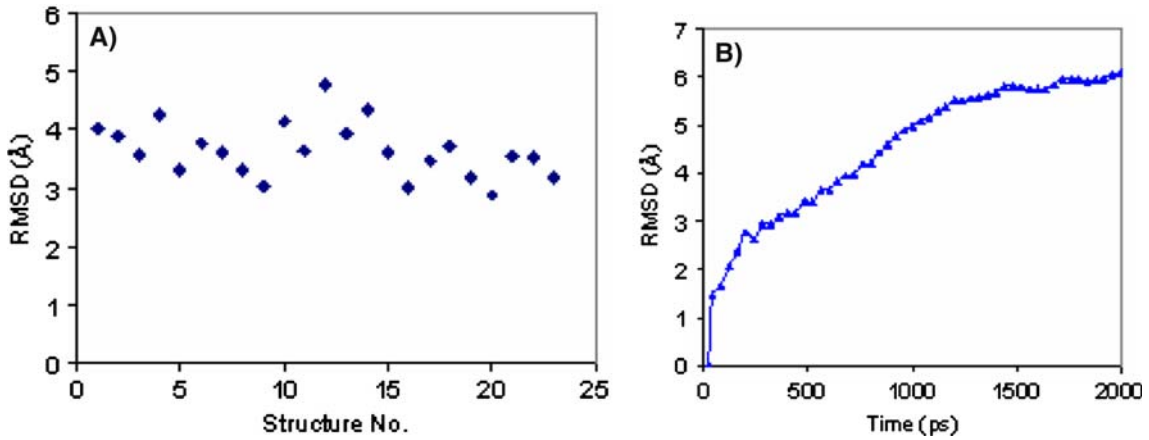

Fig. 2 The RMSD plot for 24 NMR based structures of native interferon-α 2a taken from the PDB file 1ITF [11] (**a**), and RMSD plot of the 2,000 ps stochastic simulation of native interferon-α 2a (**b**). The RMSD was calculated against the first conformation of interferon-α 2a for residues 1–158

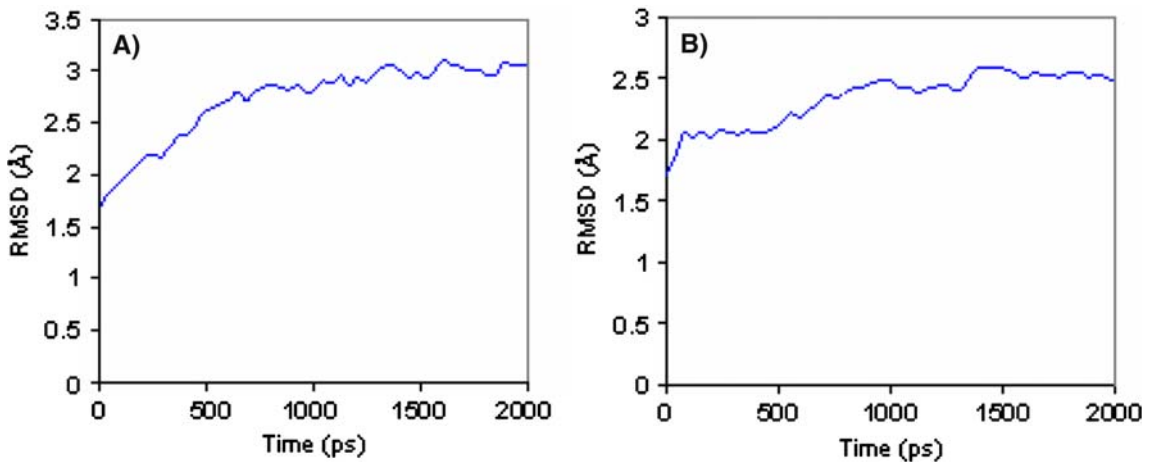

Fig. 3 Stochastic simulations of 2,000 ps of interferon-α 2a with a 3-carbon methylene bridge between the sulfurs of CYS1 and CYS98 (**a**), and interferon-α 2a with a 3-carbon methylene bridge between the sulfurs of CYS29 and CYS138 (**b**). The RMSD of the backbone atoms was calculated against the native conformation of interferon-α 2a for residues 1–158

in water were conducted and the total RMSD for backbone atoms was up to 18 Å during 1,000 ps [24]. Furthermore, molecular dynamics simulations of the native protein and the derivatised protein with the engineered surface disulfide bond were compared [25]. Unexpectedly, the disulfide engineered protein displayed greater flexibility with the RMSD of all of the atoms increasing to 22 Å. In terms of the protein's conformational flexibility, the study revealed that the engineered disulfide bridge in plastocyanin affected the dynamic fluctuations of the protein's domains. It was also shown that the engineered plastocyanin displayed lower stability. This was attributed to the increased strain on the disulfide bond, which in turn, affected the stability of the whole molecule [26].

Disulfide bonds are essential to maintain a protein's tertiary structure in the extracellular environment and they are important in the process of protein folding because they trap the protein into a limited number of conformational structures. However, once a protein has folded into its tertiary and biologically active structure, some of the disulfide bonds may no longer be required to maintain its tertiary structure. This is consistent with our results which indicate that the native conformation of interferon had a greater flexibility with both of its disulfides intact than either of the structures IFN I or IFN II which were formed when one of the disulfide bonds had been reduced. This result suggests that some disulfide bonds can be chemically utilized for modification of a protein without significantly altering its tertiary structure, and by inference, ultimately maintain its biological properties. Consequently, we speculate that the bridging of the free sulfhydrals with a 3-carbon methylene bridge will not significantly alter the protein's tertiary structure and the aim of further simulation was to examine this hypothesis.

3.3 Stochastic simulation of bridged interferon-α 2a

A 3-carbon methylene bridge (–CH_2–CH_2–CH_2–) was used to link the pair of free cysteines in both IFN I and IFN II (1–98 and 29–138, respectively). One sulfur was covalently

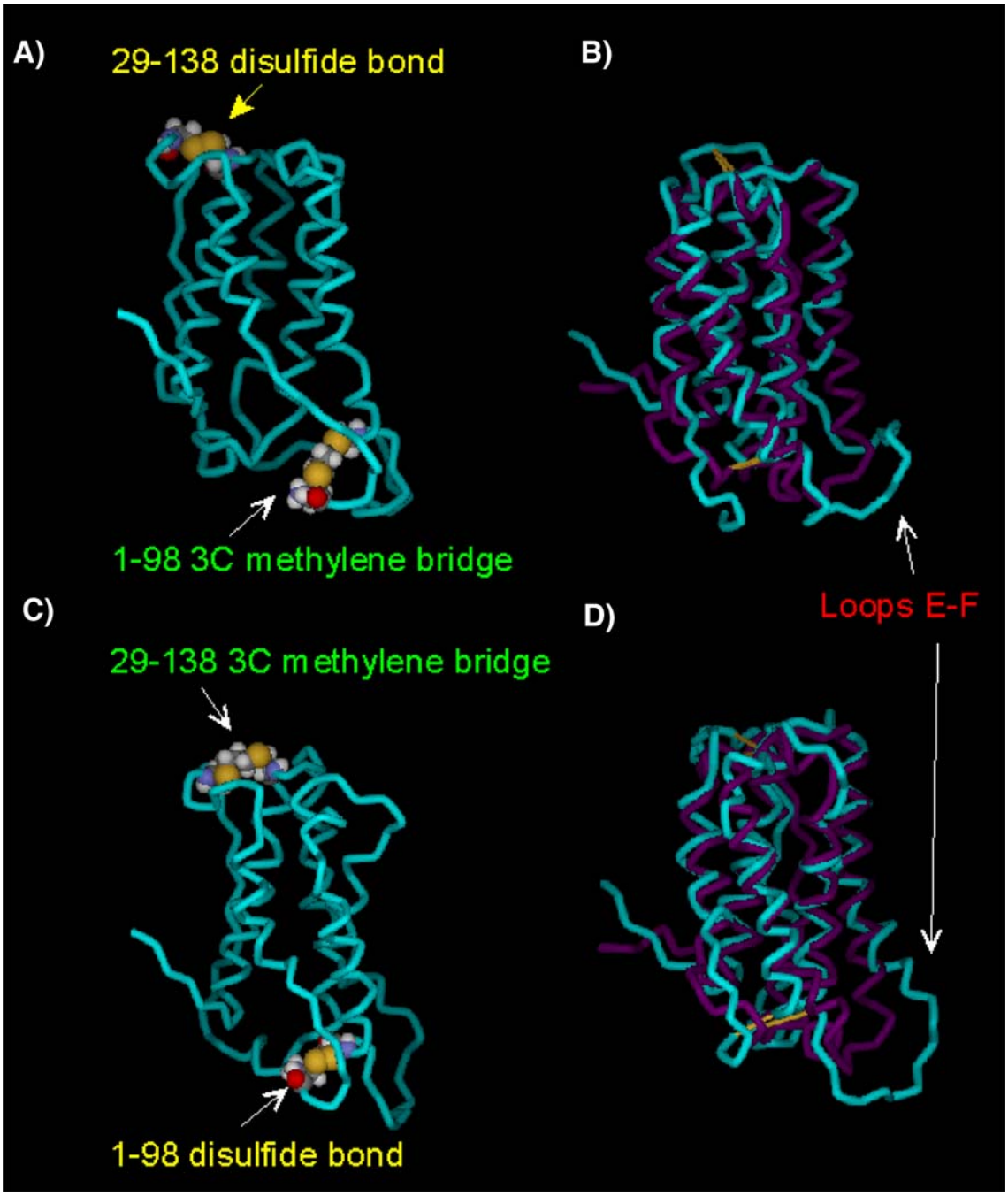

Fig. 4 Backbone plots of the chemically modified interferon-α 2a. The final conformation of the interferon-α 2a with the 3-carbon methylene bridge between CYS1 and CYS 98 residues (**a**), and overlap with the native structure (**b**). The final conformation of the interferon-α 2a with the 3-carbon methylene bridge between CYS29 and CYS 128 residues (**c**), and overlap with the native structure (**d**). CYS residues and the 3-carbon methylene bridge are shown in CPK representation. The bridged modified structures are shown in *blue* and the native structures are shown in *purple* in **b** and **d**

bound to a terminal carbon and the other sulfur was bound to the other terminal carbon (e.g., CYS1–S–CH_2–CH_2–CH_2–S–CYS98). The result was that the two cysteines derived from a native disulfide were bound to each other via two thiol ether bonds and a 3-carbon bridge rather than being covalently bound to each other as a disulfide. These bridged interferon derivatives underwent energy minimization and stochastic dynamics simulations and the RMSDs are shown in Fig. 3 (Supplementary material – http://www.aiki-dojo.f2s.com/eccc10c/ifn-sd-1-3C.mpg and http://www.aiki-dojo.f2s.com/eccc10c/ifn-sd-2-3c.mpg, respectively). In both cases, the RMSD between the modified and the native structures increased to about 2.5–3 Å for the bridged structures. This was due to the increase in distance between the two

cysteine sulfur atoms, which was 4.9 Å for the bridged structure as compared to 2.03 Å for the native disulfide.

The final conformations of the cysteine-bridged interferons (Fig. 4a, c) were then superimposed onto an NMR structure of interferon-α 2a. There was deviation of the backbone atoms of up to 3 Å when the bridge was between CYS1 and CYS98 (Fig. 4b). The maximum RMSD was only 2.5 Å for the CYS 29 and CYS 128 bridge modified interferon (Fig. 4d). In both cases, the loop E-F has exhibited the largest deviation between backbone chains of native and modified interferon-α 2a. These RMSD values suggest that the tertiary structure of the bridged modified protein had not changed beyond what had been observed for the dynamic conformational space of the native interferon-α 2a. Therefore, reduction of either disulfide bond followed by the insertion of the 3-carbon methylene bridge in interferon-α 2a should preserve the biological activity of the protein. Biological experiments are now in progress to determine if this is the case.

4 Summary

Protein based medicines are the most rapidly growing class of new therapeutic molecules that are now entering the clinic. The covalent conjugation of PEG based polymers to these proteins is often necessary to protect the protein from rapid degradation and to exploit their high selectivity index. Typically, the conjugation reagents used are based upon the conjugation of amine residues. However, these conjugation reagents show no selectivity and the consequence is that conjugation occurs along the entire protein backbone. There are some reagents that are known to be selective for free sulfhydrals. Unfortunately, clinically useful proteins do not have free sulfhydrals. Native protein disulfides can be reduced to two free cysteine sulfhydrals. Reagents are available that can undergo these chemical reactions with two sulfhydrals and which enable the subsequent formation of a 3-carbon methylene bridge between the two sulfurs. We decided to computationally evaluate the perturbation in the tertiary structure of interferon-α 2a by the incorporation of a 3-carbon methylene bridge. It was established that for the reduced form of interferon-α 2a, the stochastic simulations behaved in a manner that was similar to the molecular dynamics of the fully solvated protein. Molecular dynamics simulations for fully solvated proteins as well as the stochastic simulation with implicit representation of the solvent demonstrated that reducing either of the two disulfide bonds in interferon-α 2a did not significantly perturb the overall tertiary structure of the protein. In addition, the subsequent introduction of a 3-carbon methylene bridge did not result in any significant conformational change in the protein when compared to the native interferon-α 2a. Furthermore, the derivatised bridged protein displayed a similar RMSD, irrespective of whether the bridge was introduced between residues 1 and 98 (3.0 Å) or between residues 29 and 138 (2.5 Å). The small changes in the backbone conformation that were seen suggest that the tertiary structure of the modified protein had not changed significantly and that its biological activity should also not change. Disulfide based, site-specific insertion of a 3-carbon methylene bridge offers exciting new opportunities for making new and cost-effective proteins that are safe and that can be rapidly and economically manufactured for use as global healthcare products.

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