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Conformational disorder in phosphopeptides: solution studies by CD and NMR techniques

Abstract: In the last few years intrinsically disordered proteins (IDPs) have received great attention from the scientific community as they participate in several important biological processes and diseases. The intrinsic disorder and flexibility of IDPs grant them a number of advantages with respect to ordered proteins, such as conformational plasticity to bind several targets, a large interaction surface, involvement in high specificity/low affinity interactions, enhanced binding kinetics. It is assumed that post-translational modifications such as phosphorylation can stimulate structural rearrangement in IDPs and facilitate their binding to partners. To better understand at a structural level the multifaceted mechanisms that govern molecular recognition processes involving IDPs, we designed, synthesized by solid phase methods, and structurally characterized unstructured peptides. These molecules contain a putative disordered module, flanked at either the N- or C-terminal ends by a different phosphorylated amino acid (serine or threonine) to mimick the effects of phosphorylation. The absence of an ordered state in the designed peptides was proved experimentally by CD and NMR conformational studies that were carried out under different solution conditions.

Keywords: intrinsically disordered peptides, peptide design, Solid Phase Peptide Synthesis (SPPS), Circular Dichroism (CD), Nuclear Magnetic Resonance Spectroscopy (NMR).

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1 Introduction

Currently, intrinsically disordered proteins (IDPs) [1,2] receive great attention from the scientific community. IDPs are indeed involved in many important physiological processes related to signaling and regulation of transcription [3], moreover, they take part in several human diseases [4,5].

IDPs are natively disordered proteins that lack in whole or in part a single organized three dimensional structure [1,2]. Recent studies indicate that in eukaryotes more than a third of the proteins are provided with intrinsically disordered regions (IDRs) that are made up of at least thirty residues [2]. It is thought that disorder is actually encoded in the primary sequence and that IDPs are generally enriched in certain types of amino-acids [2,6]. Detailed studies relying on comparison of natively folded and natively unfolded proteins demonstrate that IDPs have generally a low content of "order-promoting" amino acids such as Ile, Leu, Val, Trp, Tyr, Phe, Cys, Asn, whereas, they are considerably enriched in "disorder-promoting" residues (i.e., Ala, Arg, Gly, Gln, Ser, Glu, Lys and Pro) [1]. Intrinsically disordered regions (IDRs) have in common several characteristics such as flexibility, β-sheet propensity, low average hydrophobicity and high net charge; these features are hallmarks of IDPs and have been used to develop specific predictors of disorder [1].

IDPs can be considered "promiscuous", as they can bind through several interactions, different targets [7]. Binding of IDPs to partners may result in formation of highly dynamic complexes [8-10] or be accompanied by a folding process with a generation of an ordered structure. This latter process allows IDPs to gain a particular biological function.

Interestingly, it has been reported that several peptides, such as the CCK8, to interact selectively with their receptors require an initial disordered conformation [11].

For the coupled "binding-folding" of IDPs a "fly-casting" mechanism has been proposed that is basically responsible for enhanced interaction kinetics [12,13]. In contrast to ordered proteins, the large chain flexibility in IDPs supplies a bigger capture radii; according to the "fly-casting" model, a flexible region, that represents the unfolded state, will first interact partially and weakly with its target relatively far from the binding site and then, will fold while approaching to it [13].

IDPs often contain post-translational modifications (PTM), such as phosphorylation, that can stimulate structural rearrangements. It is hypothesized that the location of PTM sites in proximity of a disordered segment may facilitate the binding of the enzyme catalyzing the PTM in several targets [1,5].

To get better understanding of the complex machineries that govern molecular recognition processes involving IDPs, we have undertaken studies of predicted unstructured peptides (See Table 1). In major detail, peptide sequences enriched in disorder promoting amino acids [1] flanked at either the N- or C-terminal end by a phosphorylated residue, were designed and synthesized by solid phase synthesis methods. In addition, the conformational behavior of the following unphosphorylated peptide sequence (IDP), NH_2-AQIREASSPSLQVDNQSDQT*g*-$CONH_2$ (*g*=Allyl-glycine), was also investigated. The absence of one single ordered conformation in these peptides was established experimentally by CD and NMR analyses that were conducted under different solution conditions (i.e.: aqueous buffer, water/trifluoroethanol (TFE) mixtures, dimethylsulfoxide (DMSO)).

These peptides may represent new disordered scaffolds which, by preserving most of the advantages of IDPs, could eventually take part in several molecular recognition processes and exert biological actions.

2 Experimental Procedures

2.1 Disorder prediction

A peptide sequence with a high disorder propensity, i.e. a region enriched in "disorder promoting residues" (A, R, Q, S, P and E) [1] was designed and checked for disorder prediction using the MeDor metaserver (http://www.vazymolo.org/MeDor/index.html) [14].

2.2 Chemicals

All solvents were reagent grade. N^{α}-Fmoc-protected amino acids and activating agents were purchased from Inbios (Pozzuoli, Italy), N^{α}-Fmoc-protected-D-phospho-Threonine (*pt*) and N^{α}-Fmoc-protected-L-phospho-Serine (*pS*) from AnaSpec, Inc. (Fremont, CA). Resin for peptides synthesis was purchased from Novabiochem (Läufelfingen, Switzerland). All other chemicals were commercially available from Sigma-Aldrich, Fluka (Buchs, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received, unless otherwise stated. HPLC chemicals were purchased from Lab-Scan (Dublin, Ireland).

2.3 Peptide synthesis

IDP-phosphopeptides and IDP-allyl-glycine (see Table 1) were synthesized in batch by using standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) procedures [15] on Rink-amide 4-methylbenzhydrylamine (MBHA) resin (0.65 mmol g^{-1}, 0.020 mmol scale). Peptide elongation was achieved by sequential addition of Fmoc-AA-OH using 1-hydroxybenzotriazole (HOBt)/ O-benzotriazole-tetramethyl-uronium-hexafluoro-phosphate (HBTU)/ diisopropylethylamine (DIPEA) (1/1/2 v/v/v) as coupling reagents, in dimethylformamide (DMF) in preactivation mode. All couplings were performed for 30 minutes, by using an excess of 5 equivalents for the single amino acid derivative. Fmoc removal was achieved by piperidine/DMF (3:7, v/v) treatment for 10 min. Peptides deprotection and cleavage from the solid support were achieved by treatment with a trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/water (95/2.5/2.5, v/v/v) mixture for 90 min at room temperature. The crude peptides were precipitated at 0 °C with ethyl ether, dissolved in a water/ acetonitrile (1:1, v/v) mixture and lyophilized. Crude products were purified by RP-HPLC chromatography. Preparative RP-HPLCs were carried out on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan)

Table 1. Peptide sequences and their molecular weight.

Peptide	Sequences	M_W (Da)
IDP1	NH_2-AQIREASSPSLQVDNQSDQ*pt*-$CONH_2$	2254.2
IDP2	NH_2-*pt*AQIREASSPSLQVDNQSDQT-$CONH_2$	2355.4
IDP3	NH_2-*pS*AQIREASSPSLQVDNQSDQT-$CONH_2$	2341.4
IDP	NH_2-AQIREASSPSLQVDNQSDQT*g*-$CONH_2$	2270.2

pt=D-phospho-Threonine; pS=L-phospho-Serine, g=Allyl-glycine

equipped with an UV lambda-Max Model 481 detector, by using a Phenomenex (Torrance, CA) -C18 Jupiter column (90 Å; 10 mm) and by using as elution solvents 0.1% TFA in water (a) and 0.1% TFA in acetonitrile (b) from 5 to 70% over 30 min (flow rate: 20 mL/min). Peptides purity and integrity were assessed by analytical LC–MS analysis with a Finnigan Surveyor MSQ single quadrupole electrospray ionization spectrometer (Finnigan/ Thermo Electron Corporation San Jose, CA), by eluting with 0.1% TFA in water (a) and 0.1% TFA in acetonitrile (b) from 5 to 70% over 15 min (0.8 mL/min). Characterization was conducted under standard conditions of peptide analysis. The expected and experimental mass of our peptides are the following: M.W. IDP1=2254.2 Da and $[M+2H^+]/2$=1128.1 m/z; M.W. IDP2=2355.4 Da and $[M+2H^+]/2$=1178.7 m/z; M.W. IDP3=2341.4 Da and $[M+2H^+]/2$=1171.7 m/z; M.W. IDP= 2270.2 Da and $[M+2H^+]/2$= 1135.1 m/z.

2.4 Circular dichroism measurements

Far-UV CD spectra of IDP1 were recorded from 190 to 260 nm on a Jasco J-810 spectropolarimeter equipped with a NesLab RTE111 thermal controller unit using a 0.1 cm quartz cell at 25 °C. Circular dichroism measurements were carried out on peptide solutions at $1{\cdot}10^{-4}$ M concentration. Solutions were prepared by promptly dissolving lyophilized pure peptide powder in net H_2O at a final pH ~ 5 (the acidic pH was due to the residual TFA used during peptide cleavage and purification). CD spectra were also recorded for an IDP1 sample dissolved in a mixture of water/trifluoroethanol (TFE) (50/50) and of water/TFE (25/75).

Other experimental settings were: scan speed, 10 nm min^{-1}; sensitivity, 50 mdeg; time constant, 4 s; bandwidth, 2 nm. Each spectrum was obtained by averaging three scans, subtracting contributions from other species in solution and converting the signal to mean residue ellipticity in units of deg cm^2 $dmol^{-1}$ res^{-1}.

2.5 Nmr measurements and solution structure calculations

NMR analysis was performed for IDP, IDP1, IDP2 and IDP3. NMR samples were prepared by dissolving about 0.5 mg of each peptide in a solution volume equal to 600 µL. NMR analysis of IDP1 was performed in H_2O containing 10% v/v D_2O (99.8% d, Armar Scientific, Switzerland) and in a mixture H_2O/trifluoroethanol-d3 (98% d, Armar Chemicals, Switzerland) 17/83 v/v. Solution conformational studies of IDP were conducted in H_2O/trifluoroethanol-d3 (98% d, Armar Chemicals, Switzerland) 17/83 v/v. NMR characterizations of IDP2 and IDP3 were carried out in DMSO-d6 (99.9% d, Armar Chemicals, Switzerland).

NMR spectra were recorded at 25 °C on a Varian Unity Inova 600 MHz spectrometer provided with a cold probe. The process of proton resonance assignments (See Supplemental Tables S1, S2, S3 and S4) was carried out with a canonical protocol [16] based on analysis of the following two dimensional [^{1}H, ^{1}H] spectra: TOCSY (Total Correlation Spectroscopy) (70 ms mixing time) [17], DQFCOSY (Double Quantum Filter Correlation Spectroscopy) [18], NOESY (Nuclear Overhauser Enhancement Spectroscopy) [19] (200 and 300 ms mixing times). Chemical shifts were referenced with respect to the TSP (Trimethylsilyl-3-propionic acid sodium salt-d4, 99% d, Armar Scientific, Switzerland) signal at 0.0 ppm.

1D spectra were acquired with a relaxation delay of 1s and 32-128 scans. 2D experiments were generally acquired with 32-64 scans, 128-256 FIDs in t_1, 1024 or 2048 data points in t_2.

The DPFGSE (Double Pulsed Field Gradient Selective Echo) sequence [20] was used to suppress water signal. Spectra were processed with the Varian software VNMRJ 1.1D (Varian by Agilent Technologies, Italy) and analyzed with the NEASY [21] program that is included in the CARA (Computer Aided Resonance Assignment) software package (http://www.nmr.ch/).

Peptide structure calculations were carried out with the software CYANA (version 2.1) [22]. A D-phospho-Threonine and L-phospho-Serine were introduced in the CYANA standard residue library [23]. Distance constraints for structure calculations were gained from NOESY experiments (300 ms mixing time). The GRIDSEARCH module of CYANA was used to generate angular constraints. Calculations started from 100 random conformers; the 20 conformers with the lowest CYANA target functions were finally checked with the program MOLMOL [24] and iCing (http://proteins.dyndns.org/cing/iCing.html) [25].

3 Results and Discussion

Four intrinsically-disordered peptides (IDP, IDP1, IDP2 and IDP3; see Table 1) were designed and analyzed. Their primary sequences include a predicted disordered region (-AQIREASSPSLQVDNQSDQ-) (See also Material and Methods for details) and a phosphorylated amino acid at C-terminus (IDP1 with phospho-Threonine) or N-terminus (IDP2 with phospho-Threonine and IDP3 with phospho-Serine, respectively) or an Allyl-glycine at the C-terminus (IDP).

The high disorder propensity of the common -AQIREASSPSLQVDNQSDQ- peptide core was evaluated

with the MeDor server [14] (Supplemental Figure S1, upper panel) while its tendency towards β-aggregation was investigated with the software TANGO [26] which indicated that its sequence is not inclined to form β-aggregates (Supplemental Figure S1, lower panel).

Conformational features of IDP, IDP1, IDP2 and IDP3 were studied in solution by CD and NMR techniques.

3.1 Circular Dichroism

CD spectra of IDP1 in water and in the water/TFE mixtures (50/50 and 25/75 v/v) were recorded between 190 and 260 nm. IDP2 and IDP3 peptide sequences were not soluble under the same experimental conditions. The shape of CD spectrum of IDP1 in water, with a negative band between 197 and 201 nm, suggests an unordered structure (see Figure 1A). The addition of TFE induces a conformational rearrangement from random coil to β-structure. This rearrangement can be better appreciated by difference spectra (Figure 1B), which show a pronounced negative maximum around 220 nm and a positive maximum around 200 nm, which are characteristic of β-sheet conformation. However, the low structural content gained by the peptide, even in TFE, supports its high flexibility. Similar CD spectra were also detected for the unphosphorylated IDP peptide at different water/TFE ratios (data not shown).

3.2 NMR studies

Detailed NMR studies of IDP, IDP1, IDP2 and IDP3, including acquisition and analysis of 1D [^{1}H] and 2D [^{1}H, ^{1}H] spectra, were conducted at 25°C.

We first investigated the conformational preferences of IDP1 in H_2O/D_2O (90/10 v/v). Under these experimental conditions, the poor spectral dispersion of the 1D [^{1}H] spectrum (Figure 2A) and the almost complete absence of signal in the 2D [^{1}H, ^{1}H] NOESY experiment indicated that IDP1 was very flexible and disordered (Supplemental Figure S2A).

Next we treated IDP1 with TFE that is a useful co-solvent to investigate the inherent conformational preferences of proteins and peptides [27] and has already been used in IDPs studies [28-30]. Since CD data indicated a gain of structure in IDP1 only at high TFE concentration, we conducted NMR characterization of the peptide in presence of 83% TFE.

A gain of ordered structure induced by TFE can already be appreciated in the H_N region of the 1D proton experiment (Figure 2A) where an improvement of the spectral dispersion is evident. Nevertheless, many cross-peaks appear in the 2D [^{1}H, ^{1}H] NOESY spectrum as well (Figure 2C) possibly indicating a decrease of flexibility in the peptide. The high quality of the spectra, recorded in presence of TFE, allowed us to carry out a complete structural characterization and obtain proton resonance assignments (Figure 2 and Supplemental Table S1). We evaluated chemical shift deviations (CSD) of Hα protons from random coil values (Supplemental Figure S3) and noticed that they did not point to any specific secondary structure element. In fact, most of the CSD were low (i.e. with absolute values lower that 0.1) and positive (positive deviations are generally indicative of extended conformations) [31]. Afterwards, we analyzed short and medium range NOEs (Figures 3A and S4). For most of IDP1

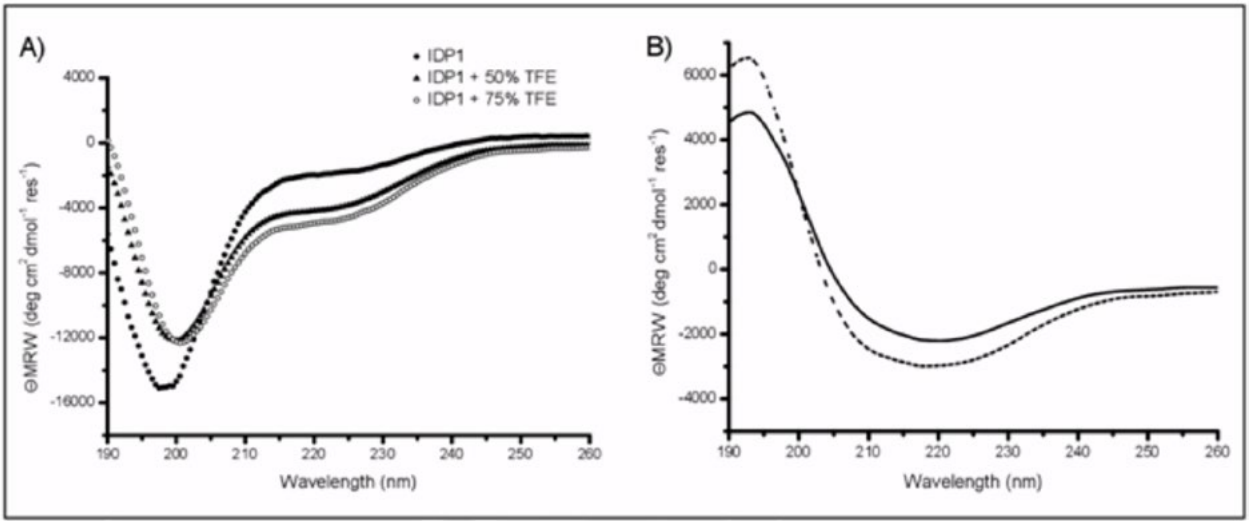

Figure 1. Far UV CD spectra of: A) IDP1 at 0, 50 and 75% of TFE and of B) IDP1 at 50% (solid line) and 75% (dash line) of TFE after subtraction of IDP1 at 0% TFE.

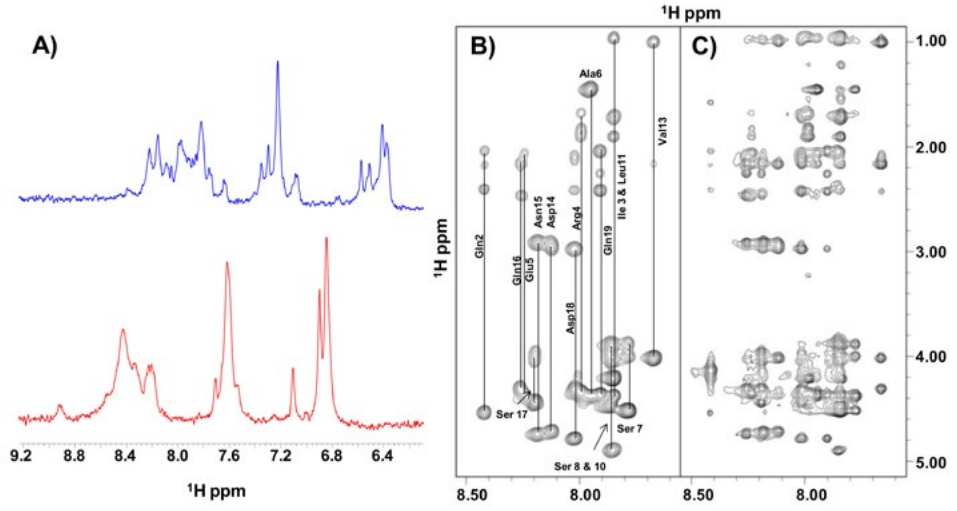

Figure 2. (A) Overlay of 1D proton spectra of IDP1 recorded in H_2O/D_2O (90/10 v/v) (red) and in H_2O/TFE-d3 (17/83 v/v) (blue). (B) 2D [^{1}H, ^{1}H] TOCSY and (C) 2D [^{1}H, ^{1}H] NOESY 300 spectra of IDP1 acquired in H_2O/TFE-d3 (17/83 v/v); the H_N/aliphatic protons correlation regions of both spectra are shown; spin system assignments are indicated in (B).

primary sequence sequential NOE contacts of the type $H\alpha_i$-HN_{i+1} (indicated as $d_{\alpha N(i,\,i+1)}$ in Fig. 3A), $H\beta_i$-HN_{i+1} ($d_{\beta N(i,\,i+1)}$ in Fig. 3A) and HN_i-HN_{i+1} ($d_{NN(i,\,i+1)}$ in Fig. 3A) are predominant and not clearly indicative of any ordered conformation [16]. Interestingly, the NOE pattern improves in the segment encompassing residues from Ser10 to Gln16, where medium range contacts typical of helical structures (i.e. $d_{\alpha\beta(i,\,i+3)}$ and $d_{\alpha N(i,\,i+3)}$) can be observed [16]. The Pro residue is mainly in trans configuration as shown by the strong NOE cross peak in between the Hα proton of Ser8 and the Hδ protons of Pro9, however, a weaker NOE between the Hα protons of Ser8 and Pro9 is evident as well and points to some proline cis-trans isomerization [16].

Indeed, structural calculations, carried out with the software CYANA [22] (See Table 2 and Figure 3), demonstrate that the peptide is rather flexible and assumes a more ordered pseudo-helical turn only between residues Val13 and Gln16 (Figure 3B,C). This small helical/turn contribution to the overall IDP1 disordered conformation in TFE cannot be appreciated by CD experiments which mainly underline the prevalence of extended structures (Figure 1).

The disorder of the peptide is represented by the high RMSD values measured for the NMR ensemble (Figure 3B and Table 2). In summary, these data, in agreement with CD results, indicate that the propensity of IDP1 to gain an ordered secondary structure is rather low even under strong structuring conditions (i.e. high percentage of TFE in solution). For comparison purpose, we also carried out similar NMR studies in aqueous solution, containing 83% TFE, of the analogue peptide: AQIREASSPSLQVDNQSDQTg (where g= Allyl-glycine) which lacks a phosphorylated residue (Supplemental Figure S5 and Table S2). This peptide exhibits identical conformational behavior as IDP1, as indicated by similar CD spectrum (data not shown) and NMR parameters such as CSD (Supplemental Figure S6) and NOE pattern (Supplemental Figure S7). These data likely demonstrate that the presence of a phosphate group does not dramatically influence the overall conformational disorder of our IDP peptides.

Attempts to carry out structural studies of IDP2 and IDP3 in water and in water/TFE mixtures were hampered by the low solubility of both peptides. Thus, we analyzed their structural features in DMSO, a solvent in which it was possible to record high quality NMR data (Figures 4 and 5) and obtain proton resonance assignments (Supplemental Tables S3 and S4). In DMSO, IDP2 and IDP3 show a very similar conformational behavior. 2D [^{1}H, ^{1}H] NOESY spectra of both peptides (Figure 4B and 5B) contain many cross-peaks and resemble typical experiments recorded for rigid folded species. Detailed analysis of NOE patterns reveals a clear prevalence of sequential contacts, such as $H\alpha_i$-HN_{i+1}, $H\beta_i$-HN_{i+1}, HN_i-HN_{i+1}, (See Figure 4C) that alone are not sufficient to indicate any specific ordered secondary structure element [16]. However, these kinds of NOEs co-exist with several medium range contacts in between side chain protons two residues apart in the sequence, and with sequential Hα-Hα contacts, the latter are concentrated in the peptide region 15-DNQSDQ-20, and are generally more characteristic of beta type structures. Moreover, in

Table 2. Structure statistics for the IDP1 NMR ensemble calculated in H_2O/TFE-d3 (17/83 v/v).

NOE upper distance limits	143
Angle constraints	88
Residual target function, Å²	0.22±0.07
Residual NOE violations	
Number > 0.1 Å#	2
Number	7
Residual angle violations	
Number	0
Atomic pairwise RMSD@, Å	
Backbone atoms (a.a. 2-19)	2.39±0.54
Heavy atoms (a.a. 2-19)	3.57±0.66

Average CYANA violation; @Calculated by iCing

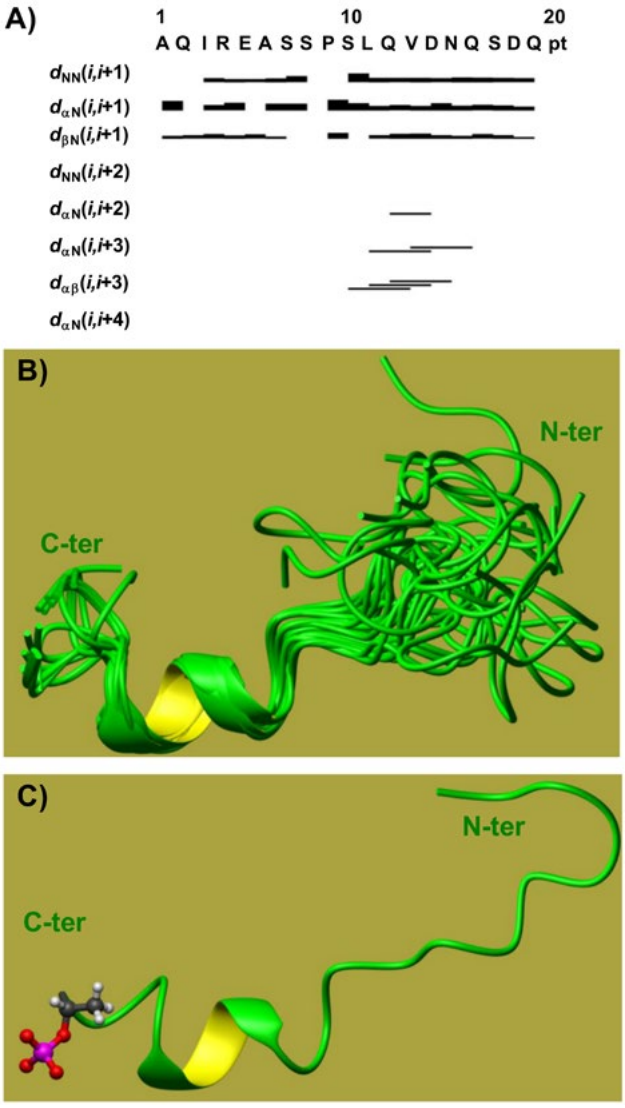

Figure 3. (A) NOE intensity pattern for IDP1 in H_2O/TFE-d3. Only principal short and medium range NOEs are reported. Different residues are specified with the one-letter amino acid code; "$d_{\xi\zeta}$ (b, c)" designates a NOE contact between the H_ξ and H_ζ protons in the b and c residues respectively. (B) Overlay on the backbone atoms (residues 12-18, RMSD=0.70 ± 0.33 Å) of 20 IDP1 structures. (C) Ribbon representation of the best IDP1 CYANA conformer (i.e. the one with the lowest target function). The D-phospho-Threonine side chain is reported in neon representation. The final CYANA calculation included 143 upl (upper distance limits) of which 60 intra-residue, 73 short- and 10 medium-range.

analogy with the IDP1 peptide, the Pro residue in IDP2 and IDP3 contributes to the overall disorder. Indeed the trans configuration is predominant but some cis-trans interconversion is evident as well [16]. Tentative complete structure calculations were carried out and demonstrated the intrinsic conformational disorder of IDP2 and IDP3 (See Figure S8). The presence of several types of NOEs in the spectra of IDP2 and IDP3 may indeed point to the occurrence of either inter-conversion of unfolded and partially ordered states or towards aggregation processes that lower the flexibility of the two peptides in DMSO. It's worth nothing that many intrinsically disordered peptides exhibit high tendency to aggregate, and in fact, under physiological conditions, are able to form beta fibrils ("amyloids") [32,33] and play roles in pathological processes like Alzheimer's and Parkinson's diseases [34-36]. Based only on the low computationally predicted tendency towards β-aggregation of the peptide core -AQIREASSPSLQVDNQSDQ- (Supplemental Figure S1, lower panel), this latest scenario appears unlikely for IDP2 and IDP3. On the other side, we cannot exclude that the addition of a phosphorylated amino acid may change the predicted aggregation propensity and/or the implemented non physiological conditions may favor peptide aggregation.

4 Conclusions

IDPs represent targets in drug discovery for several diseases (for example tumor, Parkinson's disease, Alzheimer's disease, type II diabetes) [5,6,37]. In order to create novel and efficient drug discovery strategies for such proteins, it is essential to fully unveil distinctive characteristics of intrinsically disordered regions. To this aim we designed and synthesized three intrinsically disordered peptides, made up of a predicted disordered segment flanked at either the N- and C-terminal side by a phospho-Serine or a phospho-Threonine (See Table 1). We performed NMR

and CD solution conformational studies. In particular we first analyzed the peptide IDP1, that contains a phospho-Threonine at the C-terminal end. The absence of precise secondary structure elements was proved in water and water/TFE mixtures. The addition of TFE in high percentage only slightly decreases IDP1 conformational flexibility thus stressing out the natively disordered nature of this peptide. The same conformational trend in a solution containing TFE, is exhibited by the analogue unphosphorylated IDP peptide (See Table 1); these data likely indicate that phosphorylation does not affect relevantly the structure of our peptides.

We next analyzed IDP2 and IDP3 whose primary sequences differ only in the first N-terminal residue: either a phospho-Threonine or a phospho-Serine. Solubility issues did not allow us to characterize these two peptides in aqueous buffer, we thus investigated their conformational preferences in DMSO. Under these non physiological conditions, IDP2 and IDP3 exhibited identical conformational behavior, characterized by the absence of one single ordered state and probably by aggregation phenomena.

As concerning the possible applications of the disordered IDP1, IDP2 and IDP3 peptides, we can certainly envision their use to generate novel peptide amphiphiles (PAs) to be implemented in the field of biomedicine and biocompatible materials [11,38-41].

In fact, we have very recently described novel PAs, obtained by coupling an intrinsically disordered short peptide to different alkyl-chains, that are able to self-assemble in supramolecular aggregates (i.e., unilamellar vesicles and micelles) with a consequent reduction of flexibility and a gain of structure with respect to the free peptide [41].

Moreover, we cannot exclude that our IDP1, IDP2 and IDP3 peptides, being provided with characteristic features of intrinsically disordered proteins, may take part in different protein-protein interaction networks and have a particular biological action, but of course deep studies in a cellular environment are needed to prove this scenario.

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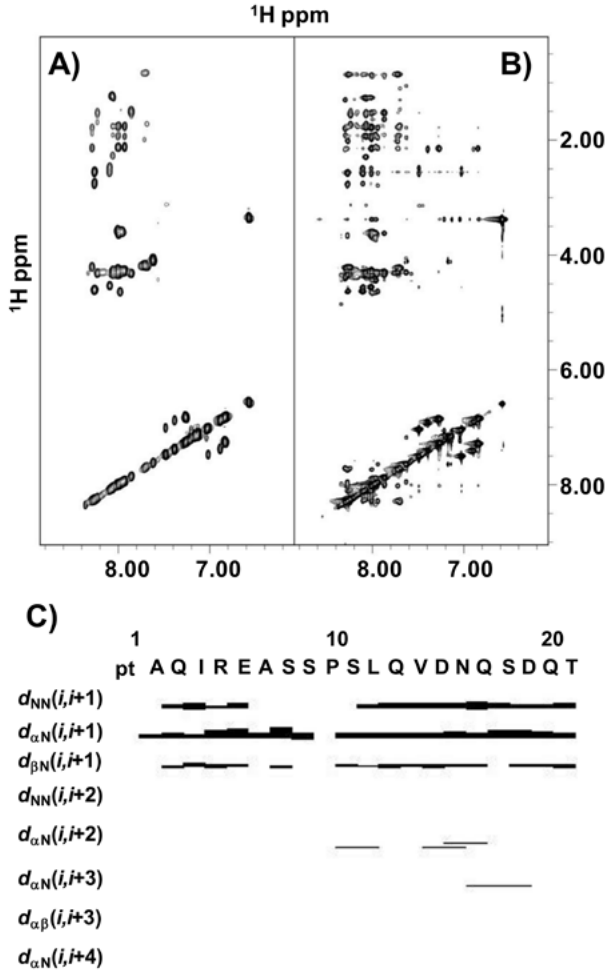

Figure 4. Comparison of (A) 2D [¹H, ¹H] TOCSY and (B) NOESY 300 spectra of IDP2 recorded in DMSO-d6. The H_N correlation regions of the spectra are shown. (C) NOE intensity pattern for IDP2.

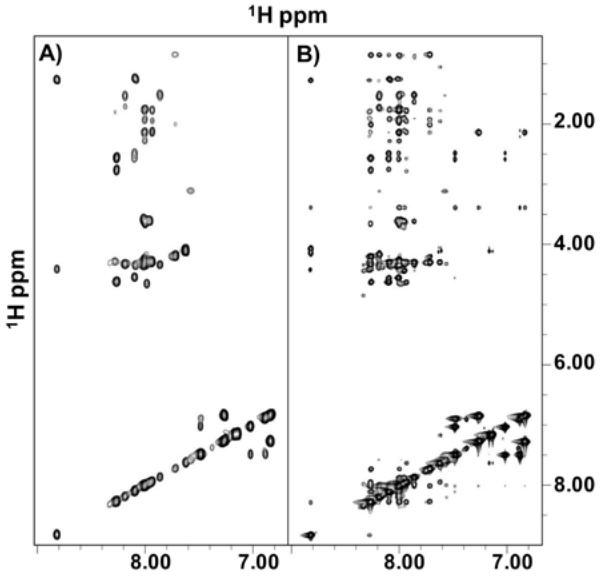

Figure 5. Expansion of a region of (A) 2D [¹H, ¹H] TOCSY and (B) 2D [¹H, ¹H] NOESY 300 of IDP3 where correlations arising from H_N protons can be observed. Spectra were acquired in DMSO-d6.

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