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Article

Wide-line NMR and DSC studies on intrinsically disordered p53 transactivation domain and its helically pre-structured segment

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Running Title : Wide-line NMR and DSC studies on p53TAD

ABSTRACT

Wide-line ^{1}H NMR intensity and differential scanning calorimetry measurements were carried out on the intrinsically disordered 73-residue full transactivation domain (TAD) of p53 tumor suppressor protein and two peptides, one a wild type p53 TAD peptide with a helix pre-structuring property and a mutant peptide with a disabled helix-forming propensity in order to characterize their water and ion binding characteristics. By quantifying the number of hydrate water molecules, we provide microscopic description for the interactions of water with a wild-type p53 TAD and two p53 TAD peptides. The results provide direct evidence that intrinsically disordered proteins (IDPs) and a less structured peptide not only have a higher hydration capacity than globular proteins but also are able to bind a larger amount of charged solute ions.

Keywords : Wide-line NMR, Differential Scanning Calorimetry (DSC), p53 Transactivation Domain (p53TAD), Hydration, Pre-Structured Motif (PreSMo)

INTRODUCTION

IDPs (intrinsically disordered proteins) are highly peculiar proteins that are devoid of tertiary structures yet are functional (1-3). These proteins have become a focus of intense research over the last decade due to their unorthodox structure-function behavior. By definition, IDPs have to contain at least one intrinsically disordered region (IDR) consisting of more than 40 amino acid residues that do not form a globular structure. The prime significance of IDPs lies in the observation that they are associated several important diseases such as cancers, mad cow diseases, viral diseases, neurodegenerative diseases to name a few (1, 3). Several disorder predictions using different bioinformatics programs have provided a consensus knowledge that IDPs occupy more than 30% of entire protein kingdom (4, 5).

The high-resolution heteronuclear multidimensional NMR spectroscopy, which has timely ripened by the mid-1990s is a robust tool that provides us with quantitative atomic-resolution structural knowledge on proteins and many other biomolecules (6, 7). During the last two decades dozens of heteronuclear NMR structural investigations have been carried out on IDPs/IDRs and revealed an interesting fact that most (at least 70%) IDPs are not just "unstructured" as one naively envisioned at the dawn of IDP research (1). It was especially noted that IDPs may be fully unfolded in a tertiary sense, but may not be fully unstructured in a secondary sense. For example, a completely unstructured (CU) state of an IDP therefore represents a structural state where an IDP is completely devoid of structures both in a tertiary and a secondary sense. On the other hand, a mostly unstructured (MU) state is a structural state of an IDP where a protein is fully unfolded only in a tertiary sense but contains transient secondary structural elements. Transient local secondary structural elements observed in target-unbound MU type IDPs "pre-exist" (8) or "pre-form" (9) and are primed for target binding. These elements are recently renamed more appropriately as pre-structured motifs (PreSMos). PreSMos are of a critical importance for our understanding of IDP-target

mechanism (1) since target binding of IDPs is expected to involve initial recognition of a PreSMo by a target followed by further structural tightening of the PreSMo into a stable secondary structure, e.g., a helix, rather than going through a full induced fit starting from a random coil.

It is of interest to explore experimental techniques other than high-resolution NMR that could distinguish the CU-type IDPs versus the MU-type IDPs. We have used two independent methods, wide-line NMR and differential scanning calorimetry (DSC), to learn that the combination of the two is an efficient tool for differentiating IDPs against globular proteins (10-12). To further test if these techniques would be able to distinguish the CU and the MU states of IDPs we carried out wide-line NMR and DSC measurements on a wild-type full 73-residue p53 TAD that contains three PreSMos, one helix (residues 18-26) and two turns (residues 40-45 and residues 49-54, respectively) (8), as well as two peptides, one wild-type helix-forming peptide and the other with helix-breaking mutations (19F→Ala, 22L→Ala, 23W→Ala, and 25L→Ala). Our aim is to gain information on the behavior of water molecules in the solutions of a wild type full p53 TAD and two "helix" peptides with and without helix-forming propensity and to characterize their structural features in terms of bound water fraction.

We use the terminology proposed by Cooke and Kuntz (14) for bound water nomination: we refer to water molecules that are in the vicinity of and interact with macromolecular surfaces and that have properties that are detectably different from those of the medium, as "bound water" and the remaining water as "bulk water". By the term "unfrozen water", we denote the actual fraction of water molecules in a mobile state at a given temperature. The unfrozen water term can therefore refer to a phase composed of either bound water molecules only or bound plus bulk water molecules. Here we aim to characterize the hydration/solvation properties of the intrinsically disordered full p53 TAD and its peptides

from proton wide-line NMR intensity and DSC measurements by taking the results of a globular protein, BSA, as a reference. Our results confirm that p53 TAD and a less structured peptide have a higher hydration potential, and also reveal an unexpected fact that p53 TAD has a large capacity and broad specificity for ion binding.

EXPERIMENTAL PROCEDURES

Protein and peptide preparation

The full-length p53 TAD (1-73) was expressed and purified as has been described previously (7). The p53 helix peptide (15-29) and its mutant peptide with helix-breaking mutations (19F→Ala, 22L→Ala, 23W→Ala, and 25L→Ala) were synthesized by a solid phase method with Multiple Peptide Synthesizer APEX 348Ω (Advanced Chemtech). Synthesized p53 TAD peptides have the following amino acid sequences; a wild type peptide = (SQETFSDLWKLLPEN-NH_2) and a mutant peptide=(SQETASDAAKLAPEN-NH_2). The C-termini of all the synthesized peptides were amidated. The peptides were purified by HPLC using Vydac C_{18} columns and their masses confirmed by MALDI-TOF mass spectrometry.

Wide-line NMR

The aqueous protein solutions were prepared by dissolving the proper amounts of bovine serum albumin (BSA, Sigma) or p53TAD, and p53 helices in double-distilled water. Detailed descriptions of the applied NMR methods have been reported (12, 14). Shortly, the intensity of the NMR signal is measured as the amplitude of free induction decay (FID) signal extrapolated to $t = 0$. The extrapolated amplitude is proportional to the number of contributing nuclei. In case of a multi-fraction system, the number of protons as existing in a given phase can be measured, especially protons in a mobile state. The measurements were done on cooled and reheated samples in the temperature range of -70°C to +30°C. All the protons are in a mobile state (water molecules in liquid state) above 0°C and we normalize the NMR intensities to this value accordingly. The effect of freezing on the protein solutions was controlled by the comparison of NMR parameters obtained before and after a freeze-thaw cycle at temperatures above 0°C. We found that the freeze-thaw cycle caused no observable

changes for the studied protein solutions as far as the measured NMR parameters are concerned.

The temperature was controlled by an open-cycle Janis cryostat with an uncertainty better than ±1 K. ^{1}H NMR measurements and data acquisition were accomplished by a Bruker AVANCE III NMR spectrometer at the frequency of 82.4 MHz with a stability of better than $\pm 10^{-6}$. The data points in the figures are based on spectra recorded by averaging signals to reach a signal to noise ratio better than 50. We varied the number of averaged NMR signals to achieve the desired signal quantity for each samples and unfrozen water quantities. The extrapolation to zero time was done by fitting a stretched exponential.

Differential scanning calorimetry (DSC)

Double-distilled water was measured as a starting material to obtain calibration data and parameters for the temperature correction. 10 μl of liquid was used in DSC measurements and 70 μl in NMR. The DSC results presented here were temperature corrected, the details of which and the enthalpy calculations are given elsewhere (15, 16). The DSC measurements were carried out at heating rate of q = 2 K min^{-1} on a home-built heat-flux differential scanning calorimeter (DSC). Prior the experiment the samples were cooled down with the same rate of 2 K min^{-1}.

RESULTS

Wide-line ^{1}H-NMR measurements can give information about the hydration of a protein, which can be related to the level of order of a disordered protein (10-12). The wide-line ^{1}H-NMR signal intensity is the measure of the number of mobile protons or equivalently, the number of mobile water molecules, which is expressed as hydration (*h*, gram water/gram protein) (see Figure 1 for example). The slopes of hydration *vs.* temperature curves give a quantitative parameter characteristic of the energetic heterogeneity of protein–water interactions. The more steeply changes the hydration with temperature, the wider are the energy distributions of these interactions. The interactions of the IDPs with water molecules are characterized with broad energy distributions, but for the globular proteins, these energies fall in a much narrower range (17, 18).

Full p53 TAD dissolved in water, wide-line ^{1}H NMR

The hydration curve or melting diagram measured for the full p53 TAD dissolved in water (Fig. 1A) shows a thermal trend and hydration values that are typical of the IDPs (see e.g. refs. 6, 8). Mobile hydrogen atoms i.e., reorienting water molecules were detected first at -43°C, at a much higher temperature than the -60°C for the globular BSA (Fig. 1A) even though the amount of this mobile water for the full p53 TAD (h = 0.45) was similar to the hydration measured for the globular protein BSA (h = 0.50 at -44 °C). The hydration curve for the full p53 TAD shows continuously growing number of mobile water molecules with increasing temperature, in contrast to the quasi constant hydration of BSA between -60°C and -5°C. The low-temperature step in the hydration curve appears when detectable molecular motion becomes active (Fig. 1A). The lower this 'melting' temperature, the weaker the interaction is between the water molecules and the protein compared to the water-water interaction in the

bulk solvent (7). Thus our results indicate that water-protein interaction for water-BSA is weaker than for water-p53 TAD.

Helices dissolved in water, wide-line ^{1}H NMR

The helix forming peptide (15-29) and its mutant (F19A, L22A, W23A, L26A) bind much lower amount of water (Fig. 1B) than the full p53 TAD, their hydrations being about the third or a half, respectively, of the hydration of the full p53 TAD (Fig. 1A). The hydration values measured for the peptides at -5°C are shown as filled circles (red and green) in Fig. 1A for comparison. The bound water molecules start to rotate for both peptides at -43°C, which is the same temperature value where the full p53 TAD begins to rotate. The melting diagrams of both peptides are very similar up to -20 °C. The hydration of the wild type peptide grows gradually with increasing temperature at a considerably slower pace above -20 °C than that of the mutant peptide. The hydration curve of the mutant peptide shows a very intensively enhancing region up to 0°C.

Full p53 TAD dissolved in buffer of 150 mM NaCl, wide-line ^{1}H NMR

In general, the hydration of the proteins dissolved in buffer solution is much higher than that in pure water as solvent (compare the ordinates in Figs. 1 & 2). The hydration curve of the full p53 TAD in buffer shows a 'hydration water melting' at the same temperature similar to the one measured in salt-free solution. In Fig. 2A the hydration values of the full p53 TAD measured between -43°C and -28°C are nearly the same (the curves are essentially superimposable) as that of BSA, although it becomes much greater at higher temperatures, i.e., above -28°C than that of BSA. This result suggests that p53 TAD has two temperature regions (-43°C and -28°C and above -28°C up to 0°C) which interact with the solute ions. The lower-temperature melting diagram for the range of -43°C and -28°C reflects a salt binding behavior

like a globular reference protein BSA, while the higher-temperature diagram for the range of above -28°C up to 0°C indicates a salt binding behavior like an IDP.

Helices dissolved in buffer of 150 mM NaCl, wide-line ^{1}H NMR

The hydration curves in the salty buffer solution both for the wild type and the mutant peptide are essentially very similar to each other (the melting curves are superimposable) but measurable differences are visible in the temperature range of -27°C ~ -5°C (Fig. 2B).

Differential scanning calorimetry

We have demonstrated previously that DSC experiments can provide information on the protein-ion interactions or the salt binding properties of biopolymers [ref: PMID: 16798808]. The proteins dissolved in a NaCl containing solvent, can bind ions to an extent determined by the protein properties. The presence of 'free' sodium and chloride ions can be detected by making use of the eutectic nature of the NaCl-H_2O system. The eutectic phase formed from the free salt ions melts at -21°C and produces an endotherm peak in the DSC curve. Fig. 3 shows that the DSC curves for pure water and for the full p53 TAD in two concentrations (25 mg/cm^3 and 12.5 mg/cm^3) dissolved in the buffer solution of 150 mM NaCl are clearly different. The low protein concentration was not sufficient to bind all salt ions present and the thermal effect of the eutectic melting was visible in the DSC curve (red line in Fig. 3). It was shown earlier that the enthalpy changes caused by a phase transition could be studied as a function of the protein concentration in order to get information on the salt binding capacities of the proteins (Fig. 4). We found that the mutant peptide bound approximately two times more salt (12.5 mg/cm^3 mutant peptide bound 150 mM NaCl) than p53 TAD (~20 mg/cm^3 protein bound 150 mM NaCl) or the wild type peptide (21.9 mg/cm^3 peptide bound 150 mM

NaCl). As it can be seen, the wild type peptide bound the least amount of salt; the full p53 TAD somewhat more salt, and the mutant peptide bound quite a large amount of NaCl.

DISCUSSION AND CONCLUSIONS

The strengths of the water-protein interactions for IDPs vary in a narrower range, and are nearer to the bulk-phase water-water interactions than that for globular proteins e.g. BSA. The "surface" of IDPs is more open to the water, and the amount of the bound water molecules involved is much greater than in the case of globular proteins. The number of the interacting protein-water connections could be quantified and the differences found are apparent for p53 TAD and the peptides (Figs. 1 and 2). The wild-type p53 TAD has highly disordered structure compared to BSA, a globular-protein reference. The degree of disorder (being less structured) known at the time when we set up this investigation increases in the following order: BSA < wild type full p53 TAD < wild type p53 TAD helix peptide < mutant p53 TAD peptide. Our results agree with this order. The DSC measurements inform about the interactions of the proteins with the other solutes, especially with sodium and chloride ions. The number of the bound ions can be calculated from the experimental DSC curves. The full p53 TAD and the peptides bound a considerably greater amount of NaCl than the globular BSA in accordance with the earlier results. Based on the current results we conclude that both of the applied methods, wide-line ^{1}H-NMR and DSC, are not only capable of distinguishing IDPs against globular proteins but also are able to differentiate two structural states of IDPs, MU (wild type p53 TAD peptide containing a helix PreSMo) and CU (mutant p53 TAD peptide with helix-breaking mutations) in terms of their interactions with water molecules or other chemical entities e.g. salt ions.

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FIGURE LEGENDS

Figure 1. Hydration measured by the ^{1}H-NMR signal intensities of the mobile water for (A) 50 mg/ml p53 TAD (blue circles) and 50 mg/ml BSA (black circles) dissolved in water. The *T* = -4°C data for the helices (green and red circles) are the same as in graph (B). (B) 30 mg/ml p53 TAD helix (green circles) and 30 mg/ml mutant helix (red circles) dissolved in water. The lines are guides to the eye.

Figure 2. Hydration measured by the ^{1}H-NMR signal intensities of the mobile water for (A) 50 mg/ml p53 TAD (blue circles) and 50 mg/ml BSA (black circles) dissolved in Tris-buffer. The *T* = -4°C data for the helix peptides (green and red circles) are the same as in graph (B). (B) 30 mg/ml wild type p53 TAD helix peptide (green circles) and 30 mg/ml mutant helix peptide (red circles) dissolved in Tris-buffer; or alternatively, the hydration given in units of mole mobile water per mole peptide. Tris-buffer: 150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH = 7.5. The lines are guides to the eye.

Figure 3. DSC curves measured in double-distilled water (black line), wild type helix peptide (residues 15-29 of p53 TAD) of concentrations 25 mg/cm^3 (blue line) and 12.5 mg/cm^3 (red line) dissolved in Tris-buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH = 7.5). The endotherm peak at -25°C (red line) represents the melting of the eutectic phase formed by that fraction of the NaCl which was not bound by the peptide.

Figure 4. Enthalpy changes associated with the eutectic melting of the H_2O-NaCl system measured as a function of protein concentration for full p53 TAD (black squares), wild type p53 TAD helix peptide (blue circles) and mutant peptide (red triangles) dissolved in the buffer

solution of 150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH = 7.5. The lines are guides to the eye.

FIGURES

Figure 1

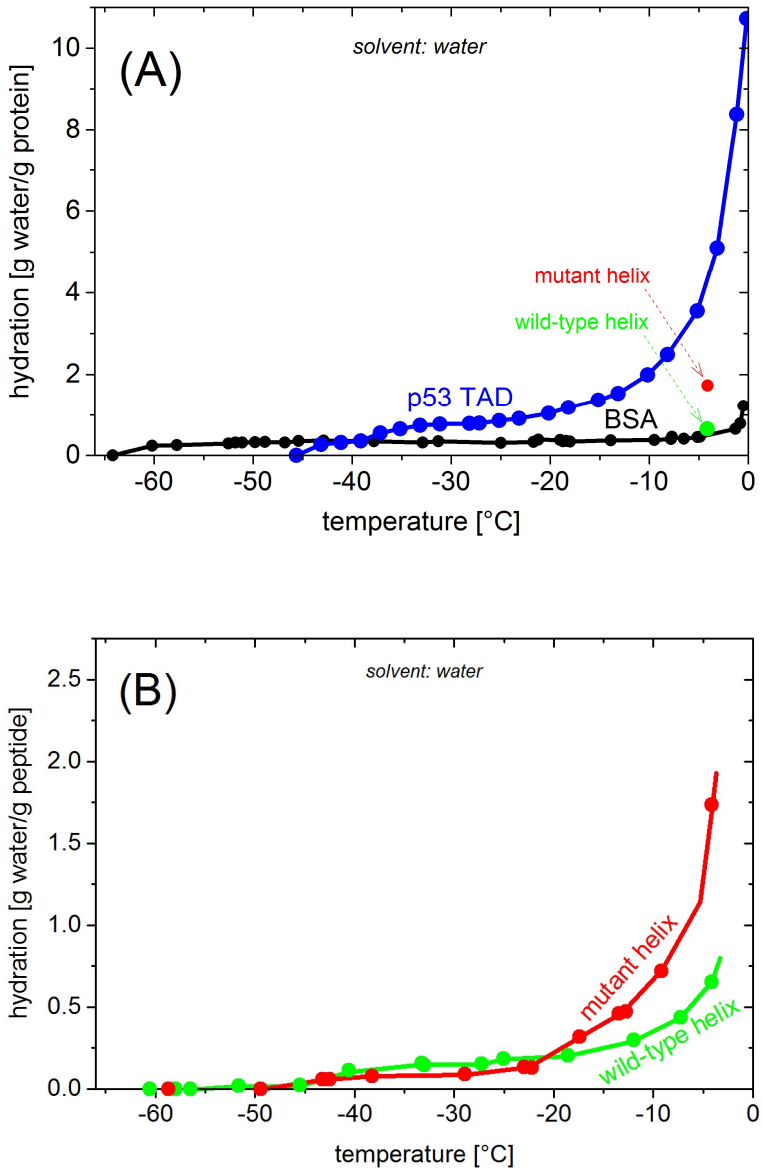
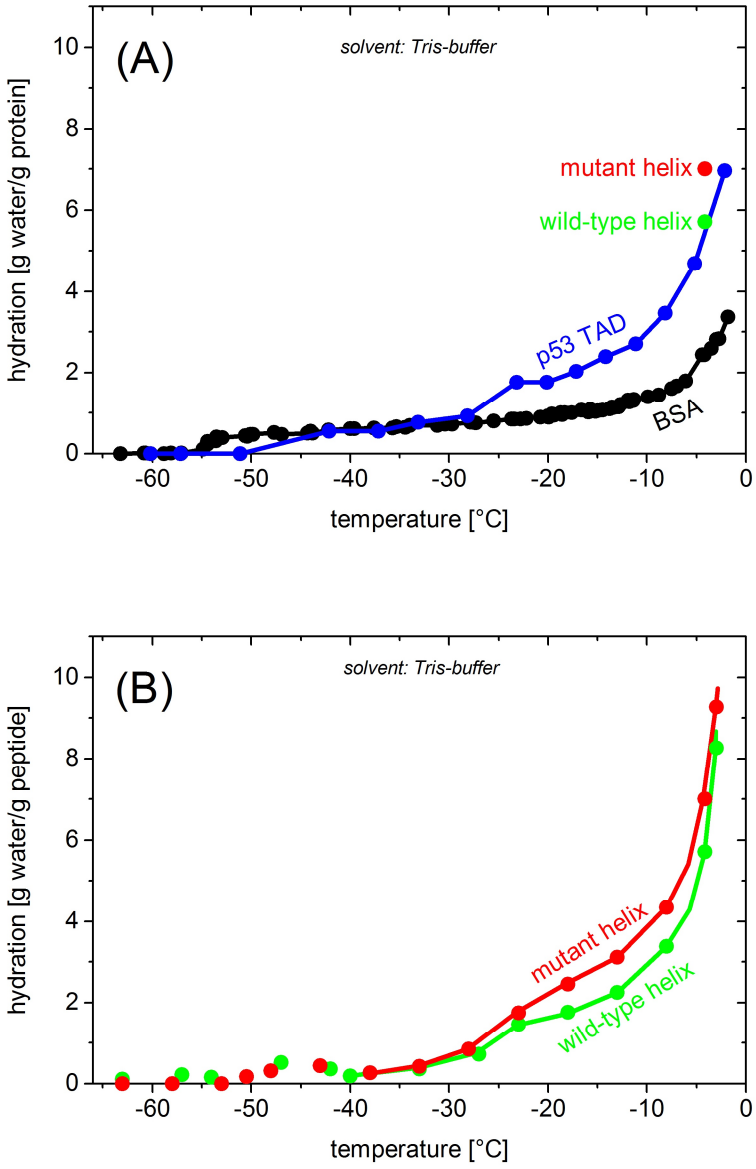


Figure 2


(A)
solvent: Tris-buffer
mutant helix
wild-type helix
p53 TAD
BSA
hydration [g water/g protein]
temperature [°C]
(B)
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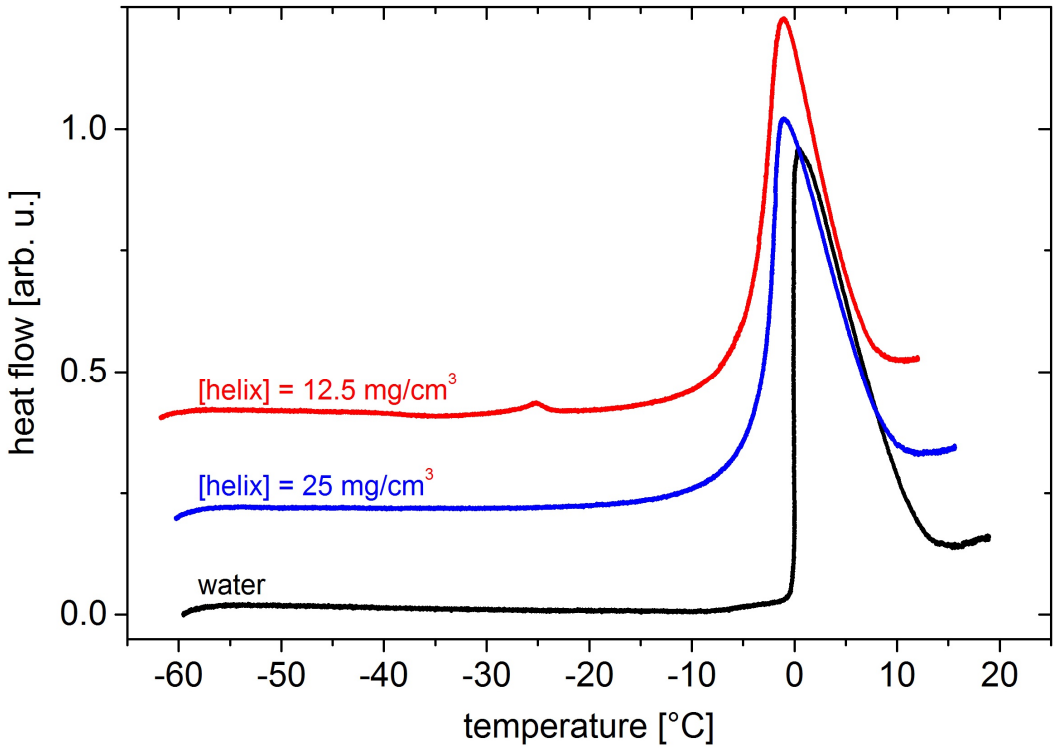
mutant helix
wild-type helix
hydration [g water/g peptide]
temperature [°C]

Figure 3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[helix] = 12.5 mg/cm3
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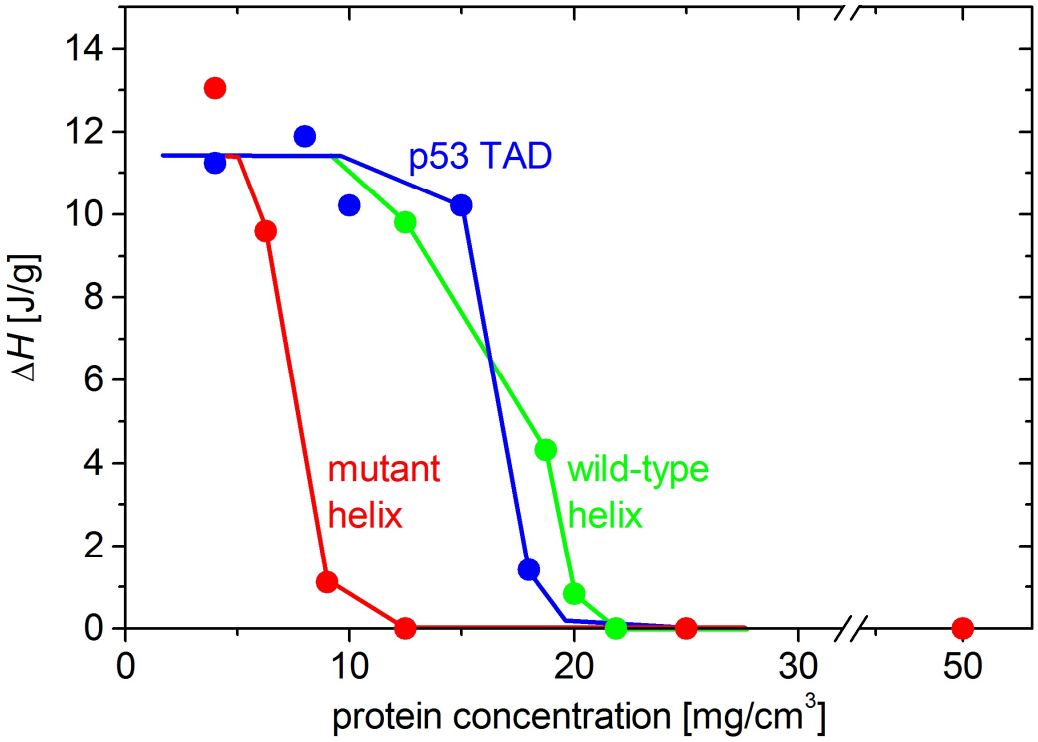
temperature [°C]

Figure 4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protein concentration [mg/cm3]
p53 TAD
mutant
helix
wild-type
helix