J. Biochem. 2010;147(2):273–278 doi:10.1093/jb/mvp178

JB THE JOURNAL OF BIOCHEMISTRY

The effect of PKA-phosphorylation on the structure of inhibitor-1 studied by NMR spectroscopy

Received July 6, 2009; accepted October 5, 2009; published online November 3, 2009

Yi-Choang Huang[1,2,3], Yi-Chen Chen[4], Huey-Jen Tsay[5], Chia-lin Chyan[6], Chun-Yu Chen[7], Hsien-bin Huang[7,*] and Ta-Hsien Lin[1,2,3,†]

[1]Institute of Biochemistry and Molecular Biology; [2]Structural Biology Program, National Yang-Ming University; [3]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 112; [4]Institute of Medical Science, Tzu Chi University, Hualien 970; [5]Institute of Neuroscience, National Yang-Ming University, Taipei 112; [6]Department of Chemistry, National Dong Hwa University, Hualien 974; and [7]Department of Life Science and Institute of Molecular Biology, National Chung Cheng University, Chia-Yi 621, Taiwan, Republic of China

*Hsien-bin Huang, Department of Life Science and Institute of Molecular Biology, National Chung Cheng University, Chia-Yi 621, Taiwan, Republic of China, Fax: +886-5-2722871, E-mail: biohbh@ccu.edu.tw

†Ta-Hsien Lin, Department of Medical Research & Education, Taipei Veterans General Hospital, Taipei 112, Taiwan, Republic of China; Fax: +886-2-8751562; Email: thlin@vghtpe.gov.tw

Inhibitor-1 is an acid- and heat-stable protein. It can be turned into a potent inhibitor of protein phosphatase-1 (PP1) after phosphorylation at Thr35 by c-AMP-dependent protein kinase (PKA). Although it has been known that pre-phosphorylation is essential for inhibition of PP1, the structure–function relationship of Thr^{35}-phosphorylated inhibitor-1, such as whether or not PKA-phosphorylation pre-triggers conformational changes in inhibitor-1, remains unclear. In this study, we performed structural characterization of Thr^{35}-phosphoroylated inhibitor-1 by using multi-dimensional heternuclear NMR spectroscopy. The result of structural comparison between Thr^{35}-phosphoroylated and non-phosphorylated inhibitor-1 indicated that PKA-phosphorylation has no significant effect on the global conformation of free-state inhibitor-1. This finding may support the inference that regulation of the interactions between inhibitor-1 and PP1 through PKA-phosphorylation mainly depends on the phosphate group instead of phosphorylation-induced conformational change.

Keywords: Inhibitor-1/phosphorylation/protein phosphatase-1/PKA/NMR.

Abbreviations: PP1, protein phosphatase-1; PKA, cAMP-dependent protein kinase; DARPP-32, dopamine and cAMP-regulated phosphoprotein, Mr. 32.000; NMR, nuclear magnetic resonance; CSI, chemical shift index.

Protein phosphatase-1 (PP1) is one of the major serine/threonine eukaryotic protein phosphatases. The catalytic subunit of PP1 in cells is associated with different binding proteins to form a variety of holoenzymes. These binding proteins target the enzyme to specific subcellular compartments in which PP1 regulates the functions of its substrates, accounting for PP1 that can modulate the diverse cellular functions, including carbohydrate metabolism, transcription, muscle contraction, neuronal signaling, protein synthesis, cardiac function and cell cycle (*1–4*). PP1 is specifically inhibited by three acid- and heat-stable protein inhibitors, including inhibitor-1, DARPP-32 and inhibitor-2. PP1 is only inhibited by inhibitor-1 and DARPP-32 when both inhibitors are pre-phosphorylated by c-AMP-dependent protein kinase (PKA); by contrast, PP1 is inhibited by inhibitor-2 without pre-phosphorylation. Inhibitor-1 and DARPP-32 share a remarkable identity in the sequence of NH_2-terminal region between residues 6 and 38 (*5*, *6*). This region containing one consensus PP1-binding motif and a PKA-phosphorylation site is required in inhibition of PP1 (*7–11*). The PKA-phosphorylation sites of inhibitor-1 and DARPP-32 are at Thr35 and Thr34, respectively. In contrast, the C-terminal sequences of inhibitor-1 and DARPP-32 are highly distinct.

Previously, we have characterized the structures of inhibitor-1 (*12*), DARPP-32 (*13*) and inhibitor-2 (*14*) by using NMR spectroscopy. These PP1 protein inhibitors share a common structural feature. They all exhibit a high propensity of random coiled structure. These intrinsically unstructured proteins may contain more flexible conformation and have an advantage to achieve the long-range and complicated interactions with PP1 (*15–17*). This structural characteristic may also explain why these PP1 protein inhibitors are stable to heat treatment and very sensitive to protease digestion. From the biochemical point of view, the inhibition of PP1 by inhibitor-1 is regulated through PKA-phosphorylation of inhibitor-1. From the structural point of view, it may be regulated by way of PKA-phosphorylation-induced conformational changes in inhibitor-1. Moreover, the binding of Thr^{35}-phosphorylated inhibitor-1 to PP1 may also induce conformational changes. The relationship between structure and function of Thr^{35}-phosphorylated inhibitor-1 remains unclear. In the present study, we focused on the effect of PKA-phosphorylation on the structure of intact inhibitor-1. To investigate this issue, we prepared Thr^{35}-phosphorylated inhibitor-1 and characterized its structure by nuclear magnetic resonance (NMR). Our results suggest that PKA-phosphorylation does not change the structure of free-state inhibitor-1.

Materials and methods

Proteins and reagents

$^{15}NH_4Cl$ and ^{13}C-glucose were purchased from Cambridge Isotope Laboratories. The recombinant stable isotope labeled inhibitor-1 and Thr^{35}-phosphorylated inhbitor-1 were prepared as described (*12*).

NMR Spectroscopy

Samples for NMR experiments contained 25 mM sodium phosphate buffers, pH 6.0, 0.02% NaN_3, 0.5 mM protein in 90% H_2O/10% D_2O. DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) was used as internal chemical shift standard (*18*). The final protein sample solutions were transferred to 5 mm Shigemi NMR tubes (Shigemi Co.) for recording NMR spectra. All NMR experiments were performed at 293 K on Bruker AVANCE-500 spectrometer equipped with a 5 mm inverse triple resonance ($^1H/^{13}C$/BB), *Z*-axis gradient probe or AVANCE-600 spectrometer equipped with a 5 mm inverse triple resonance ($^1H/^{13}C/^{15}N$), *XYZ*-axis gradient probe. All spectra were processed using the program TopSpin and analyzed using AURELIA (Bruker) on an SGI workstation. Linear prediction was used in the indirectly detected dimensions to improve the digital resolution. ^1H Chemical shifts were referenced to the ^1H frequency of the methyl resonances of DSS at 0 ppm. The ^{15}N and ^{13}C chemical shifts were indirectly referenced using the following consensus Ξ ratios of the zero-point frequencies: 0.101329118 for $^{15}N/^1H$ and 0.251449530 for $^{13}C/^1H$ (*18*). Resonance assignments were accomplished by using the following heteronuclear 3D spectra: HNCO, HN(CA)CO, HNCA, HN(CO)CA, CBCANH, CBCA(CO)NH, HBHA(CBCACO)NH, TOCSY-HSQC.

CD Spectroscopy

Circular dichroism spectra were recorded using a AVIV Circular Dichroism spectrometer Model 410. All measurements were performed in quartz cells with pathlength of 0.1 cm at protein concentration of 95 μM. Data were collected in the wavelength from 190 to 260 nm at 0.2 nm increments. Each CD spectrum reported is the average obtained from three repeated measurements of each sample. All measurements were carried out at 25.0 ± 0.1°C.

Results

Structural comparison of non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 is a key step to elucidate the effect of PKA-phosporylation on the structure of inhibitor-1. Therefore, we prepared non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 for structural studies using heteronuclear NMR spectroscopy. Figure 1A shows the SDS–PAGE of non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1, and result indicates that the purity of both proteins after three steps of purification is >95% homogeneity. The purified proteins were further verified for the phosphorylation of inhibitor-1 using the mass spectrometry. An overlay of the mass spectra of non-phosphorylated (light gray) and Thr^{35}-phosphorylated (black) inhibitor-1 is presented in Fig. 1B. As shown in Fig. 1B, the molecular weight difference between the non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 is ~80 Da, indicating that a PO_3 group is attached to the non-phosphorylated inhibitor-1.

The purified proteins of both non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 were used for further structural characterization by applying multi-dimensional heteronuclear NMR spectroscopy. Figure 2A shows the two-dimensional ^1H-^{15}N-HSQC spectrum of Thr^{35}-phosphorylated inhibitor-1 obtained at 500 MHz NMR spectrometer. It can be seen that ~100% of amide proton and ^{15}N backbone resonance was assigned for Thr^{35}-phosphorylated inhibitor-1. There is little dispersion of the cross peaks in the two-dimensional ^1H-^{15}N-HSQC spectrum of Thr^{35}-phosphorylated inhibitor-1. Except of the amino acid at the C-terminus and phospho-Thr35 of the protein, the dispersion of amide proton chemical shift is ~0.7 ppm. The ^{15}N chemical shifts of some residues in Thr^{35}-phosphorylated inhibitor-1 are clustered in a manner that is amino acid type specific. For example, the ^{15}N chemical shifts of glycine residues are clustered ~110 ppm, and those of serine and threonine residues are clustered in the range between 113 and 118 ppm. A similar observation has been obtained for

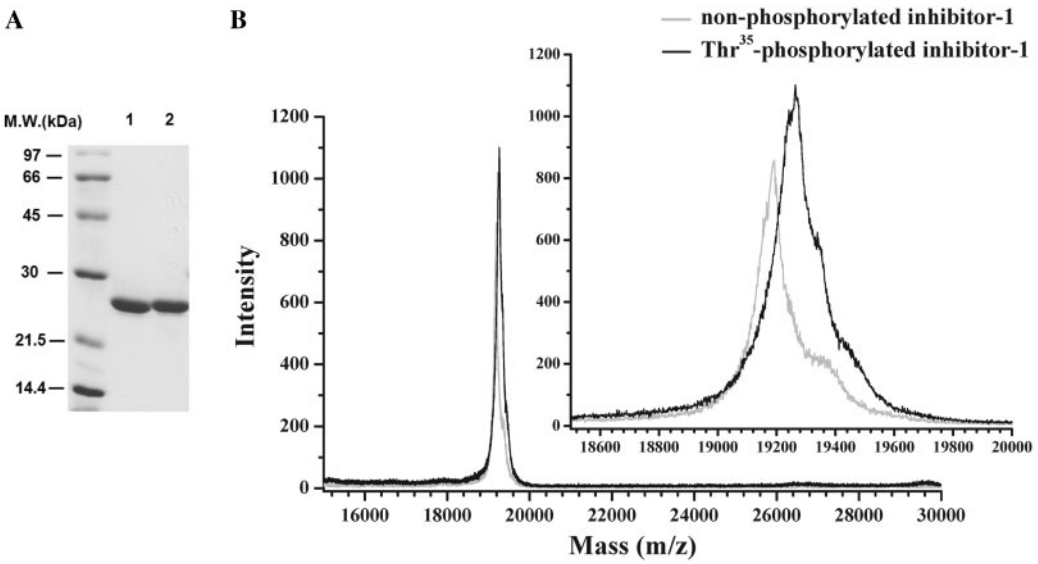

Fig. 1 (A) SDS–PAGE of non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1. Molecular weight markers are phosphorylase b (97 kDa), bovine serum albumin (76 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). (B) Overlay of mass spectra of non-phosphorylated (light gray) and Thr^{35}-phosphorylated (black) ^{15}N-enriched inhibitor-1. The calculated mass of non-phosphorylated ^{15}N-enriched inhibitor-1 is 19 181.4 Da.

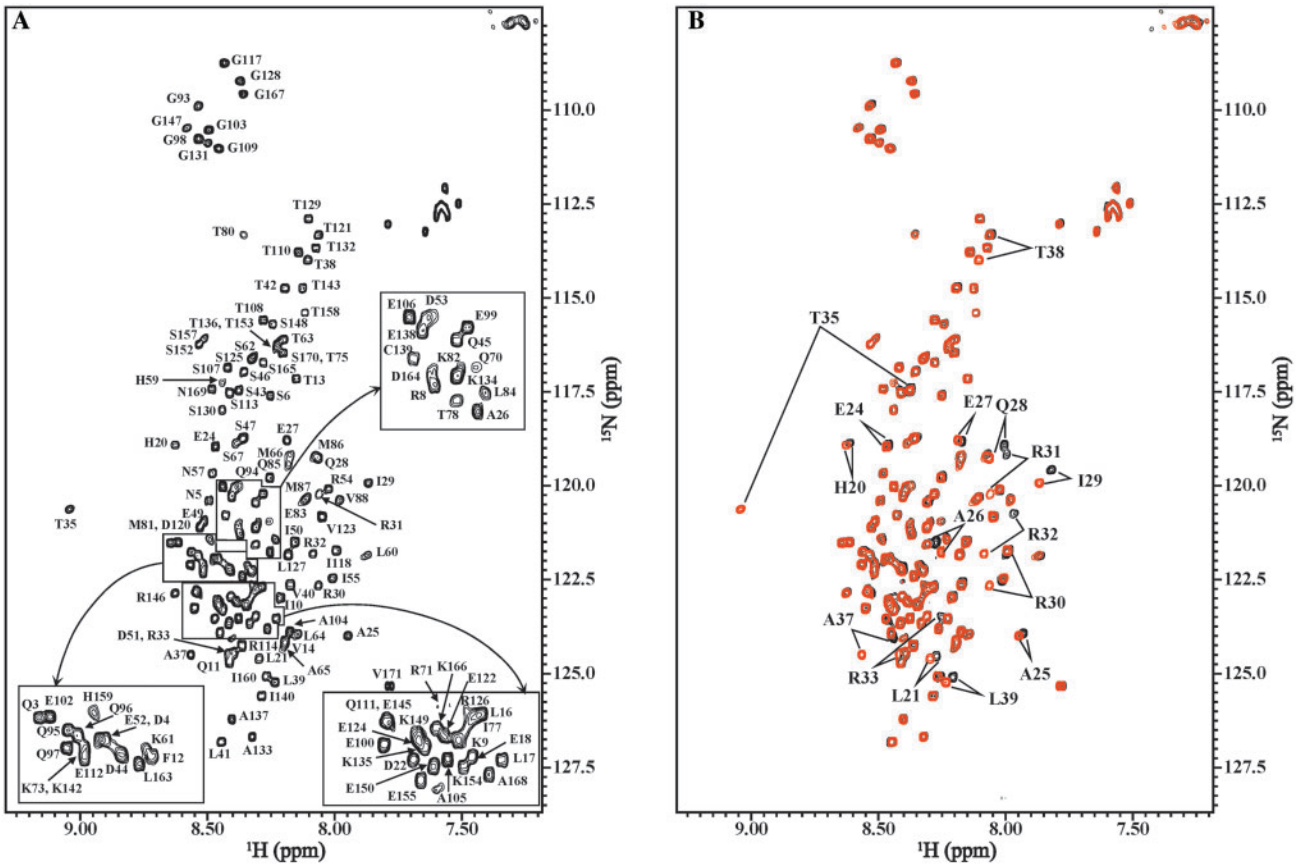

Fig. 2 (A) Two-dimensional ^{1}H-^{15}N-HSQC spectrum of Thr^{35}-phosphorylated inhibitor-1 obtained at 500 MHz NMR spectrometer. The assignments of the backbone amide proton and ^{15}N cross peaks are shown in the figure. The expanded region indicated by arrow is for the purpose of clarity. (B) Overlay of 2D ^{1}H-^{15}N-HSQC spectra of non-phosphorylated (black) and Thr^{35}-phosphorylated (red) inhibitor-1. Residues with significant chemical shift changes are labeled. Both spectra were collected under the same sample conditions. (C) The H^{α}, C^{α}, C^{β}, C' secondary chemical shifts ($\Delta\delta$) and consensus chemical shift index (CSI) for residues 20–40 of non-phosphorylated (light gray) and Thr^{35}-phosphorylated (black) inhibitor-1. The secondary chemical shift was calculated by subtracting the chemical shift of each amino acid residue in a random coil conformation (δ_{rc}) from the observed chemical shift (δ_{obs}). (D) Overlay of the CD spectra of non-phosphorylated (black) and Thr^{35}-phosphorylated (red) inhibitor-1.

non-phosphorylated inhibitor-1 (*12*), suggesting that Thr^{35}-phosphorylated inhibitor-1 may also adopt a predominantly random coil conformation and the phosporylation of inhibitor-1 at Thr35 by PKA is unlikely to induce any dramatic change of inhibitor-1 tertiary folding.

Further comparison of two-dimensional ^{1}H-^{15}N HSQC spectra for non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 is shown in Fig. 2B. It is apparent that the chemical shifts of backbone amide protons and ^{15}N resonances for most of residues of inhibito-1 remain unchanged before and after phosphorylation by PKA. Result shows that most conformations of inhibitor-1 backbone are not affected by PKA-phosporylation. The most significant change of chemical shift for the amide proton and ^{15}N cross peak was the Thr35 that shifts to the downfield as phosphorylated by PKA. Besides, a couple of residues with significant chemical shift changes were readily identified by comparison with the backbone amide proton and ^{15}N chemical shifts of non-phosphorylated inhibitor-1 (*12*). These residues are His20, Leu21, Glu24, Ala25, Ala26, Glu27, Gln28, Ile29, Arg30, Arg31, Arg32, Arg33, Ala37, Thr38 and Leu39. These residues with significant changes of chemical shift are distributed mainly in the primary sequence and a consecutive manner instead of a random manner, suggesting that the chemical shift changes of these residues may not solely result from the electrostatic effect of the phosphate group. If the PKA-phosphorylation induces conformational change in this region, it could also result in chemical shift changes of these residues.

To elucidate the effect of PKA-phosphorylation on local conformation of inhibitor-1, analyses of the secondary chemical shifts and consensus chemical shift index (CSI) (*19*, *20*) of these residues were performed. Figure 2C depicts the histograms of $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ secondary chemical shifts and consensus CSI index for residues 20–40 of non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1. According to the CSI index, the non-phosphorylated inhibitor-1 contains a short α-helix spanning residues 24–30 and adopts a random coil conformation for the rest, which is similar to those characterized in the previous study (*12*). From the sign and magnitude of the secondary chemical shifts, the structural features for residue 20–40 of Thr^{35}-phosphorylated inhibitor-1 are very

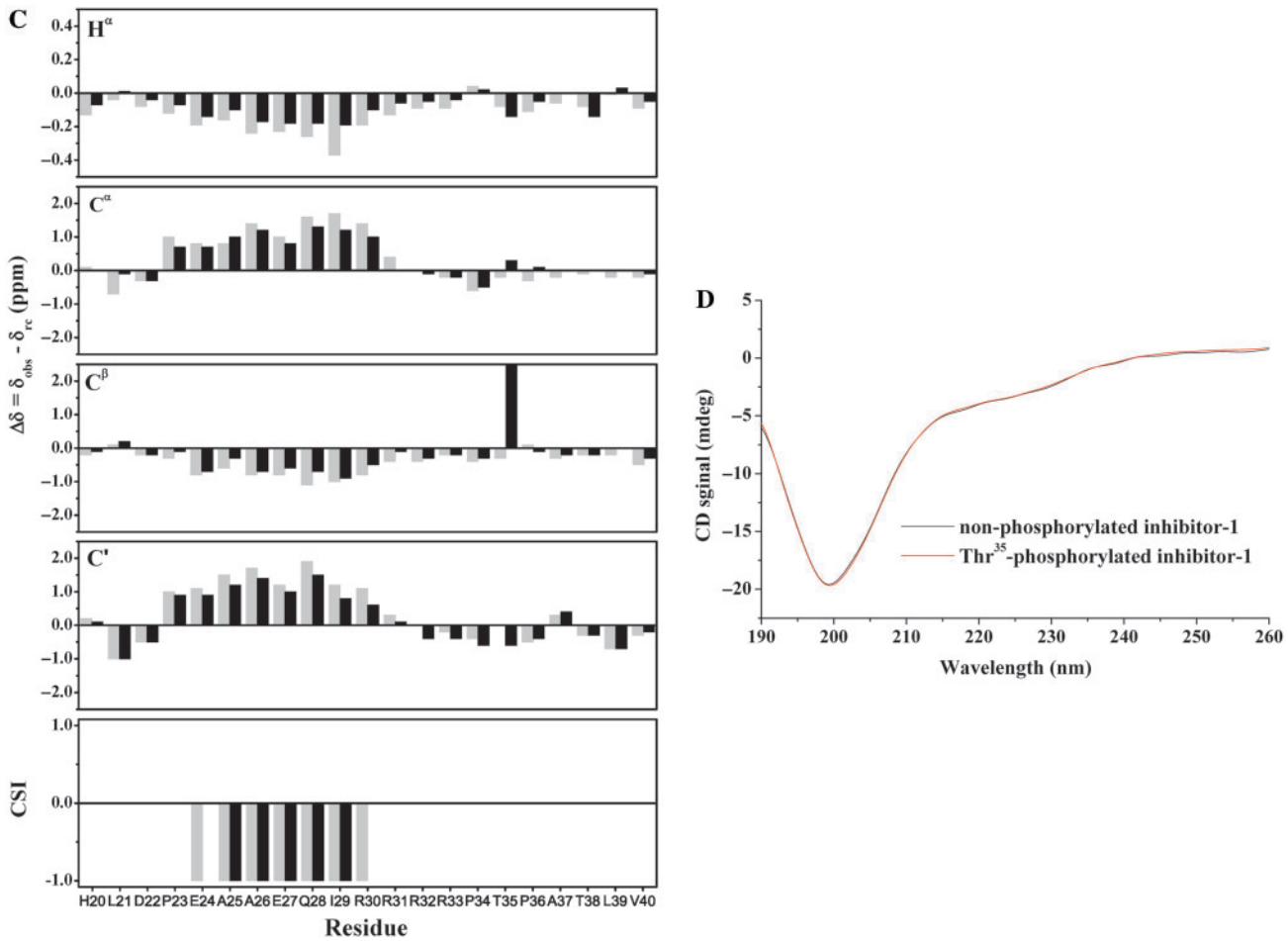

Fig. 2 Continued.

similar to those of non-phosphorylated inhibitor-1, indicating that the overall structural features of non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 should be very similar. Similar to the non-phosphorylated inhibitor-1, Thr^{35}-phosphorylated inhibitor-1 also contains a shorter α-helix spanning residues 25–29 according to the consensus CSI index. The result of structural comparison suggests that the effect of PKA-phosphorylation on the structural conformations of inhibitor-1 is insignificant. The possible reason for the chemical shift changes for the residues around phosphorylated Thr35 might be mainly due to the through-space electrostatic effects of the phosphate group. Moreover, the effect of phosphorylation on the conformation of inhibitor-1 was also studied by circular dichroism spectroscopy. Figure 2D shows an overlay of the CD spectra of non-phosphorylated inhibitor-1 and Thr^{35}-phosphorylated inhibitor-1. These two spectra are almost identical suggesting that the global conformation of inhibitor-1 is not influenced by phosphorylation. This result may further support the inference drawn from the NMR studies.

Discussion

PP1 is an important Ser/Thr phosphatase that has diverse biological functions. To process its diverse roles and maintain the specificity, the activity of PP1 is controlled by more than 100 inhibitors and targeting proteins (*21*, *22*). Among those inhibitor proteins, DARPP-32, inhibitor-1 and inhibitor-2 are the most well-studied inhibitor to PP1. Both inhibitor-1 and DARPP-32 under the phosphorylated state can suppress the function of PP1, while inhibitor-2 can only block the activity of PP1 under the non-phosphorylated state. Although inhibitor-1, DARPP-32 and inhibitor-2 undergo the inhibition at different modes, they share a common structural feature, being the class of intrinsically unstructured proteins (IUPs).

In this study, we characterized the conformation of inhibitor-1 at the non-phosphorylated and phosphorylated states using multi-dimensional heteronuclear NMR techniques. Basically, both non-phosphorylated and Thr^{35}-phosphorylated inhibitor-1 proteins adopt a largely unstructured conformation when free in solution. For non-phosphosrylated inhibitor-1, a short helical propensity at residues 24–30 is identified, while a similar short helical propensity is also characterized at residues 25–29 for Thr^{35}-phosphorylated inhibitor-1. Different from the KID domain of CREB (*23*), the phosphorylation of Thr35 might slightly reduce the population of helical conformation. Overall there is no significant tertiary-folding change while inhibitor-1 protein is phosphorylated by PKA at Thr35.

Although the comparison of tertiary folding between non-phosphosrylated and Thr^{35}-phosphorylated inhibitor-1 proteins shows no dramatic changes, a local chemical shift change has been identified along the phospho-Thr35 from residue 20 to 39. On the other hand, the chemical shift for the major PP1 binding motif, $R^{8}KIQF^{12}$, shows no changes, indicating that the phosphorylation of inhibitor-1 does not affect the local conformation of PP1 binding motif. Similar observation has been reported for DARPP-32 NH_2-terminal domain [DARPP-32 (1–118)] (*24*), however, whether or not the PKA-phosphorylation of intact DARPP-32 affects the conformation of COOH-terminal domain of DARPP-32 remains unclear. It needs further investigation.

Phosphorylation of a protein may alter its function or structure. Phosphorylation-induced conformational change may be local to the phosphorylation site. It may also accompany long-range allosteric change. From the structural point of view, phosphorylation that regulates protein function is often achieved by way of phosphorylation-induced structural change. In the case of inhibitor-1, no local conformational change was observed and the global structure remained intrinsically disordered after the Thr^{35} of inhibitor-1 was phosphorylated by PKA. According to the present result, regulation of the interactions between inhibitor-1 and PP1 through PKA-phosphorylation might mainly depend on the phosphate group instead of phosphorylation-induced structural change. However, the crystal structure of PP1-inhibitor complex has been solved recently (*25*). The structure of inhibitor-2 is largely unstructured in free solution and folds into an ordered form while binding with PP1c. Therefore; we cannot preclude the possibility that the binding of Thr^{35}-phosphorylated inhibitor-1 to PP1 may induce conformational changes.

In conclusion, the present study, we have characterized and compared the structure of non-phosphosrylated and Thr^{35}-phosphorylated inhibitor-1 protein using NMR spectroscopy. Results indicate that the conformations of non-phosphosrylated and Thr^{35}-phosphorylated inhibitor-1 show no significant difference. Our result supports the hypothesis that the interactions between inhibitor-1 and PP1 through PKA-phosphorylation may depend on the phosphate group instead of phosphorylation-induced conformational change.

Acknowledgements

The NMR spectra were obtained at High-Field Biomacromolecular NMR Core Facility supported by the National Research Program for Genomic Medicine.

Funding

National Science Council of the Republic of China (NSC 95-2311-B-010-001 and NSC 95-2320-B-194-005); Taipei Veterans General Hospital, Taiwan, Republic of China (V97C1-036).

Conflict of interest

None declared.

References

1. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453–508
2. Shenolikar, S. (1994) Protein serine/threonine phosphatases–new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10**, 55–86
3. Cohen, P.T. (2002) Protein phosphatase 1–targeted in many directions. *J. Cell Sci.* **115**, 241–256
4. Shenolikar, S. and Nairn, A.C. (1991) Protein phosphatases: recent progress. *Adv. Second Messenger Phosphoprotein Res.* **23**, 1–121
5. Aitken, A., Bilham, T., and Cohen, P. (1982) Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur. J. Biochem.* **126**, 235–246
6. Williams, K.R., Hemmings, H.C. Jr., LoPresti, M.B., Konigsberg, W.H., and Greengard, P. (1986) DARPP-32, a dopamine- and cyclic AMP-regulated neuronal phosphoprotein. Primary structure and homology with protein phosphatase inhibitor-1. *J. Biol. Chem.* **261**, 1890–1903
7. Desdouits, F., Cheetham, J.J., Huang, H.B., Kwon, Y.G., da Cruz e Silva, E.F., Denefle, P., Ehrlich, M.E., Nairn, A.C., Greengard, P., and Girault, J.A. (1995) Mechanism of inhibition of protein phosphatase 1 by DARPP-32: studies with recombinant DARPP-32 and synthetic peptides. *Biochem. Biophys. Res. Commun.* **206**, 652–658
8. Egloff, M.P., Johnson, D.F., Moorhead, G., Cohen, P.T., Cohen, P., and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16**, 1876–1887
9. Endo, S., Zhou, X., Connor, J., Wang, B., and Shenolikar, S. (1996) Multiple structural elements define the specificity of recombinant human inhibitor-1 as a protein phosphatase-1 inhibitor. *Biochemistry* **35**, 5220–5228
10. Hemmings, H.C. Jr., Nairn, A.C., Elliott, J.I., and Greengard, P. (1990) Synthetic peptide analogs of DARPP-32 (Mr 32,000 dopamine- and cAMP-regulated phosphoprotein), an inhibitor of protein phosphatase-1. Phosphorylation, dephosphorylation, and inhibitory activity. *J. Biol. Chem.* **265**, 20369–20376
11. Kwon, Y.G., Huang, H.B., Desdouits, F., Girault, J.A., Greengard, P., and Nairn, A.C. (1997) Characterization of the interaction between DARPP-32 and protein phosphatase 1 (PP-1): DARPP-32 peptides antagonize the interaction of PP-1 with binding proteins. *Proc. Natl Acad. Sci. USA* **94**, 3536–3541
12. Huang, H.B., Chen, Y.C., Lee, T.T., Huang, Y.C., Liu, H.T., Liu, C.K., Tsay, H.J., and Lin, T.H. (2007) Structural and biochemical characterization of inhibitor-1alpha. *Proteins* **68**, 779–788
13. Lin, T.H., Huang, Y.C., Chin, M.L., Chen, Y.C., Jeng, H.H., Lin, F.M., Shiao, M.S., Horiuchi, A., Greengard, P., Nairn, A.C., and Huang, H.B. (2004) 1H, 15N, and 13C resonance assignments of DARPP-32 (dopamine and cAMP-regulated phosphoprotein, Mr. 32,000)—a protein inhibitor of protein phosphatase-1. *J. Biomol. NMR* **28**, 413–414
14. Huang, H.B., Chen, Y.C., Tsai, L.H., Wang, H., Lin, F.M., Horiuchi, A., Greengard, P., Nairn, A.C., Shiao, M.S., and Lin, T.H. (2000) Backbone 1H, 15N, and 13C resonance assignments of inhibitor-2 – a protein inhibitor of protein phosphatase-1. *J. Biomol. NMR* **17**, 359–360
15. Connor, J.H., Frederick, D., Huang, H., Yang, J., Helps, N.R., Cohen, P.T., Nairn, A.C., DePaoli-

Roach, A., Tatchell, K., and Shenolikar, S. (2000) Cellular mechanisms regulating protein phosphatase-1. A key functional interaction between inhibitor-2 and the type 1 protein phosphatase catalytic subunit. *J. Biol. Chem.* **275**, 18670–18675
16. Connor, J.H., Weiser, D.C., Li, S., Hallenbeck, J.M., and Shenolikar, S. (2001) Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol. Cell. Biol.* **21**, 6841–6850
17. Weiser, D.C., Sikes, S., Li, S., and Shenolikar, S. (2004) The inhibitor-1 C terminus facilitates hormonal regulation of cellular protein phosphatase-1: functional implications for inhibitor-1 isoforms. *J. Biol. Chem.* **279**, 48904–48914
18. Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. (1995) 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **6**, 135–140
19. Wishart, D.S. and Sykes, B.D. (1994) The 13C chemical-shift index: a simple method for the identification of protein secondary structure using 13C chemical-shift data. *J. Biomol. NMR* **4**, 171–180
20. Wishart, D.S. and Sykes, B.D. (1994) Chemical shifts as a tool for structure determination. *Methods Enzymol.* **239**, 363–392
21. Bollen, M. (2001) Combinatorial control of protein phosphatase-1. *Trends Biochem. Sci.* **26**, 426–431
22. Meiselbach, H., Sticht, H., and Enz, R. (2006) Structural analysis of the protein phosphatase 1 docking motif: molecular description of binding specificities identifies interacting proteins. *Chem. Biol.* **13**, 49–59
23. Radhakrishnan, I., Perez-Alvarado, G.C., Dyson, H.J., and Wright, P.E. (1998) Conformational preferences in the Ser133-phosphorylated and non-phosphorylated forms of the kinase inducible transactivation domain of CREB. *FEBS Lett.* **430**, 317–322
24. Dancheck, B., Nairn, A.C., and Peti, W. (2008) Detailed structural characterization of unbound protein phosphatase 1 inhibitors. *Biochemistry* **47**, 12346–12356
25. Hurley, T.D., Yang, J., Zhang, L., Goodwin, K.D., Zou, Q., Cortese, M., Dunker, A.K., and DePaoli-Roach, A.A. (2007) Structural basis for regulation of protein phosphatase 1 by inhibitor-2. *J. Biol. Chem.* **282**, 28874–28883