Phosphorylation by glycogen synthase kinase of inhibitor-2 does not change its structure in free state

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Abstract Inhibitor-2 (I2) is a thermostable protein that specifically binds to the catalytic subunit of protein phosphatase-1 (PP1), resulting in the formation of the inactive holoenzyme, ATP-Mg-dependent phosphatase. Phosphorylation of I2 at Thr-72 by glycogen synthase kinase-3 (GSK-3) results in activation of the phosphatase, suggesting that kinase action triggers conformational change in the complex. In this paper, we characterize the effect of GSK-3 phosphorylation on the structure of free state I2[1–172] by nuclear magnetic resonance and circular dichroism spectroscopy, and show that phosphorylation has no significant effect on its conformation. We conclude that the conformational changes of ATP-Mg-dependent phosphatase induced by GSK-3 phosphorylation must depend on the interactions between PP1 and I2.

Key words: Nuclear magnetic resonance; Circular dichroism; Inhibitor-2; Protein phosphatase-1; Glycogen synthase kinase-3

1. Introduction

Protein phosphatase-1 (PP1), one of the major mammalian serine/threonine protein phosphatases, plays a critical role in the regulation of various cellular functions, including carbohydrate metabolism, protein synthesis, cell cycle, muscle contraction and neuronal signaling [1–4]. PP1 occurs in cells as a holoenzyme and consists of a 37-kDa catalytic subunit combined with a specific regulatory subunit that appears to target the enzyme to specific subcellular compartments. The catalytic subunit of PP1 is also regulated by several thermostable protein inhibitors, including inhibitor-1, DARPP-32 and inhibitor-2 (I2). PP1 is only inhibited by inhibitor-1 and DARPP-32 when both inhibitors are pre-phosphorylated by cAMP-dependent protein kinase (PKA); by contrast, PP1 is inhibited by I2 without pre-phosphorylation. I2 binds to PP1 to form a complex that is the ATP-Mg-dependent form of the phosphatase, and in turn inhibits enzyme activity. This inactive holoenzyme can be reactivated when glycogen synthase kinase-3 (GSK-3) phosphorylates I2 at Thr-72. The mechanism for reactivation of ATP-Mg-dependent phosphatase is complicated. GSK-3 phosphorylation does not induce the dissociation of the complex, but triggers a conformational change in PP1, which rapidly auto-dephosphorylates the phosphorylated Thr-72 site of I2. Subsequently, the inactive holoenzyme is slowly converted into an active form [5]. In its dephosphorylated form, the active complex then slowly recurs to an inactive state. Different stages of the reactivation cycle are carried out by diverse regions of I2 [6]. However, it remains uncertain whether or not GSK-3 phosphorylation pre-triggers a conformational change in I2, in turn leading to the structural change of the phosphatase complex. To investigate the conformational state of I2, we prepared GSK-3-phosphorylated I2[1–172] and characterized its structure by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. Our results suggest that GSK-3 phosphorylation does not significantly change the structure of I2 in the free state. The interactions between PP1 and I2 may play a vital role in the conformational change of the complex upon GSK-3 phosphorylation.

2. Materials and methods

2.1. Proteins and reagents

ATP, Tris, dithiothreitol, EDTA, Brij-35 and sodium azide were obtained from Sigma. $^{15}NH_4Cl$ and [^{13}C]glucose were purchased from Cambridge Isotope Laboratories. Recombinant GSK-3β was prepared from *Escherichia coli* as described [7]. One unit of GSK-3β was defined as the amount of enzyme that incorporates 1 nmol of phosphate/min into a PKA-pre-phosphorylated peptide (KRREILSRRPS(P)YR) at 50 μM.

2.2. Preparation of GSK-3-phosphorylated ^{15}N-enriched I2[1–172]

Human recombinant ^{15}N-enriched I2[1–172] was prepared as described [8]. I2[1–172] showed similar properties to wild-type I2 with respect to the IC_{50} for inhibition of PP1, the formation of an inactive complex with PP1 and re-activation of PP1 following phosphorylation by GSK-3β (data not shown). Phosphorylation of ^{15}N-enriched I2[1–172] by GSK-3β was performed in 50 mM Tris–HCl buffer, pH 7.0, containing 0.1 mM EDTA, 0,02% sodium azide, 0.05% Brij-35, 0.2 mM ATP, 2.5 mM magnesium acetate, ^{15}N-enriched I2[1–172] (5 mg/ml) and GSK-3β (2 U/ml). The reaction was carried out at 30°C for 2 days, with fresh GSK-3β (2 U/ml) and ATP (0.2 mM final concentration) added every 12 h. ^{15}N-enriched phospho-I2[1–172] was purified by FPLC using a Mono-Q column (10/10) with a linear salt gradient from 0.18 to 0.42 M NaCl in 20 mM Tris–HCl buffer, pH 7.5, containing 0.2 mM EDTA, 2.0 mM dithiothreitol and 0.02% (w/v) sodium azide. Fractions (1.5 ml/tube) were collected from 0 to

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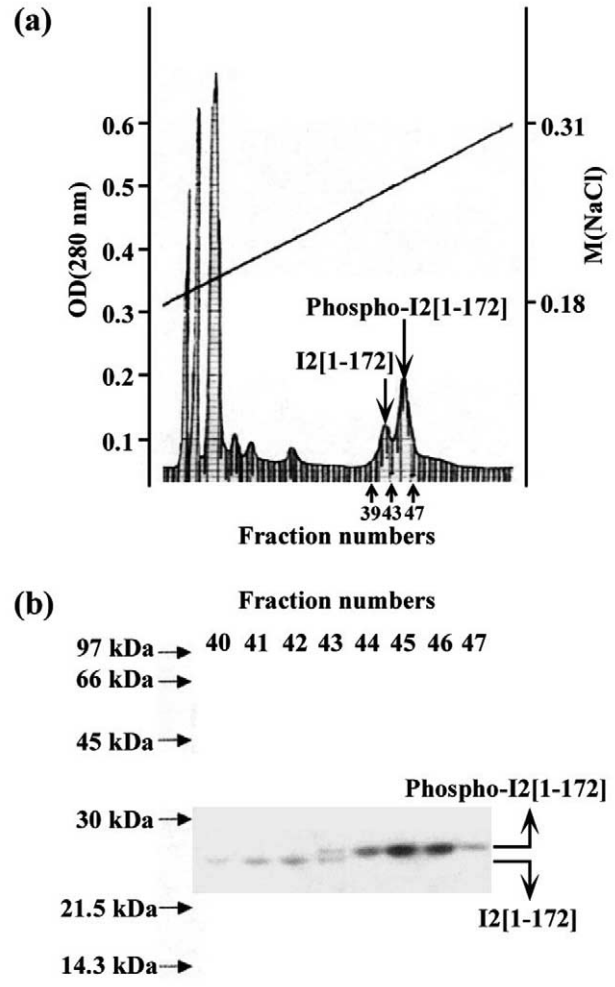

Fig. 1. a: Purification of phospho-I2[1–172] by FPLC on a Mono-Q column. b: SDS–PAGE analysis of phospho-I2[1–172] in fractions 40–47 from panel a. Molecular weight markers are phosphorylase b (97 kDa), bovine serum albumin (76 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

45 min. The fractions containing phospho-protein were pooled, dialyzed against water to remove the excess reagents and lyophilized to powder.

2.3. NMR spectroscopy

For NMR spectroscopy, samples of 0.5 mM ^{15}N-enriched phospho-I2[1–172] in 90% H_2O/10% D_2O were prepared in 100 mM phosphate buffers, pH 6.0, with 0.02% NaN_3. 2,2-Dimethyl-2-silapentane-5-sulfonic acid was used as the internal chemical shift standard [9,10]. The final protein sample solution was transferred to a 5-mm Shigemi NMR tube (Shigemi, Tokyo, Japan) to record the NMR spectra. 2D ^{1}H-^{15}N-heteronuclear single-quantum coherence (HSQC) spectra were recorded at 296 K on a Bruker AVANCE-500 spectrometer equipped with a 5-mm inverse triple resonance (^{1}H/^{13}C/BB), *z*-axis gradient probe. Water suppression was achieved by applying WATERGATE sequence [11]. Quadrature detection in the indirectly detected dimension was accomplished using the States-TPPI method [12]. Spectral width was 1500 Hz in both the direct (^{1}H) and indirect (^{15}N) dimensions. A total of 128 time increments were recorded with 32 transients for each increment. Spectra were processed using XWINNMR and analyzed using AURELIA [13] on an SGI workstation.

2.4. CD spectroscopy

CD spectra were recorded using a Jasco 715 spectropolarimeter with a thermal circulator accessory. The optical rotation was calibrated using d-10-camphorsulfonic acid at wavelengths of 192.5 and 290 nm. The wavelength was calibrated with benzene vapor. All measurements were performed in quartz cells with a path length of 0.01 cm. The protein concentration of the samples was 1.0 mg/ml. Data were collected for wavelengths from 190 to 260 nm at 1-nm increments. Reported CD spectra represent the average of at least three individual samples and three repeated measurements of each sample. Baseline corrections for the spectra were made by using solutions containing DMPC. All measurements were carried out at 25.0 ± 0.2°C. Secondary structure analysis was performed using online software, website: Dichroweb from BBRC Centre for Protein and Membrane Structure and Dynamics [14,15].

3. Results

3.1. Phosphorylation of ^{15}N-enriched I2[1–172] by GSK-3β

The two rat brain isoforms of GSK-3, GSK-3α and GSK-3β, differ in their ability to phosphorylate I2 at Thr-72 [16,17]. GSK-3β is a better I2 kinase than GSK-3α. Under the present experimental conditions, however, the yield of phosphorylated I2 by GSK-3β was low. Therefore, both fresh GSK-3β and ATP were added every 12 h during the phosphorylation reaction. After 2 days, about 70% of the ^{15}N-enriched I2[1–172] was phosphorylated, as judged by analysis of the ion exchange chromatography fractions (Fig. 1a). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) shows that ^{15}N-enriched phospho-I2[1–172] has a lower mobility on the gel (Fig. 1b), and that phospho-I2[1–172] was purified to greater than 96% homogeneity by FPLC using a Mono-Q column (data not shown).

The effect of phosphorylation on the conformational behavior of I2[1–172] was studied by CD spectroscopy. Fig. 2 shows an overlay of the CD spectra of I2[1–172] and phospho-I2[1–172]. These two spectra are almost identical, suggesting that the global conformation of I2[1–172] is not influenced by phosphorylation. Analysis of the secondary structure from the CD spectra shows that both I2[1–172] and phospho-I2[1–172] have a mostly non-structural conformation. The NMR results (Fig. 3) also support this inference. Clearly, the chemical shifts of the backbone amides and the ^{15}N resonances for most of the residues of I2[1–172] remain unchanged when Thr-72 is phosphorylated by GSK-3. Working from previously assigned backbone amide proton and ^{15}N

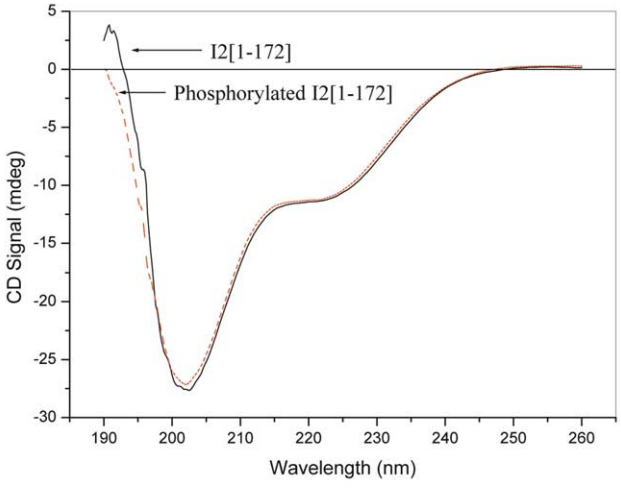

Fig. 2. An overlay of the CD spectra of I2[1–172] (black) and phosphorylated I2[1–172] (red).

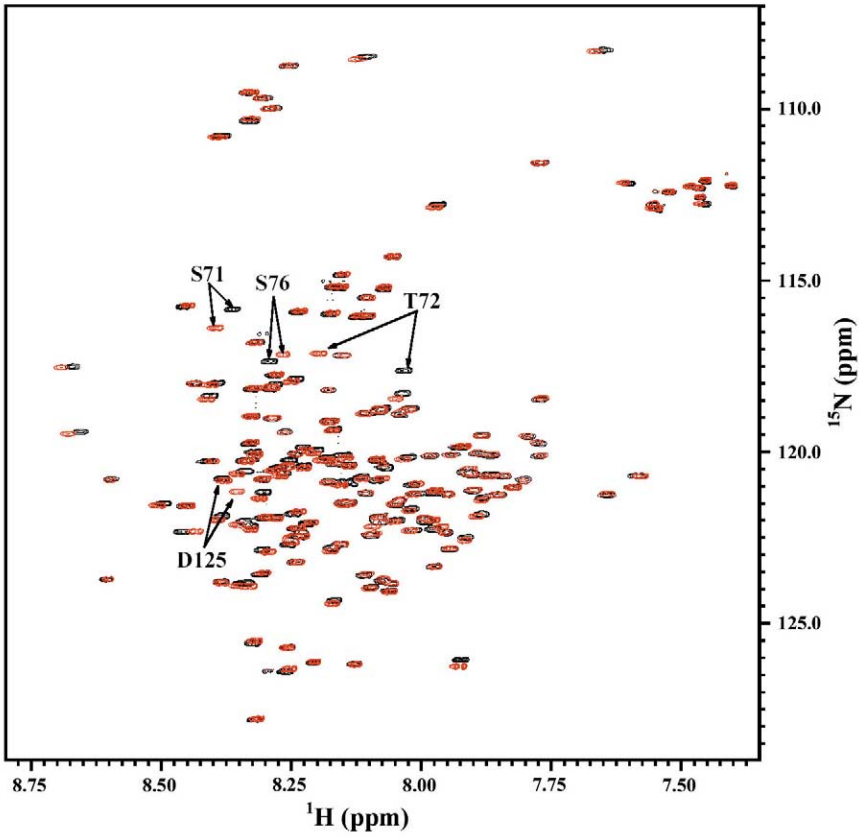

Fig. 3. An overlay of the 2D ^{1}H-^{15}N-HSQC spectra of I2[1–172] (black) and phosphorylated I2[1–172] (red). Residues with significant chemical shift changes are labeled.

chemical shifts of I2[1–172] [8], residues with significant chemical shift changes (S71, T72, S76, D125) were identified by a graphic mapping approach. The chemical shift changes of these residues may be due to the electrostatic effect of the phosphate group or to phosphorylation-induced conformational change or to a combination of both [18]. Since only a few residues exhibit significant chemical shift changes, it is unlikely that phosphorylation of I2[1–172] at Thr-72 has a significant effect on the global conformation of I2[1–172]. Besides, these residues are randomly distributed in the primary sequence, and not located consecutively, which suggests that even local conformational changes would probably occur. Our interpretation of the results is therefore that the phosphorylation-induced chemical shift changes are mainly caused by the through-space electrostatic effects of the phosphate group. If this is correct, then the residues with significant chemical shift changes should be located in the spatial vicinity of the phosphorylation site.

4. Discussion

Previous studies on the effect of phosphorylation on the conformation of proteins have yielded diverse results [19–23], with some supporting a change of conformation and others reporting no effect at all. In most cases, when phosphorylation causes conformational change, long-term allosteric changes may occur [19–22]. In the case of I2, the conformation of which is a random coil except for a short α-helix that spans the region from residue 132 to 142 [8], our NMR and CD spectroscopy data suggest that no significant conformational changes were induced after the Thr-72 site of I2[1–172] was phosphorylated by GSK-3. Using fluorescence techniques, Picking et al. [5] demonstrated that phosphorylation by GSK-3 resulted in a conformational change of I2, but this effect was induced only when I2 had formed a complex with PP1. Furthermore, they pointed out that the free phosphorylated I2 will eventually relax back to its native conformational state. Bearing in mind that our NMR and CD data were obtained for I2 in its free state and in equilibrium, the present results are consistent with those of Picking et al. [5]. We conclude that the binding of I2 to PP1 is critical, and that the interactions between PP1 and I2 may play a vital role in the conformational change of the complex upon GSK-3 phosphorylation.

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