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Phosphorylation of the regulatory domain of human tyrosine hydroxylase 1 monitored using non-uniformly sampled NMR

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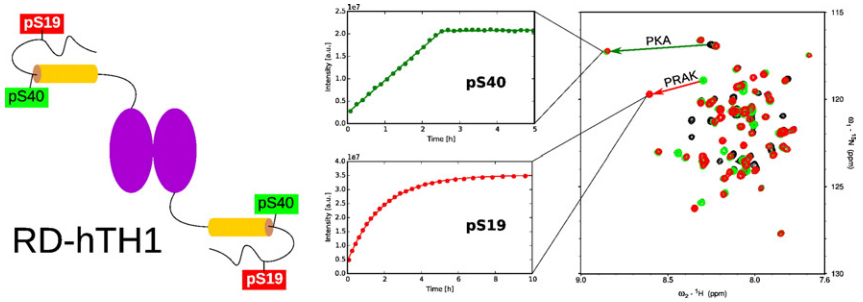
HIGHLIGHTS

- Disordered part of regulatory domain of human tyrosine hydroxylase 1 was assigned.
- Transient alpha-helices are present next to phosphorylation sites S40 and S19.
- The secondary structure does not change after phosphorylation.
- The phosphorylation kinetic rates were measured efficiently using time resolved NMR.

GRAPHICAL ABSTRACT



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ABSTRACT

Human tyrosine hydroxylase 1 (hTH1) activity is regulated by phosphorylation of its regulatory domain (RD-hTH1) and by an interaction with the 14-3-3 protein. The RD-hTH1 is composed of a structured region (66-169) preceded by an intrinsically disordered protein region (IDP, hTH1_65) containing two phosphorylation sites (S19 and S40) which are highly relevant for its increase in activity. The NMR signals of the IDP region in the non-phosphorylated, singly phosphorylated (pS40) and doubly phosphorylated states (pS19_pS40) were assigned by non-uniformly sampled spectra with increased dimensionality (5D). The structural changes induced by phosphorylation were analyzed by means of secondary structure propensities. The phosphorylation kinetics of the S40 and S19 by kinases PKA and PRAK respectively were monitored by non-uniformly sampled time-resolved NMR spectroscopy followed by their quantitative analysis.

1. Introduction

Tyrosine hydroxylase (TH) is an enzyme which converts L-tyrosine to L-DOPA. This reaction is the rate limiting step in the biosynthetic pathway producing important catecholamine neurotransmitters: dopamine, noradrenaline and adrenaline [1,2]. The human enzyme isoform 1 is a tetramer each consisting of three domains: an N-terminal regulatory domain (RD-hTH1, 1-169 aa), a catalytic domain (170-450 aa), and a short C-terminal tetramerization domain (451-497 aa) [3]. The first 65 residues of the regulatory domain form an intrinsically disordered protein region (IDP, hTH1_65) important for regulation. The activity of hTH1 is controlled by the phosphorylation of its IDP region (S19, S31, S40) and by the interaction with 14-3-3 protein [4]. Phosphorylation sites S19 and S40 are the most relevant phosphorylation sites regarding 14-3-3 binding [5,6].

Recently, the NMR structure of the ordered region (65-159) of the dimeric regulatory domain of rat tyrosine hydroxylase (the sequence

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identity between rat and human RD-hTH1 is 81.8%) was determined by Zhang et al. [7]. Authors claimed that the problems with unstable sample and low signal dispersion of the IDP region prevented structural characterization of the full RD. We overcame these problems by modifying the preparation of full length RD-hTH1 (1-169) sample in non- and phosphorylated states and by applying non-uniform sampling (NUS) NMR approaches allowing much faster data collection in comparison with the uniformly sampled experiments.

In the past, the rate of the phosphorylation of hTH1 was monitored semi-quantitatively by Toska et al. [8] using radioactively labeled ATP. Such methodology has several drawbacks, especially the necessity of working with radioactive material, laborious sample preparation and low temporal resolution. The alternative methodology for a monitoring of protein phosphorylation is NMR spectroscopy where individual NMR spectra are collected sequentially during the course of a phosphorylation reaction [9]. The time resolution of such an approach is on the order of the measurement time of one particular NMR spectra. Another possibility for monitoring is to measure one long 2D spectrum and afterwards analyze lineshapes of signals modulated by the reaction kinetics [10]. In this way, the time resolution can be reduced at cost of significant increase in difficulty of analysis. The recent advances in NMR allow us to overcome these limitations by applying non-uniform sampled time-resolved NMR spectroscopy to monitor the reaction course with time resolution down to seconds [11]. This approach was already successfully utilized for the monitoring of the phosphorylation of cytoplasmic domain of human B cell receptor protein CD79b [11].

In this study, we present the resonance assignment of the IDP region (hTH1_65) of RD-hTH1 in the non-, singly- and doubly-phosphorylated states using non-uniformly sampled NMR experiments with increased dimensionality. Next, we analyze the structural changes induced by the phosphorylation of S40 and S19 by PKA and PRAK kinase respectively and the kinetics of these processes.

2. Experimental

2.1. Protein expression and purification

The regulatory domain (residues 1-169) of human tyrosine hydroxylase 1 (RD-hTH1) in a pET15b plasmid containing a TEV-cleavable His-tag was expressed in *E. coli* BL21(DE3)RIL cells. For preparation of ^{15}N-labeled and ^{13}C,^{15}N-labeled samples, the cells were cultured in M9 medium with $^{15}NH_4Cl$, ampicillin (100 mg/ml), chloramphenicol (35 mg/ml) and with addition of $^{13}C_6$-glucose for the double labeled sample. Cells grew at 37 °C until OD_{600} ~ 0.8 then were induced with 0.5 mM IPTG and further cultured at 18 °C for ~18 h. Cells were harvested and homogenized in 50 mM Tris pH = 8, 150 mM NaCl, 3 mM NaN_3. Cell lysate was centrifuged for 1 h at 21,040*g*. Supernatant was then applied on a Ni^{2+} affinity column (HisTrap HP, GE Healthcare) equilibrated in 50 mM Tris pH = 8, 500 mM NaCl, 3 mM NaN_3. Sample was eluted by gradient of elution buffer (equilibration buffer + 1 M imidazole) at its ~70% concentration. Sample was then gel filtrated on a Superdex 75 column (HiLoad 16/600 Superdex 75 pg, GE Healthcare) equilibrated in 50 mM Tris pH = 8, 100 mM NaCl, 3 mM NaN_3. The eluted sample was treated with TEV protease (protein:protease ratio 20:1) at 4 °C overnight and then dialysed into phosphate buffer (20 mM sodium phosphate buffer pH = 6, 3 mM NaN_3). The His-tag cleaved protein was loaded on a cation exchange column (Resource S, GE Healthcare) equilibrated in phosphate buffer (pH = 6.0), and sample was eluted by a gradient of elution buffer (phosphate buffer, pH = 6.0 + 1 M NaCl) at conductivity 23–45 $mS \cdot cm^{-1}$. Fractions containing our protein sample were again dialysed into phosphate buffer (pH = 6.0) and then concentrated for final gel filtration on a Superdex 75 column equilibrated in phosphate buffer.

2.2. NMR samples and phosphorylation

The NMR backbone assignment of the IDP region (1–65 aa) was performed on [^{15}N, ^{13}C] labeled samples of non- and doubly phosphorylated RD-hTH1 at 1.0 mM concentration in 20 mM sodium phosphate buffer pH = 6 with 8% D_2O and 3 mM NaN_3. The phosphorylation kinetic studies were performed with [^{15}N] samples at 0.3 mM concentration in phosphorylation buffer containing 50 mM sodium phosphate buffer pH = 6, 10 mM ATP, 10 mM $MgCl_2$ with 8% D_2O and 3 mM NaN_3. For phosphorylation of S40, PKA (the catalytic subunit of cAMP-dependent protein kinase, New England BioLabs Inc.) was used in 0.5 μg/ml (13.2 nM) concentration. Afterwards, S19 phosphorylation using PRAK (p38 regulated/activated protein kinase, obtained from University Dundee, Scotland) was performed. The concentration of PRAK was 0.11 mg/ml (2.02 μM). Both phosphorylation reactions were monitored over the course of 40 h.

2.3. NMR experiments

The assignment of non-phosphorylated RD-hTH1 was performed using a Bruker 850 MHz US^2 spectrometer equipped with cryogenic triple-resonance probe head (5 mm CPTCI 1H/19F-13C/15N/D). The kinetic measurements as well as assignment of phosphorylated RD-hTH1 were performed using a Bruker 600 MHz spectrometer equipped with a cryogenic triple-resonance probe head (5 mm CPQCI 1H-31P/13C/15N/D). Both probe heads are equipped with z-axis gradient coils. All measurements were done at a temperature of 293.2 K.

For the assignment of non-phosphorylated and doubly S19_S40-phosphorylated RD-hTH1, 3D HNCO [12], 5D HN(CA)CONH and 5D HabCabCONH [13,14] were measured. All experiments were carried out with non-uniform sampling of the indirectly detected domains. The time schedule was generated using Poisson disk sampling on a grid, introducing distance constraints between points. The density of points was set according to a Gaussian distribution ($\sigma = 0.5$).

The phosphorylation was monitored using 2D HSQC experiments. In both cases, the maximal evolution times were set to 102 and 64 ms, respectively. The time resolution was achieved using non-uniform sampling in the indirect domain. The sampling schedule comprised 16,000 points in total for both phosphorylations. The size of the schedules exceeded the size of regular Nyquist grid 125 times. The total measuring time was 40 h.

2.4. Data processing

The non-uniform 3D HNCO spectra were processed using a Multidimensional Fourier Transform [15], while 5D HN(CA)CONH and 5D HabCabCONH spectra were processed using a Sparse Multidimensional Fourier Transform (SMFT) algorithm [16]. The direct dimension was square cosine weighted and zero-filled to 4096 complex points, followed by a standard FFT. The 5D spectra were processed by the SMFT algorithm using the program *reduced*, using fixed frequencies of $^{13}C'$, ^{15}N and $^{1}H^{N}$ identified in the 3D HNCO spectrum, providing sets of 2D slices. The assignment and visualization of the NMR spectra was performed in the software Sparky 3.115 [18].

The secondary structure propensities were calculated by the program SSP [19] using chemical shifts of $^{1}H^{\alpha}$, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$. The data from RefDB [17] were used for random coil referencing. These values were used for calculation of the phosphorylated state as well.

The time-resolved HSQC spectra were processed using a coprocessed Multidimensional Decomposition (co-MDD) [11]. The initial window size was set to 64 points. In the case of PRAK phosphorylation, the window size was incremented by a factor of 1.05 to reduce the fitting errors in subsequent analyses. The processing yielded 250 individual frames for PKA and 52 frames for PRAK phosphorylation.