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Probing nucleotide binding site of annexin A6

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Abstract

A putative consensus site for GTP/ATP binding has been recently identified within human annexin A6 (AnxA6) by using molecular modeling, infrared (IR), and fluorescence spectroscopies. In this report, we describe the structural changes of human recombinant AnxA6 caused by binding of a non-hydrolyzable GTP analog, GTP-γ-S photoreleased from GTP-γ-S[1-(4,5-dimethoxy-2-nitrophenyl)-ethyl] (caged GTP-γ-S). Recording the infrared spectra of AnxA6 before and after photorelease of GTP-γ-S and measuring directly their differences revealed time-dependent structural changes caused by the photorelease of GTP-γ-S and its binding to AnxA6. The infrared difference spectra consisted of a decrease in the intensity of the 1660–1655-cm^{-1} band and a concomitant increase of the 1622–1617-cm^{-1} component band. This suggests that carbonyl groups of peptide backbone are affected due to a small distortion of secondary structure of AnxA6 upon nucleotide binding. The infrared spectra measured as function of time, served to generate 2D IR correlation spectra. Our findings indicated that both bands were correlated, suggesting that they belong to the same molecular species.

Keywords: Annexin; GTP; Nucleotide; Correlation spectroscopy; Infrared spectroscopy

1. Introduction

Annexins belong to a family of homologous Ca^{2+}- and membrane-binding proteins. Twelve various subfamilies were identified in different vertebrate tissues. Among them the eight repeat domain annexin A6 (AnxA6) isoforms are the largest members of the family (M_r 68 kDa). It has been proposed that AnxA6 could participate in nucleotide-dependent processes such as membrane traffic [1] and signal transduction pathways [2]. Experimental evidences suggested that AnxA6 may bind in N-terminal region of the protein [3,4]. A putative nucleotide binding site was proposed based on a combination of infrared (IR) spectroscopy, fluorescence spectroscopy and molecular modeling [5]. It was inferred from the molecular simulations that two identical sequences in human AnxA6 with the motif F-X-X-K-Y-D/E-K-S-L, located at 291–299 residues and at 640–648 residues formed one nucleotide-binding site, where the phosphate groups of nucleotide can interact [5]. To ascertain band assignments and to investigate time-dependent structural changes caused by GTP binding to AnxA6, we used reaction-induced infrared difference spectroscopy (RIDS) [6–8] in combination with 2D IR correlation spectroscopy [9–11]. The difference infrared spectra of AnxA6 were obtained after photorelease of GTP-γ-S (a non-hydrolyzable nucleotide), from GTP-γ-S[1-(4,5-dimethoxy-2-nitrophenyl)-ethyl] (caged GTP-γ-S). Photolytic release of GTP-γ-S from inactive caged GTP-γ-S enables investigation of nucleotide-protein reaction directly in the cuvette, minimizing experimental errors due to sample to sample variation. Recording the infrared spectra of AnxA6 before and after photorelease of nucleotide and measuring directly their differences revealed time-dependent structural changes caused by the photorelease of GTP-γ-S and its binding to AnxA6. The analysis of 2D cross-correlation functions indicated that the structural changes were associated with the same molecular species or with the same protein domains.

2. Experimental procedure

2.1. Materials

GTP-γ-S[1-(4,5-dimethoxy-2-nitrophenyl)-ethyl] was obtained from Molecular Probes Inc. (Eugene, OR, USA).

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Isopropyl-β-D-thiogalactopyranoside (IPTG) was provided by BIO 101, Inc. (Vista, CA, USA). All other chemicals of the highest purity were commercially available.

2.2. Preparation of human recombinant AnxA6

Human recombinant AnxA6 (isoform 1) was expressed in *Escherichia coli* strain B121(DE3) after induction with IPTG, and purified to homogeneity, as described for AnxA5 [12], with small modifications [13,14]. Purified protein was dialyzed against large excess of 5 mM Tris–HCl, pH 7.5, 0.1 mM EGTA and lyophylized. Protein concentration was determined by the method of Bradford [15] with bovine serum albumin as a standard.

2.3. FTIR difference spectroscopic measurements

Lyophylized AnxA6 was dissolved in ^2H_2O buffer containing 100 mM Tris–HCl (p^2H 7.5), 1 mM EGTA and 2 mM caged GTP, corresponding to a maximum of 1.6 mM photoreleased ligand after UV illumination. The protein concentration of AnxA6 was 100–150 μM. The p^2H was determined with a glass electrode and was corrected by a value of 0.4 [16]. The same buffer without protein was used to determine its infrared absorption. All samples were freshly prepared and incubated for 5 min in the dark at room temperature before infrared spectra measurement. Infrared data were acquired with a Nicolet 510M FTIR spectrometer equipped with a DTGS detector, using a temperature controlled flow-through cell (model TFC-M25, Harrick Scientific Corp., Ossining, NY, USA) with 50-μm spacers and CaF_2 windows. The infrared spectra were recorded at 25 °C with 64 interferograms each and at 4-cm^{-1} resolution, Fourier transformed. During data acquisition, the spectrometer was continuously purged with dry filtered air (Balston regenerating desiccant dryer, model 75-45 12 VDC). Once the infrared spectrum was recorded, the sample was exposed to 120-s UV illumination (150 W Xe–Hg lamp) that induced the photorelease of the nucleotide from respective caged compound, allowing binding to AnxA6. Immediately after illumination, infrared spectra were recorded under the same conditions for up to 30 min (total of 13 spectra were measured).

3. Results and discussion

The RIDS of sample containing AnxA6 and caged GTP-γ-S, measured as function of time (from 4 to 30 mn) are shown in Fig. 1. The RIDS of the protein sample were obtained by subtracting the first infrared spectrum (before illumination) from the infrared spectra after illumination recorded with time. Positive bands indicated the formation of new structures or molecular interactions due to the binding of photo-released GTP-γ-S, while negative bands reflected the disappearance of structures or molecular interactions present in the protein before binding of GTP-γ-S.

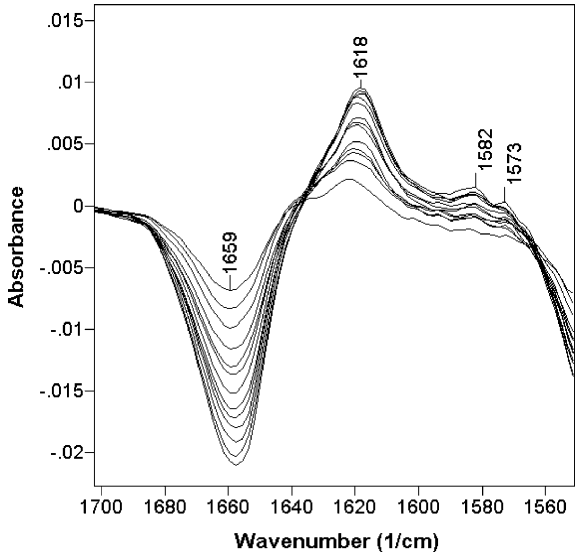

Fig. 1. RIDS of AnxA6 (isoform 1) and caged GTP-γ-S in ^2H_2O Tris-buffer as function of time. The RIDS of AnxA6 and caged GTP-γ-S were obtained by subtracting the first infrared spectrum (before illumination) from the infrared spectra after UV illumination and photorelease of GTP-γ-S from caged GTP-γ-S recorded with time.

The positive band located at 1622–1617 cm^{-1} and the negative band located at 1660–1656 cm^{-1} resulted from the effects of photorelease of GTP-γ-S in the presence of AnxA6. The 1622–1617-cm^{-1} and 1660–1655-cm^{-1} bands are characteristic for carbonyl groups of the peptide backbone of AnxA6 [17,18], reflecting slight structural changes caused by the phosphate binding moiety of GTP-γ-S to the protein [5]. The time dependence of the difference absorbance of the 1660–1655-cm^{-1} band (Fig. 2) indicated slow conformational relaxation, one order of magnitude slower than that observed in the case of nucleotide binding to Ca-ATPase [19] or similar than that reported in the case of nucleotide binding to RecA protein cooled at −8 to −10 °C [20]. Due to very small changes induced by the GTP-γ-S binding, small artifacts could contribute to the RIDS. For

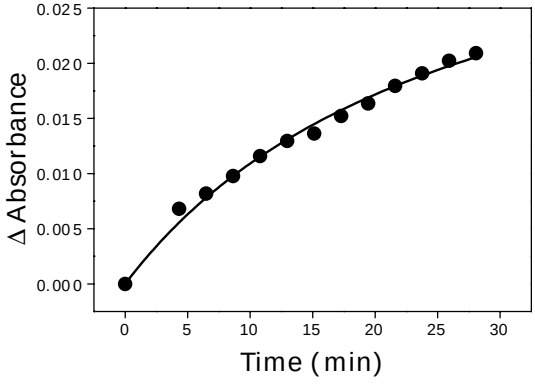

Fig. 2. Time-dependent changes of the 1660–1655-cm^{-1} absorbance, induced after photorelease of GTP-γ-S from caged GTP-γ-S and during GTP-γ-S binding to AnxA6.

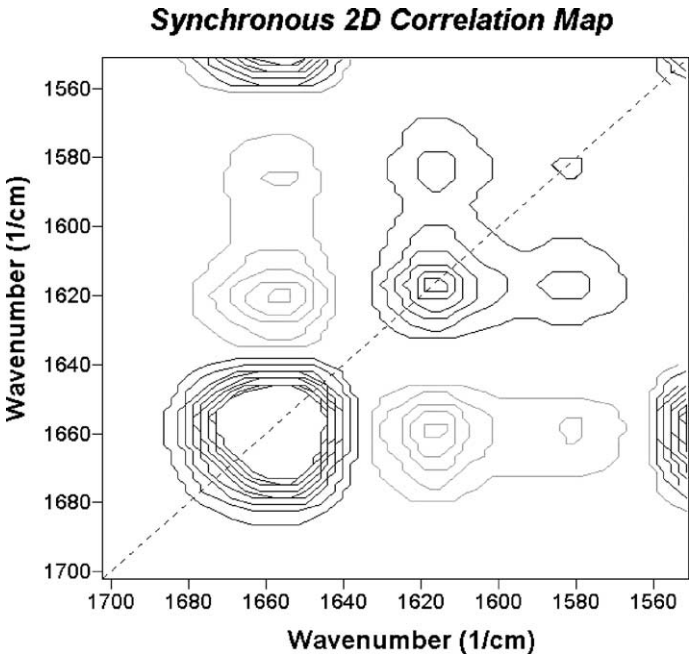

Fig. 3. 2D IR synchronous correlation map in the amide-1 region generated from time-dependent RIDS of AnxA6 (isoform 1) and caged GTP-γ-S in ^2H_2O Tris-buffer. Grey contours indicate negative values, while black contours show positive values of the synchronous correlation function.

example, one can not exclude the possibility that caged GTP-γ-S or its photoproducts interacted with AnxA6. Eventually, the decrease in the intensity of the 1660–1655-cm^{-1} band could be caused by the disappearance of bounded H_2O molecules induced by moving protein domain, increasing ^1H :^2H exchange with bulk ^2H_2O. The environment of carboxylate groups of EGTA, may be affected during photorelease of GTP-γ-S and nucleotide binding to AnxA6. Therefore, it is necessary to assign without any ambiguity all the infrared bands by performing additional controls [3,5]. One such control is to check whether both bands located at 1622–1617 cm^{-1} and 1660–1655 cm^{-1} belong to the same chemical species. Therefore, we performed a 2D IR correlation analysis [9–11] on the time-dependent RIDS of AnxA6 and caged GTP-γ-S. The 2D synchronous correlation map in the amide-1 region (Fig. 3) obtained from time-dependent RIDS of AnxA6 and caged GTP-γ-S (Fig. 1), indicated that 1622–1617 cm^{-1} and 1660–1655 cm^{-1} are correlated. This is exemplified schematically in Fig. 4. Additional correlation between the amide-I bands and a carboxylate group located at 1582 cm^{-1} is observed, suggesting that these bands belong to the same chemical species. Although, one can not rule out false correlation [21], this finding is consistent with structural changes associated to the AnxA6, since the protein contains amide-I and carboxylate bands.

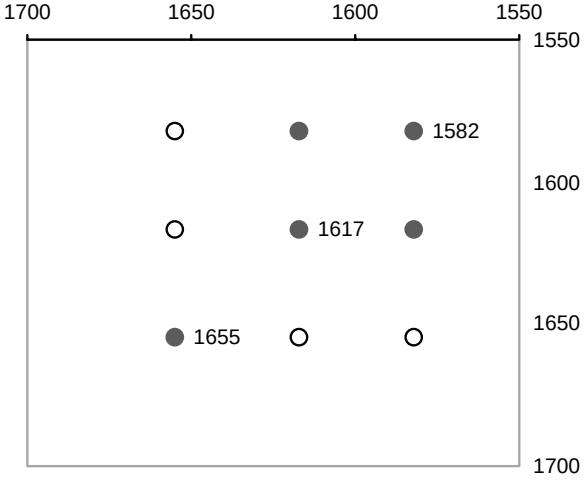

Fig. 4. Diagonal and off diagonal cross peaks indicating schematically the correlation between the two amide-I bands located at 1622–1617 cm^{-1} and 1660–1655 cm^{-1} with a carboxylate group located at 1582 cm^{-1}. Positive maxima are indicated with filled circles, while negative minima are shown with open circles. The positions of cross peaks were obtained from 2D IR synchronous correlation function.

Acknowledgements

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