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β-Synuclein exhibits chaperone activity more efficiently than α-synuclein

Daekyun Lee[a], Seung R. Paik[b], Kwan Yong Choi[a,*]

[a]*Division of Molecular and Life Sciences, National Research Laboratory of Protein Folding and Engineering, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea*
[b]*School of Chemical Engineering, College of Engineering, Seoul National University, Kwanak-Ku, Seoul 151-744, Republic of Korea*

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Abstract β-Synuclein exhibits high sequence homology and structural similarity with α-synuclein, a protein implicated in the pathogenesis of Parkinson's disease. We investigated the chaperone function of β-synuclein and its anti-fibrillar activity in comparison with α-synuclein. β-Synuclein suppressed the heat-induced aggregation of aldolase, alcohol dehydrogenase, and citrate synthase, and its anti-aggregative activity was remarkably higher than that of α-synuclein. Heat-induced inactivation of citrate synthase was significantly protected by β-synuclein. Moreover, β-synuclein inhibited the amyloid formation of both $A\beta_{1-40}$ and α-synuclein. It is, therefore, suggested that β-synuclein can prevent abnormal protein aggregations more effectively than α-synuclein by acting as a molecular chaperone.

Keywords: β-Synuclein; Chaperone activity; Protein aggregation; Fibril formation

1. Introduction

Synuclein was first isolated in *Torpedo californica* [1] and found to be a neuron-specific protein in rat [29]. The human homolog of the protein was named α-synuclein and a related protein known as β-synuclein was reported to be another human homolog of bovine phosphoneuroprotein 14 [2]. γ-Synuclein is the third member of the synuclein family and known as breast cancer-specific gene 1 [3]. These three synucleins are intrinsically unstructured in their native states and share a high degree of sequence identity especially within their N-terminal regions.

Among the synuclein family, much more research efforts have been focused on α-synuclein because of its major presence in Lewy bodies, which are abnormal protein inclusions associated with Parkinson's disease (PD) and several other neurodegenerative disorders [27]. Missense mutations of α-synuclein were discovered in a few pedigrees of familial PD [4–7], and PD-like phenotypes were developed in the transgenic mice and flies with α-synuclein overexpression [8,9]. The fact that α-synuclein bound to synthetic membranes and brain vesicles along with its presynaptic localization [10,11] led to the suggestion of its physiological function in synaptic plasticity. α-Synuclein was implicated in the regulation of dopamine neurotransmission [12] and maintenance of the reserve or resting pools of presynaptic vesicles [13,14]. α-Synuclein also exhibited chaperone activity, which protects proteins from being aggregated thermally or chemically in vitro [15,16].

Unlike α-synuclein whose structural and functional properties were extensively investigated, relatively minor attention was paid to β-synuclein regarding its physiological roles and any implications in the pathogenesis. Both α- and β-synucleins have been abundantly expressed and localized in the presynaptic nerve terminal of human brain [2]. While mRNA level of β-synuclein is high in a normal brain, the level has been decreased in the brain of Lewy body disease patients, especially in the area of substantia nigra. α-Synuclein level, on the other hand, was found to be increased significantly in the substantia nigra, where selective loss of the dopaminergic neurons occurred up to 70–80% in PD [17]. Since β-synuclein inhibited the accumulation of αsynuclein in transgenic mice and cell lines, and this inhibitory effect was further demonstrated in in vitro fibrillization of α-synuclein, it was hypothesized that the protein might be a negative regulator of the amyloid formation of α-synuclein [18,19]. β-Synuclein has been also known to display antiapoptotic function [20] and to protect neurons from neurotoxin by regulating protein kinase B/Akt activity [31].

Despite putative physiological roles of β-synuclein as mentioned above, the exact function of β-synuclein is still elusive. In this report, we propose that β-synuclein is a molecular chaperone which exhibits more effective chaperone activity than α-synuclein against heat-induced protein aggregations. Moreover, β-synuclein inhibits the in vitro amyloid formation of both amyloid β/A4 protein ($A\beta_{1-40}$) and α-synuclein. Our findings suggest that β-synuclein be one of the effective anti-aggregative agents inside the cell. Therefore, β-synuclein exhibited a potential for therapeutic or prophylactic treatment of neurodegenerative diseases involved in misfolding and subsequent aggregations of proteins.

2. Materials and methods

2.1. Purification of α- and β-synucleins

Both α- and β-synucleins were produced and purified according to the procedures as described previously [21] with minor modifications. Briefly, heat-treated cell lysate containing α-synuclein was loaded onto a DEAE Sephacel anion-exchange (Pharmacia Biotech, Sweden) column, which had been equilibrated with an equilibrium buffer containing 0.1 M NaCl and 20 mM Tris–Cl, pH 7.5, and eluted with an elution buffer containing 0.2 M NaCl and 20 mM Tris–Cl, pH 7.5.

* Corresponding author. Fax: +82-54-279-8066.
E-mail address: kchoi@postech.ac.kr (K.Y. Choi).

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Subsequently, Sephacryl S-200 (Pharmacia Biotech, Sweden) size-exclusion chromatography was performed with an elution buffer of 20 mM MES, pH 6.5, and the eluate was applied onto a Mono S HR 5/5 column (Pharmacia Biotech, Sweden) equilibrated with 20 mM MES, pH 6.5. The protein was eluted with a linear gradient of 0–0.5 M NaCl. The collected fractions were concentrated and desalted using a Fast desalting column (Pharmacia Biotech, Sweden). β-Synuclein was isolated by ion-exchange chromatography using DEAE and Mono Q anion-exchangers as described previously [30]. The purified synucleins were dialyzed against 20 mM MES, pH 6.5, and stored in aliquots at −20 °C.

2.2. Chaperone activity of synucleins against heat-induced protein aggregation

Prevention of heat-induced protein aggregation by the synucleins was investigated according to the methods described previously [15,16]. The protein aggregation was induced at 60 °C in 25 mM sodium phosphate, pH 7.4. Various amounts of synucleins were added in the reaction mixture containing 2.5 μM aldolase or alcohol dehydrogenase (ADH) (Sigma, USA). Light scattering at 405 nm was measured during the incubation by use of a spectrophotometer (UV-2501 PC, Shimadzu, Japan) with a built-in water circulator for constant temperature.

The aggregation of ADH was induced by heat treatment in the presence or absence of β-synuclein. 2.5 μM ADH was incubated with 14.1 μM β-synuclein at 60 °C for 10 min and the reaction mixtures were then centrifuged at $15\,000 \times g$ for 30 min. The supernatant was removed completely and a sample buffer containing 0.125 M Tris–Cl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M DTT, and 0.02% bromophenol blue was added to the pellets before heating at 100 °C. Inhibition of ADH precipitation by β-synuclein was assessed by 15% SDS–PAGE.

2.3. Effect of synucleins on the aggregation and inactivation of citrate synthase

The porcine heart citrate synthase (CS) (Sigma, USA) dissolved in a buffer containing 2.2 M $(NH_4)_2SO_4$, pH 7.0, 60 mM phosphate, and 0.5 mM citrate was dialyzed against a solution containing 50 mM Tris–Cl, pH 8.0, and 2 mM EDTA. The dialyzed solution was centrifuged at $15\,000 \times g$ for 10 min to remove any precipitate, and then was divided into aliquots and stored at −20 °C. To determine synuclein effects on the protein aggregation and inactivation of the enzyme, 0.5 μM CS was incubated in 40 mM HEPES, pH 7.5, at 43 °C in the absence or presence of indicated concentrations of either α- or β-synuclein. The thermal aggregation of CS was assessed by monitoring the change of light scattering using a spectrofluorophotometer (RF-5301 PC, Shimadzu, Japan) with both excitation and emission wavelengths at 360 nm. To determine the CS activity during the thermal inactivation, enzymatic assays were performed as described previously [22] with the minor modifications in which 20 μl aliquot was taken from 0.5 μM CS mixture during the thermal inactivation process and added to the assay mixture containing 0.1 mM oxaloacetic acid, 0.15 mM acetyl-CoA, and 0.1 mM 5,5′-dithio-bis (2-nitrobenzoic acid) in a buffer containing 50 mM Tris–Cl, pH 8.0, and 2 mM EDTA. The assay was carried out at 25 °C and the CS activity was determined by measuring the absorbance of the assay mixture at 412 nm utilizing 5,5′-dithio-bis (2-nitrobenzoic acid) as described previously [22].

2.4. β-Synuclein-mediated inhibition of the fibrillizations of $A\beta_{1-40}$ and α-synuclein

$A\beta_{1-40}$ (Sigma, USA) was initially dissolved in 100% dimethyl sulfoxide, and then diluted in the buffer containing 10 mM phosphate, pH 7.4, and 150 mM NaCl. 50 μM $A\beta_{1-40}$ was incubated in the absence or presence of equimolar concentration of either α- or β-synuclein at 37 °C. The concentration of dimethyl sulfoxide in the reaction mixture was maintained at less than 5% in order to exclude any effect of the organic solvent. To determine the effect of β-synuclein on the α-synuclein fibrillization, 100 μM α-synuclein was incubated with equimolar β-synuclein at 37 °C. At each designated time point, an aliquot (5 μl) of the solution from each reaction mixture was taken for the thioflavin-T binding fluorescence assay as described previously [23]. Fluorescence intensities were measured using a spectrofluorophotometer at 25 °C with a 1 cm pathlength quartz cell. The excitation wavelength was 440 nm and the emission was monitored between 400 and 500 nm. The relative fluorescence at 485 nm was used to quantify the amount of fibrillar aggregates.

3. Results and discussion

α-Synuclein has been suggested to have a molecular chaperone activity as a potential physiological function [15,16,24]. Since the amino acid sequence of β-synuclein is almost identical to that of α-synuclein in the N-terminal region while exhibiting a distinct feature in the C-terminus, which has been suggested to be responsible for the chaperone activity of α-synuclein, we examined the capability of β-synuclein to prevent heat-induced protein aggregations of aldolase and ADH. Like α-synuclein, β-synuclein prevented the aggregation of denatured aldolase and ADH during the incubation at 60 °C (Fig. 1). The presence of 14.1 μM β-synuclein in the mixture containing 2.5 μM aldolase resulted in 46% reduction in the protein aggregation after 500 s of the incubation, and the inhibitory effect appeared to be dose-dependent as it was decreased up to 80% in the presence of 28.2 μM β-synuclein (Fig. 1A). The chaperone activity of β-synuclein was even more dramatic when examined using ADH as a substrate. When 2.5 μM ADH was incubated with 14.1 μM β-synuclein, almost 90% of the aggregation of ADH was prevented after 500 s of the incubation (Fig. 1B). Thus, β-synuclein exhibited different efficiency of chaperone activity depending on the kinds of the substrate. On the other hand, when the concentration of α-synuclein was adjusted to 13.9 μM or 27.8 μM in the reaction mixture containing 2.5 μM ADH, the heat-induced aggregation of the protein was suppressed by only 26% and 32%, respectively (data not shown). Therefore, β-synuclein exhibited higher chaperone activity than α-synuclein. The chaperone activity of β-synuclein was further confirmed by analyzing the amount of precipitated ADH after the incubation at 60 °C for 10 min by SDS–PAGE (Fig. 1C). When ADH was incubated without any additional protein or with a non-specific protein like BSA, a significant amount of ADH was found in the precipitate after centrifugation for 30 min. However, the amount of precipitated ADH was remarkably decreased in the presence of β-synuclein in agreement with the result from the light scattering measurement.

CS is also a very sensitive substrate for examining chaperone activity. The enzyme loses its activity and forms protein aggregates within minutes during the incubation at 43 °C. 0.5 μM CS was incubated at 43 °C and the changes of light scattering at 360 nm were monitored during the incubation periods (Fig. 2A). Light scattering was increased after 4 min of the lag period. The aggregation of CS was clearly suppressed by β-synuclein, while α-synuclein did not affect the aggregation at all. To achieve 50% suppression of the CS aggregation, the equimolar concentration of β-synuclein was sufficient. Addition of 2-fold molar excess of β-synuclein suppressed 70% of the CS aggregation. Thus, this inhibitory effect of β-synuclein was dose-dependent (Fig. 2A). In concert with the overall decrease in the CS aggregation, the lag phase in the CS aggregation kinetics was extended in a dose-dependent manner as well.

Since CS readily loses its activity during the incubation at elevated temperature, the effect of synucleins on the heat-induced inactivation of CS was investigated. As shown in Fig. 2B, the enzyme activity of CS decreased rapidly. After incubation for 6 min at 43 °C, less than 20% of the enzyme activity was retained. However, the presence of the equimolar β-synuclein dramatically slowed down the inactivation rate of CS; over 60% of CS activity still remained after 6 min of heat

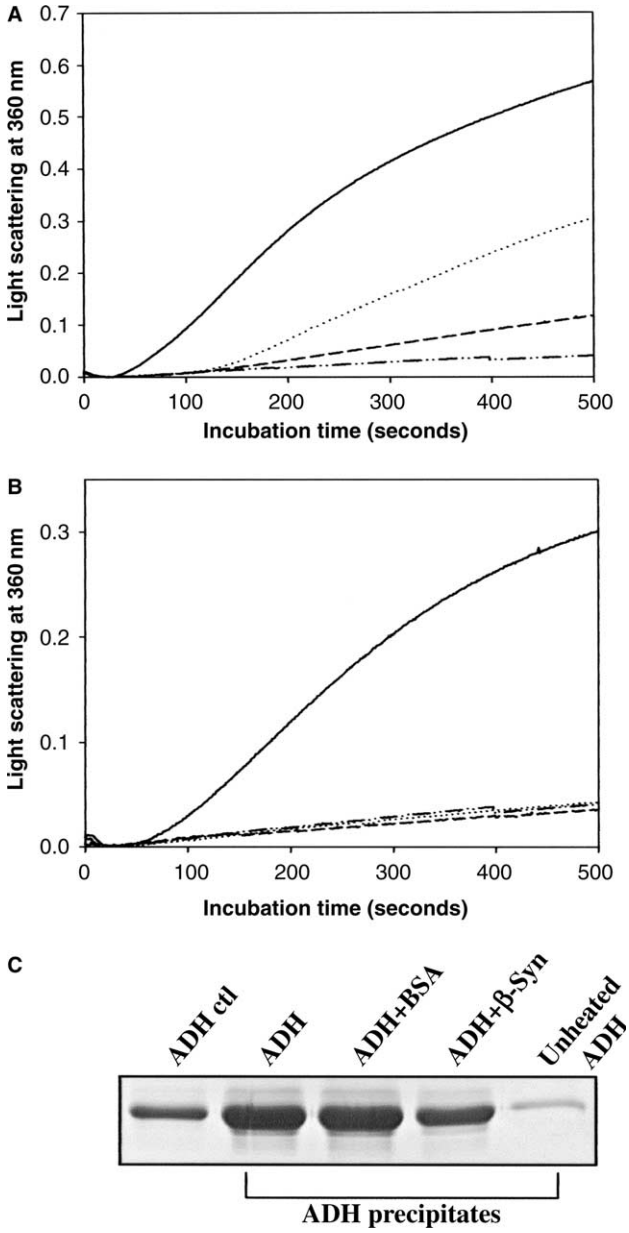

Fig. 1. Chaperone activity of β-synuclein against the heat-induced protein aggregation of aldolase and alcohol dehydrogenase (ADH). (A) 2.5 μM aldolase was incubated at 60 °C in the absence (—) or presence of either 14.1 μM (···) or 28.2 μM (––) β-synuclein. (B) 2.5 μM ADH was incubated at 60 °C in the absence (—) or presence of either 7 μM (···) or 14.1 μM (––) β-synuclein. The aggregation kinetics was monitored by measuring light scattering at 360 nm. The line (–··) represents the aggregation of β-synuclein by itself. (C) 2.5 μM ADH was incubated in the absence or presence of 14.1 μM β-synuclein or BSA for 10 min at 60 °C. After immediate centrifugation, the precipitation of ADH was analyzed by 15% SDS–PAGE. The ADH standard and the unheated ADH in the precipitate were shown as controls.

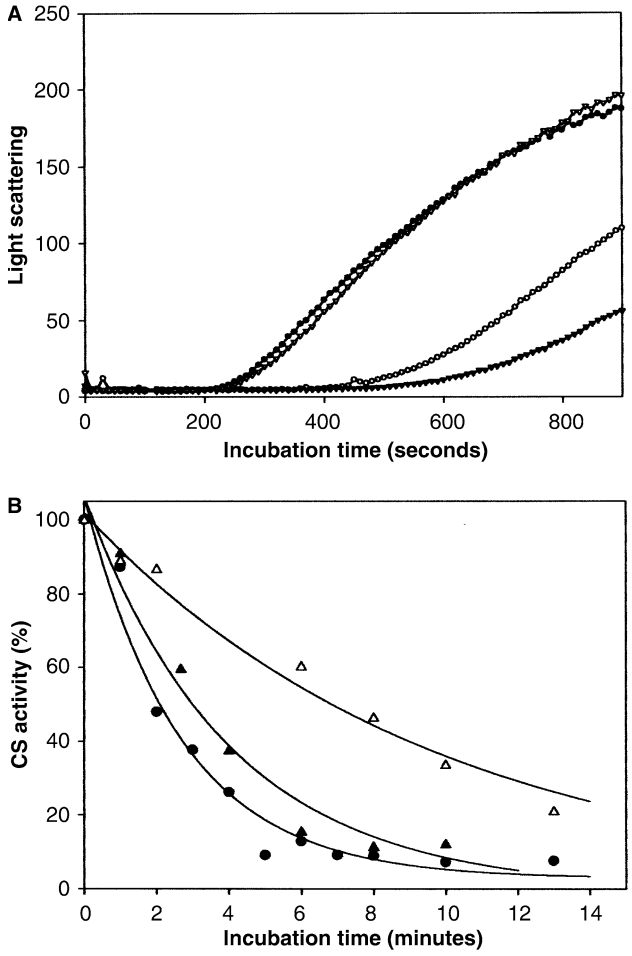

Fig. 2. Influence of α- or β-synuclein on the heat-induced citrate synthase (CS) aggregation and inactivation. (A) 0.5 μM CS was incubated at 43 °C in the absence (●) or presence of 0.5 μM α-synuclein (▽), and 0.5 μM (○) and 1 μM (▼) β-synuclein, respectively. (B) During the heat-induced CS aggregation at 43 °C, 20 μl of the reaction mixture at each time point was subjected to CS activity assay at room temperature. The remaining activities of CS at each time point were plotted in the absence (●) or presence of equimolar α-synuclein (▲) and β-synuclein (△).

inactivation, indicating that β-synuclein protects CS from both the protein aggregation and the thermal inactivation of the enzyme activity. The rate constants for the inactivation of the CS activity were estimated to be 2.11×10^{-5} or 0.8×10^{-5} s^{-1} in the absence or presence of β-synuclein, respectively (Fig. 2B). The rate constant was decreased by 2.54-fold in the presence of β-synuclein. However, α-synuclein contributed to only marginal reduction in the inactivation process of the CS activity, since the CS activity retained after 6 min incubation with α-synuclein was similar to that in the absence of additional synucleins (Fig. 2B).

Based on the anti-aggregative property of β-synulein, we investigated whether β-synuclein could exhibit an anti-fibrillar activity to suppress the fibril formation of both amyloid β/A4 protein (Aβ) and α-synuclein, which are known to play significant roles in Alzheimer's disease (AD) and PD pathogeneses, respectively. The Aβ with 39–43 amino acids are peptides derived from the amyloid precursor protein (APP). These peptides are the major constituent of extracellular amyloid plaques in the brain of AD patients and also have capability to form fibrils in certain conditions in vitro [27]. β-Synuclein was found to suppress the fibrillization of $A\beta_{1-40}$ significantly as shown in Fig. 3A. By incubating with an equimolar β-synuclein at 37 °C, the aggregate formation of $A\beta_{1-40}$ was significantly suppressed during the incubation

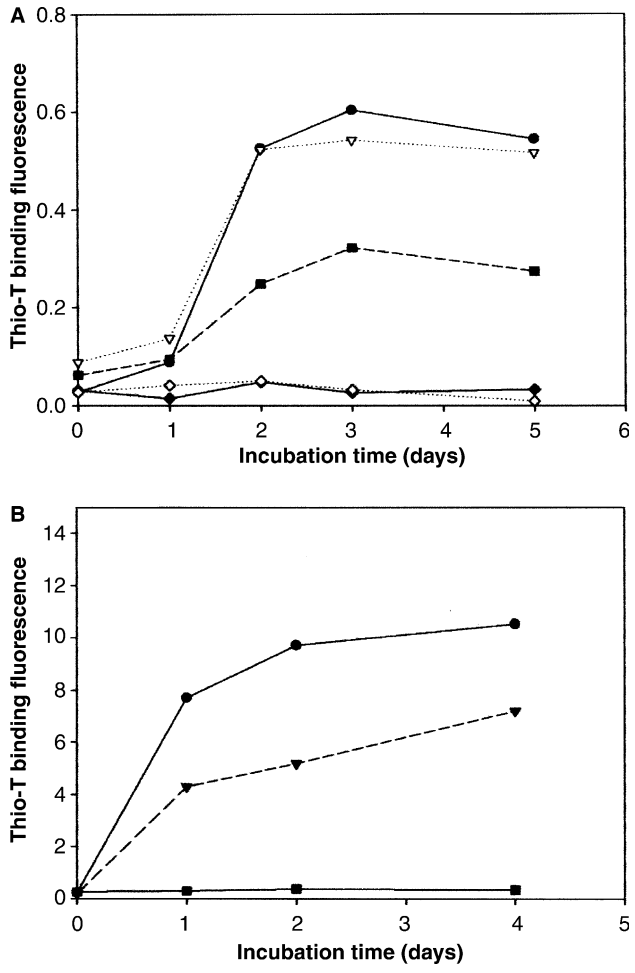

Fig. 3. Inhibitory effect of β-synuclein on the amyloid formation of amyloid β/A4 protein ($A\beta_{1-40}$) and α-synuclein. (A) 50 μM $A\beta_{1-40}$ was incubated at 37 °C in the absence (●) or presence of either 50 μM α-synuclein (▽) or β-synuclein (■). As controls, the extent of the aggregation of α-synuclein (◇) and β-synuclein (◆) is also presented during the same incubation period. (B) 100 μM α-synuclein was incubated with equimolar β-synuclein (▼). Aggregation of α-synuclein (●) or β-synuclein (■) is shown as controls. The extent of aggregation was determined by the thioflavin-T binding fluorescence assay [23].

period as judged by the increased fluorescence upon binding thioflavin T. In contrast, α-synuclein had no such inhibitory effect. The suppressive activity of β-synuclein continued during prolonged incubation over 3 weeks (data not shown). In agreement with previous reports [18,19], the fibrillization of α-synuclein was also inhibited in the presence of equimolar amount of β-synuclein (Fig. 3B). β-Synuclein itself did not influence the fluorescence intensity caused by the binding of the amyloid to thioflavin-T. Although the mechanism of β-synuclein action on the $A\beta_{1-40}$ aggregation is unknown and remains to be further investigated, β-synuclein was found to not only act as an inhibitor of α-synuclein fibrillization but also exhibit the similar anti-fibrillar activity against another amyloidogenic molecule of Aβ. Thus, it is tempting to propose that β-synuclein can have a potential to be widely applied for controlling pathologically abnormal protein aggregations.

β-Synuclein was shown to be more effective than α-synuclein in terms of its molecular chaperone activity. Despite their sequence homology and structural similarity, β-synuclein lacks the hydrophobic middle region of α-synuclein (residues 71–82) and also the acidic C-terminal regions of α- and β-synucleins are significantly distinct [2,19,25]. Thus, these structural differences in primary sequences seem to be the major reason why the chaperone activities of the two synucleins are different. Since the N-terminus of α-synuclein including non-Aβ component (NAC) of AD amyloid has been suggested to serve as a substrate binding domain for the chaperone function [16], the lack of 11 residues of the NAC within β-synuclein might lead to the chaperone activity of β-synuclein different from that of α-synuclein by improving its ability to bind and suppress aggregations of denatured proteins. Furthermore, although both synucleins have highly charged and flexible regions in their C-termini, the sequence variation in these regions might be also responsible for the enhanced chaperone activity of β-synuclein. Based on the previous report that the C-terminus of α-synuclein is particularly important in performing the chaperone function [16], it can be hypothesized that any differences in the C-terminal composition of β-synuclein can increase its ability to prevent the protein aggregation. Moreover, the C-terminus of β-synuclein shows a certain degree of sequence homology with the charged region found in the protein family of Hsp 90, an abundant heat shock protein in eukaryotic cells (Fig. 4). The charged region in the eukaryotic Hsp 90 followed by the N-terminal chaperonic site has been reported to give Hsp 90 a higher affinity for denatured polypeptides [26], suggesting that the C-terminus of β-synuclein might be important during the recognition of the denatured protein.

β-Synuclein is abundantly expressed along with α-synuclein throughout the central nervous system of normal physiology. However, it was observed that the mRNA level of α-synuclein was increased, while the level of β-synuclein was decreased in the substantia nigra region of Lewy body diseases such as PD and diffuse Lewy body disease [17]. The relevance of the decreased level of β-synuclein during the pathogenesis has not been clarified yet. However, it was reported that overexpression of α-synuclein in mice and flies resulted in PD-like phenotypes [8,9] and double expression with β-synuclein ameliorated the motor deficit with the reduced formation of inclusion bodies [18]. These observations suggest that changes in the levels of both synucleins may be important for the progress of the disease. Therefore, balance between the two

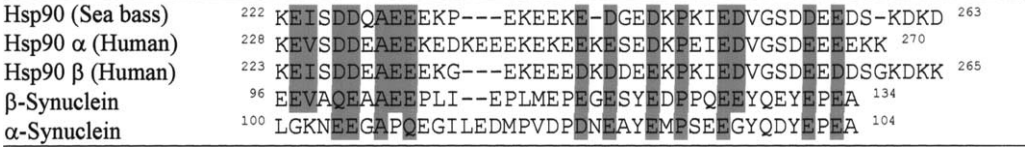

Fig. 4. Alignment of amino acid sequence between various Hsp 90 proteins and β-synuclein. The alignment was carried out using the MultAlin program (http://prodes.toulouse.inra.fr/multalin/multalin.html). Conserved amino acids are shaded.

synucleins may be critical in the pathogeneses and β-synuclein could be hypothesized to play a crucial role on cellular protection [17,28]. In this point of view, functional study of β-synuclein is needed in order to identify physiological and pathological roles of synucleins. Since α-synuclein could be readily damaged by environmental stresses and undergo abnormal protein aggregation, it is speculated that the proportionally high level of β-synuclein inside the cell could complement the deficiency of α-synuclein in addition to the fact that β-synuclein can prevent the protein aggregation through direct molecular interaction.

In conclusion, anti-aggregative activity of β-synuclein is remarkably higher than that of α-synuclein against the heat-induced aggregation of different proteins and also β-synuclein inhibits the amyloid formation of both $A\beta_{1-40}$ and α-synuclein. It is suggested that β-synuclein can prevent abnormal protein aggregations more efficiently than α-synuclein by acting as a molecular chaperone, although it remains to be investigated whether β-synuclein can function in the same way under normal cellular environment.

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