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ORIGINAL PAPER

Protective effect of 3,5,3′-triiodothyroacetic and 3,5,3′,5′-tetraiodothyroacetic acids on serum albumin fibrillation

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Abstract Inhibition or reversion of protein self-aggregation has been suggested as a possible preventive mechanism against amyloid diseases, and many efforts are underway to found out molecules capable to restrain the protein aggregation process. In this paper, the inhibitory effects of thyroid hormone analogues on heat-induced fibrillation process of serum albumin are reported. Among the analogues tested, 3,5,3′,5′-tetraiodothyroacetic and 3,5,3′-triiodothyroacetic acid showed the most important inhibitory effects on amyloid formation. Thyroxine exhibits a lesser protective effect, while 3,5,3′-triiodothyronine showed no significant inhibition. The gaining of a negative charge together with a size reduction of the hormone molecule could play an essential role in the inhibition of fibrils formation. According to infrared spectroscopy results, the thyroid hormones analogues protective effects proceed via the stabilization of the protein native structure. The current work demonstrates the effectiveness of naturally occurring molecules in the inhibition of albumin fibril formation.

Keywords Thyroid hormones · Serum albumin · Fibrillation · 3,5,3′-Triiodothyroacetic acid · TRIAC · 3,5,3′,5′-Tetraiodothyroacetic acid · TETRAC · Thyroxine · Amyloid inhibition

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Introduction

Protein misfolding and the subsequent aggregation is associated with various, often highly debilitating, diseases for which no sufficient cure is available yet (Kelly 1996; Sipe 1992). The number of pathologies associated to tissue deposition of amyloid fibrils in humans and animals dramatically increased in the last decade. The outstanding importance of protein self-aggregation in human and bovine diseases supports the extensive effort directed towards obtaining better understanding of fibrils formation mechanism. Under in vitro solution conditions where the native state is destabilized, many proteins present an abnormal structure with a strong tendency to self-aggregation into a polymeric amyloid fibril structure (Dobson 2003). At present, it is accepted that the ability to form amyloid structures is not an unusual feature of the small number of proteins associated with these diseases but is, instead, a generic feature of any polypeptidic chain (Ahmad et al. 2004; Stefani and Dobson 2003). For an increment number of proteins, environmental stress conditions like high temperature and extreme pH trigger off an alternative folding pathway and lead proteins to partial folded state, followed by formation of a cross β-sheet structure (Harper and Lansbury 1997; Holm et al. 2007; Kelly et al. 1997; Lai et al. 1996; McLaurin et al. 2000; Whittingham et al. 2002). Considerable early evidence has indicated that amyloid fibrils are toxic principally when they associate to very high levels in, e.g. systemic amyloidoses, causing organ failure (Dische et al. 1988). However, the notion that oligomeric intermediates are the most

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harmful forms of these proteins for living cells due to their ability to permeabilize cell membranes is currently accepted (Kayed et al. 2003; Volles and Lansbury 2003; Volles et al. 2001). In this way, effective therapeutic strategies should ideally target both oligomeric and fibrillar species. In this regard, many efforts are underway to screen compounds that interfere with the conversion to these structures by favouring the transformation kinetics towards native stable states (Arora et al. 2004; Miroy et al. 1996; Morshedi et al. 2007). However, a few of those inhibitors are currently undergoing clinical trials as potential drugs to treat neurodegenerative disorders (Conway et al. 2001; Murphy 2002; Tjernberg et al. 1996). In vitro studies shows that the T_4 binding to Transthyretin stabilizes the protein tetrameric state reducing the amyloid fibril formation (Miroy et al. 1996). Recently, it was demonstrated that iodine atoms inserted in TTR binding compounds is a crucial factor for design novel highly potent TTR fibrillogenesis inhibitors (Mairal et al. 2009). In order to study the role of iodinated compounds on the fibrillation inhibition of other thyroid hormones carrier, the ability of thyroid hormones analogues to inhibit in vitro amyloid-like fibrils formation from bovine serum albumin (BSA) was reported. Our results could shed light on the mechanism of amyloid fibrillation inhibition reporting natural occurring compounds, which have protective action against the interchain β-sheets formation. We also suggest some molecular requirements of iodinated compounds to enhance their effectiveness as amyloid fibrils inhibitors, and thus could be useful in rational drug design.

Materials and methods

Materials

Bovine serum albumin fraction V (BSA), Congo red (CR), Thioflavin T (ThT), 3,5,3′,5′-Tetraiodo-L-thyronine (L-T_4), 3,5,3′-Triiodo-L-thyronine (L-T_3), 3,5,3′,5′-Tetraiodo-D-thyronine (D-T_4), 3,5,3′-Triiodo-D-thyronine (D-T_3), 3,5,3′-Triiodothyroacetic acid (TRIAC) and 3,5,3′,5′-Tetraiodothyroacetic acid (TETRAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of the highest purity available. The BSA sample purity was electrophoretically controlled.

In vitro BSA fibril formation

To drive BSA towards amyloid fibrils formation, the protein was incubated at 65°C and pH 7.40 according to Holm et al. (2007). Stock solution of BSA was made at 5 mg/ml in Hepes buffer (40 mM Hepes, 60 mM NaCl, 4 mM KCl, 3.3 mM Ca_2Cl, pH 7.40). It was then diluted to 2.5 mg/ml (38 μM) with solutions containing TH analogues or with buffer for the control experiments. TH analogues solutions were prepared according to Petersen et al. (1996). The BSA:TH molar ratio was ranged from 1:1 to 1:5. Higher hormones concentration could not be used due to the low hormone solubility in aqueous media. The heating treatment was done in sealed test tubes (0.2 ml) to prevent any possible evaporation. The samples were taken out at different times and stored on ice before adding CR or ThT. For longer storage before measurement, tubes with samples were removed and frozen at −20°C.

Thioflavin T fluorescence measurements

A quantity of 80 μl of sample was mixed with 2 ml of 20 mM Tris-HCl, pH 7.4 containing 30 μM ThT and incubated 30 min at 37°C in a quartz cuvette. All fluorescence measurements were carried out with a ISS (Champaign, IL, USA) PC1 spectrofluorometer according to LeVine (1993). The excitation wavelength was set at 450 nm with emission measured at 482 nm with excitation slit width at 0.5 nm and emission slit width at 1.0 nm. Each reading was done in triplicate and the average result was used for data analysis.

Congo red absorption measurements

A quantity of 50 μl of sample was mixed with 1 ml PBS-buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 pH 7.4) containing 20 μM CR and incubated 30 min at room temperature. The absorbances were measured at 403 and 541 nm using a 1-cm path length cell (Nilsson 2004). The concentration of fibrils bound to CR was calculated according to [Fibril] (mM) = A_{541}/47.8 + A_{403}/38.1.

Fourier transform infrared (FTIR) spectroscopy measurements

Around 50 μl of sample containing 2.5 mg/ml of BSA in buffer Hepes-D_2O pD 7.4 (pD = pH + 0.4 pH unit) was loaded onto a demountable liquid cell (Harrick Scientific, Ossining, NY, USA) with calcium fluoride windows and 100 μm spacers. A BSA:TH molar ratio of 1:5 was used. The samples were recorded in a Nicolet 5700 spectrometer equipped with a DTGS detector (Thermo Nicolet, Madison, WI, USA). The sample chamber was purged with dry, CO_2-free air. Spectra were collected by using OMNIC (Nicolet) software. Usually 256 scans/sample were taken, averaged, apodized with a Happ-Genzel function, and

Fourier transformed to give a final resolution of 2 cm^{-1}. The contribution of D_2O in the amide I′ region was eliminated by subtracting the buffer spectra from the solution to obtain a flat baseline of between 2,000 and 1,700 cm^{-1}. The deconvolution, band position determination and bandwidth measurement, together with the curve fitting of the original amide I band were performed as reported previously (Arrondo et al. 1993; Chehin et al. 1999). Briefly, band component positions were obtained from deconvolution and derivation. Since the results obtained after iterations may not be unique, the following restrictions were applied: (1) from initial guesses, the band position could not diverge more than the distance between data points and (2) the width of the bands should be less than one-half of the amide I bandwidth. The use of several spectra recorded at different temperatures below the thermal denaturation reduces the error of the quantification procedure to around 3% (Banuelos et al. 1995).

Results and discussion

Influence of TH analogues concentration on the BSA fibrils formation

Congo red absorption and Thioflavin T fluorescence intensity measurements have been widely used as specific tests to study the cross β-structure found in amyloid fibrils (Eisert et al. 2006; LeVine 1999). Despite that BSA is able to form well-ordered β-sheet rich aggregates, they do not possess the same structural rigidity as classical fibrils (Holm et al. 2007). However, the BSA heat-induced fibrils formation could be evidenced by an increase in the ThT fluorescence intensity and CR absorption which is accompanied by a small redshift from 495 to 500 nm (Fig. 1a, b). In order to determinate whether TH analogues are capable to affect the fibrillation process, a BSA solution (38 μM) was preincubated with different concentrations of L-T_3, L-T_4, D-T_3, D-T_4, TRIAC and TETRAC (from 3.8 to 190 μM), and the heat-induced fibrillation was performed according to "Materials and methods". Figure 1 shows that several TH analogues were able to inhibit the amyloid-like fibrils formation as suggested by the observed decrease of both ThT fluorescence intensity and CR absorption after 3 h at 65°C. The fibrillation process was highly dependant on the TH analogues concentration and the magnitude of the inhibitory effect was TETRAC > TRIAC > T_4 (D-T_4, L-T_4) while T_3 (L-T_3, D-T_3) did not show any effect (Fig. 1c, d). It is important to note that the isomers D and L showed the same performance, and thus, in the following experiments D-T_4, L-T_4 and L-T_3, D-T_3 will be referred to as T_4 and T_3, respectively. TETRAC and TRIAC showed the highest effectiveness at molar ratio from 1:2 to 1:5.

Thyroid hormones analogues effects on BSA fibrils formation kinetic

The effect of TH analogues on the kinetics of BSA amyloid-like fibrils formation was investigated through monitoring the maximal ThT emission intensity over the course of 5 h. The BSA aggregation process proceeds without a lag phase, depends on the protein concentration, is not accelerated by seeding, and the kinetics of the process could be fitted to a hyperbolic function (Holm et al. 2007). Figure 2 shows that the presence of TH analogues did not change this kinetics reaching the plateau within the first 200 min. The apparent half-life of aggregation ($t_{1/2}^{agg}$) is around 25 min for BSA alone as well as in the presence of all TH analogues tested. However, the maximal aggregation is reduced in about 80% in the presence of TETRAC, regarding to the control. TRIAC and T_4 also diminished the total amount of fibrils formed but with less efficiency (70 and 30%, respectively). These results indicate that the TH analogues protective effects are due to the reduction of the total amount of fibrils formed and not to a delay in reaching the maximal aggregation.

In general, the kinetics of a typical fibrillation process involve a lag phase followed by a relatively rapid elongation phase, which stabilizes at a plateau level (Choo-Smith et al. 1997; Kihara et al. 2005; Kim et al. 2007; Yagi et al. 2005). This lag period can be shortened by the presence of a "seed" of pre-formed amyloid fibrils from the same protein or from another protein with a short or null lag period. The absence of lag period in the BSA aggregation process was maintained also in the presence of TH analogues. In this context, the inhibition of the fibrillation of proteins like BSA, which could be putative seeds of other amyloidogenic proteins, acquire significance.

Thyroid hormones analogues effects on heat-induced BSA structural changes

Partially folded intermediates, obtained under stress treatment like high temperature or low pH, are critical in the amyloid fibril formation process (Millican and Brems 1994; Nielsen et al. 2001). In this way, some inhibitors are known to stabilize native protein structures against the changes preceding amyloid formation (Chiti et al. 2001; Soldi et al. 2006). To explore whether TH analogues brought about conformational changes in the BSA native state, FTIR studies were performed with BSA alone and in the presence of TH analogues. The band components and area percentages obtained from the fittings of amide I region are listed in Table 1. The infrared spectrum of BSA (Fig. 3a) confirms their predominantly alpha helical structure, since α-helix (1,653 cm^{-1} bands) and short segments connecting α-helices (1,631 cm^{-1} bands) dominates the spectrum as

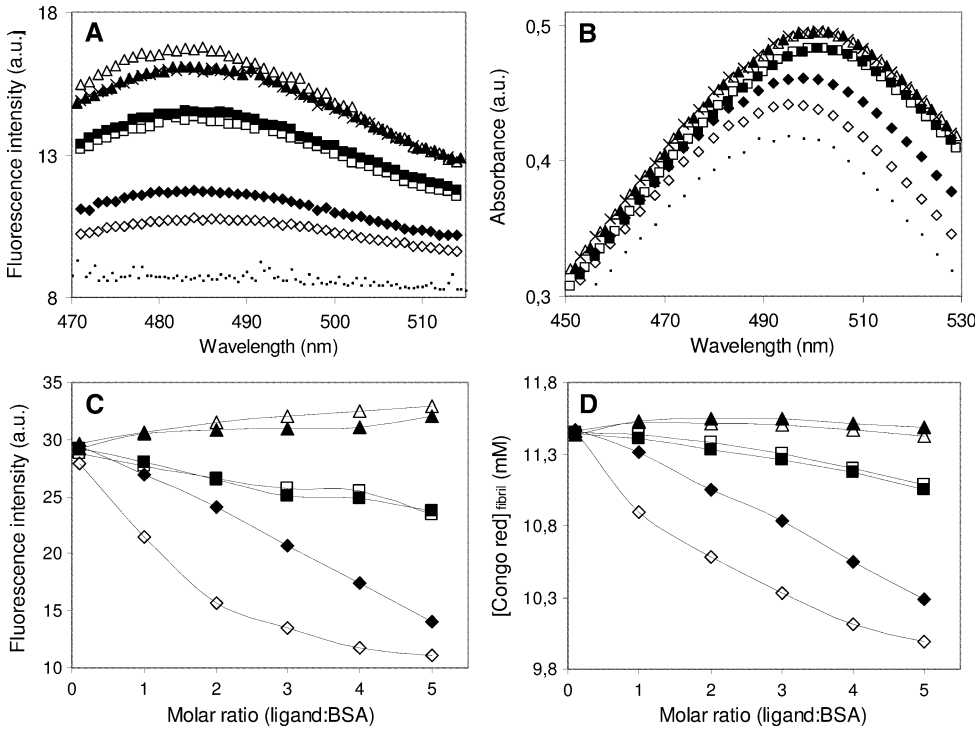

Fig. 1 Effect of various TH analogues on BSA amyloid-like fibrils formation. Emission spectra of ThT (**a**) and absorption spectra of CR (**b**) bound to BSA after 180 min of incubation at 65°C using a molar ratio of 5:1 (ligand:BSA). Fluorescence intensity of ThT (**c**) and concentration of CR (**d**) bound to BSA as a function of ligand:BSA molar ratio after incubation at the same condition as in **a** and **b**. In all cases the protein concentration was 2.5 mg/ml. BSA alone (*multiplication symbol*) and in the presence of L-T3 (*filled triangle*), D-T3 (*open triangle*), L-T4 (*filled square*), D-T4 (*open square*), TRIAC (*filled diamonds*) and TETRAC (*open diamonds*).The spectrum of BSA alone before incubation is represented by *stippled line*

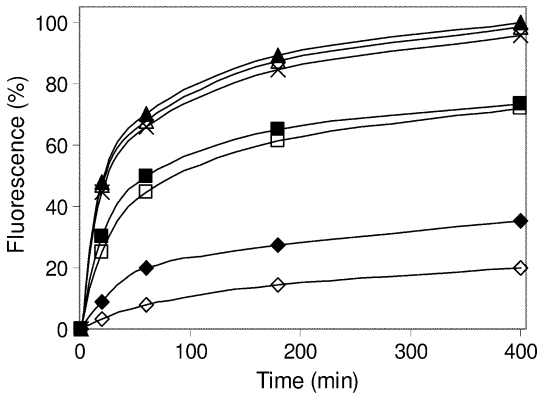

Fig. 2 Kinetics of BSA (2.5 mg/ml) fibrillation process in the absence and the presence of different TH analogues followed by changes in ThT fluorescence intensity after incubation at 65°C and a molar ratio of 5:1 (ligand:BSA). BSA alone (*multiplication symbol*) and with L-T_3 (*filled triangle*), D-T_3 (*open triangle*), L-T_4 (*filled square*), D-T_4 (*open square*), TRIAC (*filled diamonds*) and TETRAC (*open diamonds*)

Table 1 Band position (cm^{-1}) and percentage area (%) corresponding to the components obtained after curve fitting the Amide I band depicted on Fig. 2a, b

Band position	Temperature							
	25°C			65°C				
	1,631	1,653	1,676	1,613	1,628	1,649	1,673	1,683
	Area (%)							
BSA	26	69	5	5	20	56	8	11
BSA plus L- or D- T_3	26	69	5	4	21	56	8	11
BSA plus L- or D- T_4	26	69	5	4	26	61	7	2
BSA plus TRIAC	26	69	5	1.8	29	59	10	0.2
BSA plus TETRAC	25	70	5	0.9	26	64	9	0.1

previously described (Murayama and Tomida 2004). The minor band at 1,676 cm^{-1} is associated with turn structures. The addition of TH analogues did not show any change in the BSA structure after 3-h incubation at 25°C. After heating treatment, the FTIR spectrum showed significant changes (Fig. 3b; Table 1) like the appearance of two characteristic bands located around 1,613 and 1,683 cm^{-1}, arising from interchain β-sheets hydrogen bonds, achieving hardly 15% of the Amide I band area; the presence of a band located at 1,628 cm^{-1} characteristic from antiparallel intramolecular β-sheets (Arrondo et al. 1993); the absence of the band assigned to short segments connecting α-helices; the slight increment of turns and the decrease of the alpha helix structure. In presence of T_3, the same conformational changes were observed confirming that this hormone is unable to protect the protein against heat-induced aggregation. On the contrary, the presence of TRIAC and TETRAC showed a clear protective effect, since the amount of intermolecular β-sheet were nearly insignificant. Moreover, the alpha-helix band area diminished only about 6%

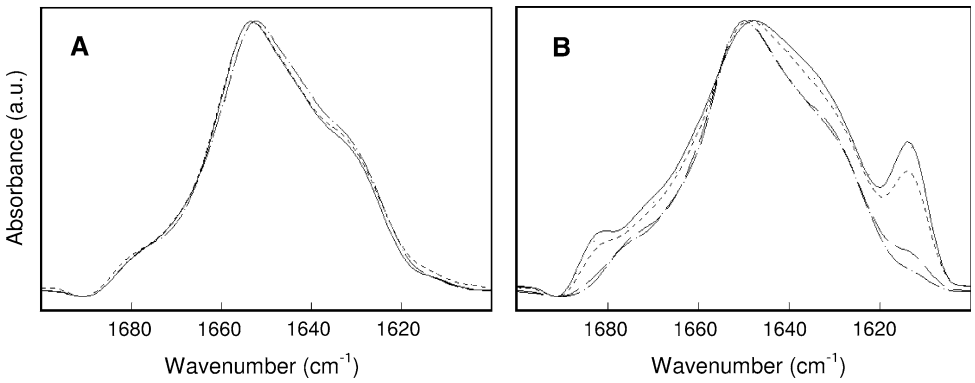

Fig. 3 FTIR absorption spectra in the amide I region of BSA alone (*solid line*) and in the presence of T_4 (*dotted line*), TRIAC (*dashed line*) and TETRAC (*point dashed line*) before (**a**) and after (**b**) 180 min of incubation at 65°C. The protein concentration was 2.5 mg/ml and the BSA:ligand molar ratio was 1:5. The spectra have been deconvolved with a halfwidth of 18 and a $K = 1.75$

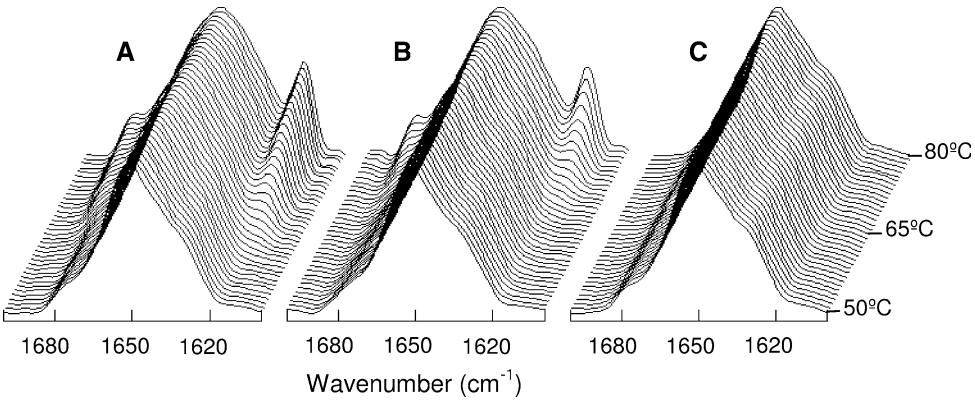

Fig. 4 Stacked FTIR spectra of BSA (2.5 mg/ml) in the absence (**a**) and in the presence of TRIAC (**b**) and TETRAC (**c**), recorded at regularly increasing temperature from 50 to 80°C with a heating rate of 1°C/min. The BSA:ligand molar ratio was 1:5. The spectra have been deconvolved with a halfwidth of 18 and a $K = 1.75$

indicating that the hormone is able to protect the native structure supporting its alpha-helix content. A lesser protective effect was observed in the presence of T_4, since the intermolecular β-sheet signal represents the 8% of the Amide I band area.

Changes in the amide I band using a heating rate of 1°C/min in the temperature range from 50 to 80°C are depicted in Fig. 4. The two characteristic bands associated with intermolecular β-sheet and located at 1,613 and 1,683 cm^{-1} appeared on the BSA spectra around 64°C. The presence of T_4 and TRIAC delayed the occurrence of these two bands until 68°C (not shown) and 70.5°C, respectively. In this way, TETRAC was more efficient to inhibit the formation of interchain β-sheets in all the temperature range studied, since no new band was detected in the FTIR spectra.

In this paper, ThT fluorescence, CR absorption and FITR studies demonstrated that several TH analogues could protect BSA against heat-induced amyloid-like fibrils formation. On the basis of the chemical structure of the different analogues used, it may be proposed that their protective effect depends on: (1) the number of iodine atoms in the outer ring since, in the presence of T_4, the amount of fibrils formation was reduced by about 30% as compared with T_3 (Fig. 2b) and (2) the presence of acetate instead of L- or D-alanine, since greater effects of TETRAC versus T_4 and TRIAC versus T_3 were observed. The

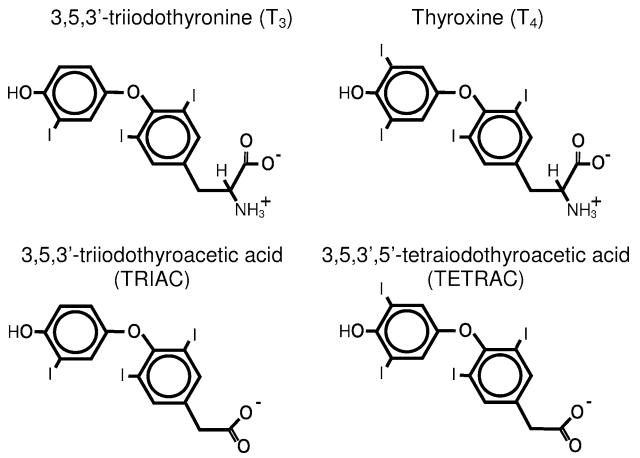

Fig. 5 Structure of thyroid hormones and analogues

replacement of a zwitterionic group like alanine [$-CH_2-HC(NH_3^+)-COO^-$] by acetate group [$-CH_2-COO^-$] resulted in gaining negative charge and a chain size reduction (Fig. 5). Thus, the acetate moiety together with the presence of a 5′ iodine atom showed a nearly additive effect, resulting in about 80% inhibition of BSA fibrillation. In this context, crystallographic analyses described specific interactions between human serum albumin residues (76% sequence identity with BSA) and the carboxylate and the phenolic group of T_4 (Petitpas et al. 2003).

The results presented herein suggest the molecular requirements of thyroid hormones analogues to inhibit the BSA amyloid-like fibrils formation and could shed light on de novo fibrillation inhibitors design.

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