FEBS Letters 580 (2006) 491–496

Chromium(III) ion and thyroxine cooperate to stabilize the transthyretin tetramer and suppress in vitro amyloid fibril formation

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Received 3 November 2005; revised 29 November 2005; accepted 15 December 2005

Available online 22 December 2005

Edited by Jesus Avila

Abstract Transthyretin (TTR) amyloid fibril formation, which is triggered by the dissociation of tetrameric TTR, appears to be the causative factor in familial amyloidotic polyneuropathy and senile systemic amyloidosis. Binding of thyroxine (T_4), a native ligand of TTR, stabilizes the tetramer, but the bioavailability of T_4 for TTR binding is limited due to the preferential binding of T_4 to globulin, the major T_4 carrier in plasma. Here, we show that Cr^{3+} increased the T_4-binding capacity of wild-type (WT) and amyloidogenic V30M-TTR. Moreover, we demonstrate that Cr^{3+} and T_4 cooperatively suppressed in vitro fibril formation due to the stabilization of WT-TTR and V30M-TTR.

Keywords: Amyloid; Transthyretin; Thyroxine; Cr^{3+}; Familial amyloidotic polyneuropathy

1. Introduction

Transthyretin (TTR), which is present in human plasma and cerebrospinal fluid, is a homotetrameric protein of 55 kDa. TTR binds and transports thyroxine (T_4) and the retinol binding protein [1]. In certain individuals, TTR is converted into an insoluble fibrillar structure called amyloid. The precise mechanisms underlying the conversion of TTR into amyloid fibrils are unknown, but the extensive β-sheet structure of TTR might be responsible for its amyloidogenic potential [2]. These amyloid fibrils putatively cause senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathy (FAP) by virtue of the neurotoxic effects of amyloid or by means of physical interference with normal organ function [3]. Tetrameric TTR is not itself amyloidogenic, but dissociation of the tetramer into a compact non-native monomer with low conformational stability can lead to amyloid fibril formation [4]. Several of the 50 FAP-associated TTR single-site mutations have a normal tetrameric structure under physiological conditions [5]; however, these mutations significantly destabilize the tetramer [6,7]. Preventing the conformational changes which initiate amyloid fibrillization could intervene in the pathogenesis of FAP [8]. Small inhibitor molecules that bind to unoccupied T_4-binding sites of TTR and the halide ions, chloride and iodide, were shown to enhance tetrameric TTR stability [9,10]. We screened a number of metal ions that can affect in vitro amyloid formation and we report here that Cr^{3+} enhanced the effect of T_4 on the thermostability of both normal- and V30M-TTR tetramers and suppressed tetramer dissociation induced by low pH.

2. Materials and methods

2.1. Purification of wild-type TTR and amyloidogenic V30M TTR from human plasma

Serum wild-type TTR (s-WT-TTR) was purified as described previously [11]. Serum amyloidogenic V30M-TTR (s-V30M-TTR) was prepared using fraction IV obtained from human plasma of homozygotic V30M FAP patients by Cohn's ethanol fraction method. The subsequent purification was essentially as described by Ando et al. [11].

2.2. Preparation of recombinant WT-TTR (r-WT-TTR)

Two primers, 5′-AACATATGGGTCCGACCGGTACCGGTGA-3′ and 5′-AAGTCGACTTATTCTTTCGGGTTGGTAA-3′, were designed to amplify the wild-type TTR gene by PCR. The PCR product was digested and ligated into *Nde*I/*Sal*I pre-digested pET-22b(+) vector (Novagen, Darmstadt, Germany). The TTR plasmid was used to transform *Escherichia coli* strain, BL21(DE3) STAR (Invitrogen, Carlsbard, CA). Protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h before harvesting cells. Cell pellet was resuspended in 20 mM phosphate buffer saline (PBS, pH 7.0), and lysed by sonication at 4 °C. The supernatant was obtained, filtered and applied onto a DEAE Sepharose Fast Flow column (Amersham). Bound proteins were eluted with a 20 mM phosphate (pH 7.0)/200 mM NaCl buffer. TTR was further purified by reverse-phase high-performance liquid chromatography (Cosmosil 5C4-AR-300 column, Nacalai Tesque, Japan) with an acetonitrile gradient. The fractions containing TTR were pooled and dialyzed extensively against 20 mM NH_4HCO_3 solution, followed by lyophilization. Purity of TTR was assessed by SDS–PAGE.

2.3. $[^{125}I]T_4$ and $^{51}Cr^{3+}$ binding studies

Serum WT-TTR and V30M-TTR having a concentration of 100 μg/mL in PBS buffer were used for T_4 and Cr^{3+} binding analyses. The concentration of $[^{125}I]T_4$ used was 0.6 nM for binding with s-WT-TTR and

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0014-5793/$32.00
doi:10.1016/j.febslet.2005.12.047

6.0 nM for s-V30M-TTR. Binding of [^{125}I]-labeled T_4 (1500 μCi/μg, Amersham Biosciences, NJ) to TTR was carried out at a total volume of 25 μL at 4 °C for 14–18 h in the absence or presence of $CrCl_3$ (300 or 500 μM). Free and protein-bound [^{125}I]T_4 were separated using Centri-Sep columns (Princeton Separations, NJ) and the bound [^{125}I]T_4 was determined using an auto-well gamma counter (ARC-2000, Aloka). The affinity constants and the number of binding sites were calculated using Scatchard analysis. For binding assay with Cr^{3+}, 5 μM of [^{51}Cr]Cl_3 (872 μCi/μg, Perkin–Elmer, MA) was incubated with TTR protein solution in the absence or presence of 360 nM T_4, in a manner as described above. All incubations were done in duplicate.

2.4. Thioflavine T-binding assay

Serum or recombinant WT-TTR, or serum V30M-TTR was incubated at 37 °C for 5 days in PBS (pH 4.0) with the indicated concentration of Cr^{3+} and 360 nM to 10 μM T_4 or 0 to 10 μM diflunisal. TTR samples were prepared to a final concentration of 0.2 mg/mL. Thioflavine T-binding assays were performed on 2.5 μg/mL TTR samples by adding freshly prepared 10 μM thioflavine T to 50 mM glycine buffer (pH 9.0). Fluorescence emission spectra were obtained with excitation and emission wavelengths of 450 and 482 nm, respectively. Fluorescence measurements were performed with a F-4500 Hitachi spectrofluorometer (Hitachi, Tokyo, Japan).

2.5. High-sensitivity differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed with a differential scanning calorimeter (MC-2, MicroCal, Northhampton, MA) with cell volumes of 1.22 mL using heating rates of 1 K/min, as described previously [12]. TTR samples were prepared at a concentration of 25 μM in 20 mM sodium phosphate and 150 mM NaCl at the indicated pH in the presence or absence of Cr^{3+}. The data obtained from DSC were applied to non-linear fitting algorithms to calculate the thermodynamic parameters, thermal denaturation temperature (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_v), from the temperature dependence of excess molar heat capacity, C_p, by employing Origin™ scientific plotting software (OriginLab Co.).

2.6. Far circular dichroism

Far circular dichroism (CD) was monitored with JASCO J-720 (Nihon Bunko, Tokyo, Japan). Serum WT-TTR samples were incubated at 37 °C for 2 days in PBS buffer (pH 4.0) with or without Cr^{3+} (500 μM). Samples were then analyzed at 25 °C using a bandwidth of 1.0 nm, a time constant of 1 s, a step resolution of 0.5 nm and a scan speed of 5 nm/min. CD measurements were carried out in triplicate and spectra were reported as the means of three scans in the range of 220–240 nm.

3. Results

3.1. Effect of Cr^{3+} on the binding of T_4 to serum WT-TTR and V30M-TTR

It has been shown that binding of the natural ligand T_4 stabilizes the tetrameric TTR [13] and that certain metal ions affect amyloidogenesis [14], therefore, we first determined the effect of Cr^{3+} on T_4 binding to TTR. Scatchard analysis of purified s-WT-TTR and radiolabeled T_4 resulted in a rectilinear curve with a measured affinity (K_a) of 149.5 L/mmol. The number of binding sites per mol TTR in the eluate was 1.16 (Table 1). Surprisingly, Cr^{3+} dose-dependently increased the concentration of bound T_4. In the sample containing 500 μM Cr^{3+}, the bound T_4 and the number of binding sites per mol TTR were approximately twice than that without Cr^{3+} (Table 1 and Fig. 1A). However, the binding affinity for T_4 was diminished by the addition of Cr^{3+} (Table 1). We also examined the effect of Cr^{3+} on T_4-binding to V30M-TTR and found that the K_a was slightly increased upon the addition of 500 μM Cr^{3+}. Moreover, the number of bound T_4 per mol V30M-TTR was increased 3-fold in the presence of Cr^{3+} (Table 1). These data suggested that Cr^{3+} could increase the maximal binding capacity of WT-TTR and V30M-TTR for T_4.

We next asked whether Cr^{3+} itself could possibly bind to WT-TTR. In Scatchard analysis using purified s-WT-TTR and [$^{51}Cr^{3+}$], we observed that the molar ratio of Cr^{3+} binding sites to TTR was 2.16. The presence of T_4 had no effect on the number of bound ligands per mol TTR (Table 1 and Fig. 1B). However, the K_a was diminished from 1.40 (T_4, 0 nM) to 0.60 (T_4, 360 nM). These results indicated that Cr^{3+} might bind to WT-TTR and that T_4 negatively affects the binding affinity but not the binding capacity of WT-TTR towards Cr^{3+}.

3.2. Cr^{3+} and T_4 cooperatively suppressed amyloid fibril formation

To investigate the effect of Cr^{3+} and T_4 on TTR amyloid fibril formation, we performed thioflavine T-binding assay. It was previously shown that a T_4 concentration of more than 10 μM was needed for T_4 alone to show a suppressive effect on amyloid fibril formation of WT-TTR [13]. However, 0.36 μM T_4 together with low Cr^{3+} concentrations (10–50 μM) significantly suppressed amyloid fibril formation of s-WT-TTR, when incubated at pH 4.0 for 5 days (Fig. 2A). Amyloid fibril formation of r-WT-TTR was also suppressed by Cr^{3+} in combination with T_4 (0.36 μM), albeit at a higher concentration of Cr^{3+} (500 μM) (Fig. 2B). To understand the discrepant effect of Cr^{3+} on s-WT-TTR and r-WT-TTR, we determined the level of T_4 remaining in the TTR samples purified from human plasma used in this study. We found that s-WT-TTR samples contained 40 nM T_4 in bound form (data not shown). The presence of this natural ligand most probably contributed to the stabilization of the tetrameric s-WT-TTR.

Table 1
Summary of scatchard analysis of T_4 and Cr^{3+} binding in serum TTR

	Bound ligands (μM)	Bound ligands/tetramer TTR	K_a (L/mmol)
T_4 binding			
WT-TTR	2.16	1.16	149.5
WT-TTR with Cr^{3+} (60 μM)	2.37	1.28	105.8
WT-TTR with Cr^{3+} (300 μM)	3.06	1.65	86.0
WT-TTR with Cr^{3+} (500 μM)	3.97	2.14	47.2
V30M-TTR	0.29	0.16	18.8
V30M-TTR with Cr^{3+} (500 μM)	1.00	0.54	29.7
Cr^{3+} binding			
WT-TTR	4.00	2.16	1.40
WT-TTR with T_4 (360 nM)	4.17	2.25	0.60