Molecular interconversion of cold-sensitive cytosolic 3,3',5-tri-iodo-L-thyronine-binding proteins from human erythrocytes: effect of cold, heat and pH treatments

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Cytosolic 3,3',5-tri-iodo-L-thyronine-binding proteins (CTBP I, II and IV species) from human red blood cells undergo rapid loss of activity at low temperatures. Cold treatment of CTBPs was accompanied by dissociation of the polymeric protein to the 60 kDa inactive monomer. Re-activation of the cold-inactivated

CTBP IV by warming resulted in association of the monomer to the active polymeric form. A similar association–dissociation phenomenon was also obtained isothermically, though pH changes. We conclude that CTBP I and CTBP II are polymeric forms of CTBP IV.

INTRODUCTION

A recent report from our laboratory indicated the presence of cytosolic 3,3',5-tri-iodo-L-thyronine-binding proteins (CTBPs) in human erythrocytes [1]. These CTBPs were divided into two groups: one cold-insensitive (CTBP III) and another cold-sensitive with three 3,3',5-L-tri-iodothyronine (L-T₃)-binding-activity species (CTBP I, II and IV). The latter CTBPs are inactive at 4 °C and could be re-activated at 20 °C or 37 °C. After heat treatment, CTBP II showed a high affinity site with K_d 10^{−10} M, able to bind L-T₃ at concentrations similar to those observed for the free hormone in plasma [1]. In the present work, we show that a molecular interconversion between cold-sensitive CTBP species occurs under different experimental conditions and that the CTBP association–dissociation event correlates with L-T₃ binding activity. CTBP I and CTBP II appear to be polymeric forms of CTBP IV.

MATERIALS AND METHODS

Isolation of CTBPs

CTBPs from human red blood cells were prepared as previously described [1]. The CTBPs were isolated at room temperature from a DEAE-cellulose column by using an NaCl gradient. CTBP I, CTBP II, CTBP III and CTBP IV were eluted at 50, 150, 280 and 350 mM NaCl respectively. Peak CTBP I represents 2–10 % of the activity of peak CTBP II in different preparations, whereas CTBP IV represents 25–30 % of the activity of CTBP II. Fractions containing cold-sensitive CTBPs activities were pooled, dialysed and stored at 4 °C for not more than 1 week or frozen at −20 °C for 1 month without loss of binding activity.

Sephacryl S-200 HR filtration

CTBP fractions were applied separately to a column (1.7 cm × 80 cm) of Sephacryl S-200 HR, equilibrated and eluted as specified in each case; 1.5 ml fractions were collected and assayed for ¹²⁵I-L-T₃ binding activity. Linear regression of log M_r

versus K_{av} was used for calculating the molecular mass of CTBPs.

¹²⁵I-L-T₃-binding assay

This was performed at 4 °C for 30 min as previously described [1], by using a Dowex resin for separation of free and bound hormone populations. Non-specific binding was determined as radioactivity bound in the presence of 1 μM unlabelled L-T₃. Specific binding was calculated by subtracting non-specific binding from total L-T₃ binding activity.

Materials

¹²⁵I-L-T₃ (sp. radioactivity 3300 μCi/μg) was obtained from Dupont/New England Nuclear. Other reagents were purchased from Sigma Chemical Co.

RESULTS

Temperature effects

Figure 1 shows the changes in activity of the cold-sensitive CTBPs stored overnight at 4 °C after heating and cooling cycles. Thus the specific L-T₃-binding activity of cold-sensitive CTBPs was rapidly decreased by cold exposure and rapidly increased by heat treatment at 20 °C.

A previous report indicated that apparent molecular masses for CTBP I and CTBP II were about 200 kDa, and 60 kDa for CTBP IV [1]. The possibility that heat and cold treatments might trigger a molecular association–dissociation phenomenon between cold-sensitive CTBP forms was investigated by filtration on Sephacryl S-200 HR. Preliminary experiments showed that when CTBP IV (molecular mass 60 kDa) was previously heated for 60 min at 20 °C before chromatography at room temperature, the L-T₃-binding activity was eluted with an apparent molecular mass of 200 kDa. Besides, when CTBP II (molecular mass 200 kDa) was preincubated at 4 °C for 60 min and then passed through the gel-filtration column equilibrated at 4 °C, the binding activity was eluted with an apparent molecular mass of 60 kDa.

Figure 1 Reversible cold inactivation and heat activation of CTBPs

Samples (3 ml) of isolated CTBPs (CTBP I, 0.8 mg/ml; CTBP II, 1.0 mg/ml; CTBP IV, 0.25 mg/ml) which had been dialysed with 150 mM NaCl/10 mM sodium phosphate buffer, pH 7.6, and stored at 4 °C were preincubated in the same buffer and, successively, at 20 °C for 30 min, 4 °C for 60 min, 20 °C for 60 min and overnight (15 h) at 4 °C. At given time intervals, 0.2 ml samples were removed and assayed for specific L-T₃ binding as indicated in the Materials and methods section. Symbols: ○, CTBP I; ●, CTBP II; ▲, CTBP IV.

Figure 2 Gel filtration of cold-inactivated and heat-activated CTBPs

Samples (2 ml) of CTBP II (a) or CTBP IV (b) were preincubated at 4 °C (○) or 20 °C (●) respectively for 60 min. Each sample was applied separately to a Sephacryl S-200 HR column equilibrated with 150 mM NaCl/10 mM sodium phosphate buffer, pH 7.6, at 4 °C (○) or at room temperature (●). Fractions (1.5 ml) were collected and the specific binding activity was determined after heating at 20 °C for 60 min. Abbreviations: Amy, amylase; Cyt, cytochrome c.

Figure 2 shows the transformation from CTBP II to CTBP IV and from CTBP IV to CTBP II at 4 °C and 20 °C respectively. When the chromatography was carried out at 4 °C, no binding activity was detected without previous warming [1]. To detect L-T₃-binding activity the fractions were preincubated for 60 min at 20 °C. From Figures 1 and 2 it is possible to conclude that (i) cold preincubation of CTBPs is accompanied by dissociation

Figure 3 Effect of pH on cold inactivation and stability of CTBP

Two 2 ml portions of stored CTBP were heated at 20 °C for 60 min in 150 mM NaCl/10 mM sodium phosphate buffer and preincubated separately for another 60 min at 20 °C (●) or at 4 °C (○) at various pH values as indicated. pH changes were obtained by addition of 1 M NaOH or 1 M HCl. The specific L-T₃ binding was determined as indicated in the Materials and methods section after neutralization to pH 7.6 by addition of NaOH or HCl. CTBP activity is expressed as percentage of the original activity (100%) determined initially after heating at 20 °C for 60 min.

Figure 4 Effect of pH under isothermic conditions

(a) Portions of CTBP stored at 4 °C were successively preincubated at pH 7.6 for 2 h, at pH 6.6 for 3 h and at pH 7.6 for 2 h. (b) Portions of stored CTBP were heated at 20 °C and successively preincubated at pH 7.6, 8.6 and 7.6 for 2 h. At time intervals 0.2 ml samples were removed and assayed at pH 7.6 for specific L-T₃ binding as indicated in the Materials and methods section. pH changes were performed by addition of NaOH or HCl. L-T₃-binding activity was expressed as percentage of the activity determined after heating at 20 °C for 60 min.

from a 200 kDa active form to a less active or inactive form of 60 kDa and (ii) the re-activation of cold-inactivated CTBPs by heat treatment resulted in association of the inactive form to an active form with an apparent molecular mass of 200 kDa. Changes in molecular mass from 200 kDa to 60 kDa were also found with CTBP I by preincubation at 4 °C (results not shown). The latter result indicates that CTBP I is also a polymeric form of CTBP IV. CTBP I and CTBP II differ in their binding to a DEAE-cellulose column. The low yield of CTBP I hampered the study of the nature of this behaviour. To perform the following experiments, pools of isolated CTBP II and CTBP IV were used.

Figure 5 Effect of pH on CTBP elution from a gel-filtration column

Two 2 ml samples of CTBP were preincubated separately for 60 min in 150 mM NaCl/10 mM sodium phosphate buffer at 20 °C and pH 8.6 (●) or at 4 °C and pH 6.6 (○). Each sample was applied to a Sephacryl S-200 HR column equilibrated at room temperature with pH 8.6 buffer (●) or at 4 °C with pH 6.6 buffer (○); 1.5 ml fractions were collected and the specific L-T₃ binding was determined after neutralization to pH 7.6 by addition of NaOH or HCl and heat treatment at 20 °C by 60 min. Abbreviations as in Figure 2.

Effect of pH

The experiments shown in Figure 1 indicate that CTBPs at pH 7.6 became inactive 1 h after changing the preincubation temperature from 20 °C to 4 °C. If the pH was changed from 7.6 to 6.6, the CTBP maintained the original activity for at least 24 h after the temperature shift. As previously shown [1], cold-sensitive CTBPs were stable at room temperature for at least 24 h when the preincubation pH was 7.6. On raising the pH from 7.6 to 8.6 the CTBP became inactive after 2 h at 20 °C. Figure 3 shows the effect of pH on L-T₃-binding activity after preincubation at 4 °C and 20 °C. In order to determine whether the inactive forms of CTBP obtained at pH 7.6 and 4 °C or pH 8.6 and 20 °C were recovered after pH changes, the experiments shown in Figure 4 were performed. At 4 °C the pH changes from 7.6 to 6.6 and from 6.6 to 7.6 resulted in activation and inactivation of CTBP activity respectively (Figure 4a). At 20 °C, the L-T₃-binding activity of CTBP decreased when the pH of the preincubation medium was changed from 7.6 to 8.6, reaching the minimum value within 120 min. The reverse pH change, from 8.6 to 7.6, resulted in a total recovery of CTBP activity after 120 min (Figure 4b). Thus it appears that, under isothermal conditions, the pH influenced the reversible association–dissociation phenomenon. The latter was confirmed by analysis of CTBP by gel filtration at 4 °C or room temperature at different pH values (Figure 5). CTBP yielded a L-T₃-binding peak corresponding to a molecular mass of 60 kDa at room temperature and pH 8.6 and 200 kDa at pH 6.5 and 4 °C.

DISCUSSION

The results obtained in the present work show that the cold-sensitive CTBPs have high and low apparent molecular masses at 20 °C and 4 °C respectively. These CTBP forms were interconverted by cold and heat preincubation treatments (Figure 2). Raising the pH promoted dissociation, whereas acidification stimulated CTBP association. This effect took place at different pH values at 4 °C and 20 °C. Assuming a single ionizable group or several groups with close pK_a values, it is possible to deduce

from Figure 3 that the $pK(s)$ of the group(s) involved in the association–dissociation is approx. 7.0 at 4 °C and 8.0 at 20 °C. These results indicate that the protonated form of the CTBP monomer is involved in the interaction leading to association. Under isothermal conditions, changes in the apparent molecular mass could also be obtained by changes in pH of the preincubation medium (Figure 5). The tetrameric form of CTBP appears to be the only form able to bind L-T₃ [2]. A semi-logarithmic plot of the dissociation event at 4 °C and pH 7.6, and 20 °C and pH 8.6, gives similar straight lines, indicating that a unique species, the oligomer, is involved in the slowest step of the dissociation process, independently of the experimental conditions. The value of k_{diss} was 0.018 min⁻¹ at 4 °C and 0.014 min⁻¹ at 20 °C. On the other hand, the association event did not exhibit a straight line in a semi-logarithmic representation, suggesting that association followed at least second-order kinetics. Assuming an association process of two steps in which the first step is the association of two monomers and the second the association of two dimers to form the active tetrameric form, and assuming that the first step is rate-limiting, the plot of the reciprocal of the monomer concentration as a function of time should give a straight line. The latter is true for the first points of the kinetics of association, when the oligomer concentration is low, resulting in a second-order rate constant of association of 0.003 min⁻¹ per monomer and 0.020 min⁻¹ per monomer at 4 °C and pH 6.6, and at 20 °C and pH 7.6 respectively. This difference in the rate constants provides support for the protonated form of the CTBP being involved in the association phenomena. The association constants calculated from the second part of the association curves were very similar (k_{ass} of 0.073 min⁻¹ per monomer and 0.107 min⁻¹ per monomer for 4 °C and pH 6.6, and 20 °C and pH 7.6, respectively).

The reversible changes in activity associated with the molecular association–dissociation phenomenon of CTBP mediated by cold, heat and pH treatments may not be significant under physiological conditions, the main reason being that in red blood cells the temperature and pH are subject to homeostasis. However, it is possible that cold-sensitive CTBPs may undergo inactivation (dissociation) and activation (association) reactions under isothermic conditions, which are mimicked *in vitro* by cold, heat and pH treatments. Recent results from our laboratory suggest that the cold-sensitive CTBPs and pyruvate kinase (homotetrapolymer of about 240 kDa) from human blood red cells could be the same entity, since similar cold-sensitive properties, values of dissociation constant and regulatory effectors were found for both activities [2]. The mature mammalian erythrocytes may play a role in blood transport of L-T₃ [1–4]. The erythrocyte compartment contains 25 times higher concentration of L-T₃ than that found at free L-T₃ in rat plasma [4]. In washed erythrocyte suspensions, the intra- and extra-cellular concentrations of L-T₃ were about 80 times that of the extracellular compartment [5]. The equilibrium distribution of thyroid hormones between blood plasma and organs depends mainly on the ratio of hormone–protein binding between both phases [6–8]. It was suggested that the CTBPs could be responsible for the intracellular retention of L-T₃ in red blood cells [1]. The present paper suggests that changes in the molecular mass of CTBP by dissociation–association may influence L-T₃ retention by the red blood cell.

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