Temperature-responsive release of thyroxine and its environmental adaptation in Australians

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The hormone thyroxine that regulates mammalian metabolism is carried and stored in the blood by thyroxine-binding globulin (TBG). We demonstrate here that the release of thyroxine from TBG occurs by a temperature-sensitive mechanism and show how this will provide a homeostatic adjustment of the concentration of thyroxine to match metabolic needs, as with the hypothermia and torpor of small animals. In humans, a rise in temperature, as in infections, will trigger an accelerated release of thyroxine, resulting in a predictable 23% increase in the concentration of free thyroxine at 39°C. The in vivo relevance of this fever-response is affirmed in an environmental adaptation in aboriginal Australians. We show how two mutations incorporated in their TBG interact in a way that will halve the surge in thyroxine release, and hence the boost in metabolic rate that would otherwise occur as body temperatures exceed 37°C. The overall findings open insights into physiological changes that accompany variations in body temperature, as notably in fevers.

1. Introduction
Thyroxine is the hormone that most directly controls mammalian activity, with its immediate derivatives regulating cellular oxygen consumption and the metabolism of body and brain [1]. Consequently, tissue concentrations of thyroxine are precisely defined: too much leads to hyperactivity and too little to dormancy. The storage and transport of thyroxine in blood has been well documented in humans [2,3]. The steady-state concentration of thyroxine in blood is set centrally by the secretion of thyroid stimulating hormone (TSH) but the maintenance of this concentration throughout the tissues is owing to the equilibrated release of thyroxine from its carrier protein in the blood, thyroxine-binding globulin (TBG) [2–4]. The binding affinity of TBG for thyroxine is exceptionally tight such that in humans only 0.03% of the total blood thyroxine is in the free form, at picomolar concentration. Other thyroxine carriers in blood, albumin and transthyretin, contribute to the equilibration of plasma concentrations but their influence is minor [5]. The overwhelming proportion of the circulating thyroxine is bound as a ligand to TBG, which acts as both a store and a buffer to give an equilibrated release of thyroxine to the tissues. The binding capacity of circulating TBG is only partially saturated, and it is the percentage saturation, 20% or more, that by the law of mass action determines the concentration of free thyroxine in the tissues. Although the steady-state concentration of free thyroxine and hence the percentage saturation of TBG is determined centrally we demonstrate here, as we have similarly shown with the closely related corticosteroid-binding globulin (CBG) [6,7], how...
the release of thyroxine from TBG is further adjusted within the circulation by a temperature-sensitive modulation of its binding affinity.

TBG and CBG are members of the serpin family of serine protease inhibitors, with homologous hormone-binding sites. Although both hormone carriers have lost any inhibitory activity, they retain the ability to undergo the remarkable conformational change, from a stressed to a relaxed form, that characterizes the serpins [8,9]. Recent structure-based studies [4,10–13] show how the initiating stage in this change, the temperature-sensitive movement of the reactive loop into and out of the A-sheet (red) of TBG directly affects the binding site magnified in (b) showing the interactions that stabilize thyroxine (skeletal form) in the binding site. Entry of the reactive centre loop will cause a steric perturbation and the expansion of the A-sheet will displace the connecting loops (green) that surround the bound thyroxine. The Australian mutations, A191T and L283F, flank the binding site. (c,d) The proportional loss of hormone-binding affinity with increasing temperature (Kd/Kd37°C): (c) shown with the homologous CBG, from Chan [7] circles and Mickelson [14] crosses; (d) with TBG and fluorophore–thyroxine data from table 1. The plot of the L283F variant of TBG (interrupted line) is superimposable on that of the wild-type, including the inflection at 37°C.

Figure 1. Temperature-responsive release of thyroxine from TBG. (a) Thyroxine, in space-filling form. Movement of the reactive centre loop (yellow) into and out of the A-sheet (red) of TBG directly affects the binding site magnified in (b) showing the interactions that stabilize thyroxine (skeletal form) in the binding site. Entry of the reactive centre loop will cause a steric perturbation and the expansion of the A-sheet will displace the connecting loops (green) that surround the bound thyroxine. The Australian mutations, A191T and L283F, flank the binding site. (c,d) The proportional loss of hormone-binding affinity with increasing temperature (Kd/Kd37°C): (c) shown with the homologous CBG, from Chan [7] circles and Mickelson [14] crosses; (d) with TBG and fluorophore–thyroxine data from table 1. The plot of the L283F variant of TBG (interrupted line) is superimposable on that of the wild-type, including the inflection at 37°C.

2. Material and methods

Wild-type recombinant human TBG and its engineered variants, A191T, L283F and A191T/L283F and the fluorophore-adduct of
Table 1. Variation of TBG binding affinities and free-thyroxine concentrations with temperature. (a) Kd, and Kd/Kd_{37°C} ratios of recombinant TBG variants with the thyroxine–fluorophore. Kd measurements were repeated more than three times in duplicate; data are means ± s.d. (b) The fluorophore Kd/Kd_{37°C} derived changes in free thyroxine (FT4) with temperature (shown in bold) are in agreement with previous independently derived values with thyroxine and isolated plasma TBG [20] (non-bold), and the assay of free thyroxine in plasma at 21°C and 37°C, (19,20) (figure 2). Calculations based on FT4 of 20 pM and TBG saturation of 20% at 37°C.

(a) TBG: thyroxine – fluorophore binding affinity with temperature

| Temp (°C) | Kd nM | Kd/Kd_{37°C} | Kd nM | Kd/Kd_{37°C} | Kd nM | Kd/Kd_{37°C} | Kd nM | Kd/Kd_{37°C} |
|-----------|-------|--------------|-------|--------------|-------|--------------|-------|--------------|
| 7         | 1.1 ± 0.3 | —       | 0.20  | —           | —     | —           | —     | —           |
| 22        | 2.2 ± 0.2 | 0.40    | 3.7 ± 0.3 | 0.47   | 2.4 ± 0.3 | 0.43   | 3.1 ± 0.3 | 0.45   |
| 37        | 5.6 ± 0.1 | 1       | 7.8 ± 0.3 | 1     | 5.6 ± 0.2 | 1     | 6.9 ± 0.5 | 1     |
| 39        | 6.9 ± 0.5 | 1.23    | 8.6 ± 0.4 | 1.10  | 6.9 ± 0.3 | 1.23  | 7.7 ± 0.2 | 1.12  |
| 42        | 7.5 ± 0.3 | 1.34    | 9.5 ± 0.3 | 1.21  | 7.5 ± 0.4 | 1.34  | 8.6 ± 0.2 | 1.24  |

(b) Free thyroxine (FT4) from fluorophore ratios and direct T4 Kd values

| temperature (°C) | 5 | 7 | 21 | 22 | 25 | 37 | 39 | 42 |
|------------------|---|---|----|----|----|----|----|----|
| Kd/Kd_{37°C} (%) | — | 20 | 41 | 40 | —  | —  | 100 | 123 |
| Kd T4 (pM)       | 20 | —  | 41 | 40 | —  | —  | 75 | 110 |
| FT4 (pM)         | 3.6 | 4  | 8  | 8  | 11 | 12 | 20 | 25 |

*Serum FT4 21°C/37°C ratio, Ross & Benraad [19].
Kd T4: *Korcek et al.* [18], *Qi et al.* [13].

Thyroxine indices: free thyroxine and % saturation of TBG and its variants over a range of temperatures were calculated using a free-thyroxine concentration at 37°C of 20 pM based, with recent updating [16], on a plasma range of 12–26 pM. The binding constant Kd of thyroxine with TBG at 37°C and pH 7.4 has been variously reported (bracketed, inverse Ka \(10^{10}\) M-1): in 1972 [17] as 60 pM (Ka 1.68), more definitively as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33). We have adopted here the mean of these values as 75 pM (Ka 1.33).

Thyroxine–fluorophore was used to determine the proportional change in binding affinity, Kd/Kd_{37°C} that takes place over a range of temperatures in human TBG and its engineered variants (table 1b).

The use of the fluorophore–thyroxine Kd/Kd_{37°C} ratios to calculate the proportional changes that will occur to the binding affinity of thyroxine to TBG in plasma is validated by the agreement with values independently determined with thyroxine [18] and with the direct assays by others of free-thyroxine concentrations in the blood at room and body temperature [19,20]. The plot of Kd/Kd_{37°C} ratios versus temperature in figure 1d and of consequent blood free-thyroxine concentrations (table 1b) demonstrate how changes in the binding affinity of TBG provide an inherent adjustment of thyroxine levels to match metabolic needs.

3. Results and discussion

Thyroxine–fluorophore was used to determine the proportional change in binding affinity, Kd/Kd_{37°C} that takes place over a range of temperatures in human TBG and its engineered variants (table 1b).

(a) Small mammals and hypothermia

Although our data here and almost all the detailed knowledge of the physiology of thyroxine transport are derived from the human, there is clear evidence that the system is strongly conserved in all mammals. This is seen not only in
the conservation of TBG sequence in diverse mammals [21]
but also in the identity of molecular mechanisms based on
structure crystals from the mouse [11] and the human
[12,13]. Hence, the findings here (figures 1d and 2) have
direct relevance to the hypothermia and torpor that occur
in small animals [22,23]. Based on a saturation of TBG in
the human of 20%, there will be a homoeostatic decrease in
the concentration of free thyroxine as body temperature
falls, with a fivefold drop from 20 pM at 37°C to a baseline
4 pM at 7°C. Similarly, as body temperature is restored, the
thyroxine, stably stored in the TBG, will be increasingly
released, rising to meet the needs of full activity at 37°C.

(b) Humans and fever
The critical metabolic demands of a much larger brain make
humans especially sensitive to changes in the release of thyroxine and even mild hypothermia, if prolonged, is fatal. A
physiological change in body temperature does, however,
occur in humans with the fevers that are induced by inflammation and infection. The accelerated decrease in binding
affinity that will accompany the increase in body temperature
from 37°C to 39°C and, exceptionally, to 42°C is seen in
figure 1d. Evidence that this fever-induced decrease in binding
affinity is specific and purposeful and comes from a similar but even greater acceleration of hormone release in the closely related CBG (figure 1c). The loss of affinity in TBG at fever temperatures has, however, even more direct physiological impact than that of CBG because of the much
tighter hormone-binding affinity of TBG and the precisely
defined limits of free-thyroxine concentration in the blood.
Any changes in the Kd of TBG will be directly reflected in
changes in free-thyroxine levels in the blood with a rise in
body temperature to 39°C predictably giving a 23% increase in
concentration, temporarily moving into the range seen in the
clinical disorder of hyperthyroidism (figure 2).

(c) Adaptation in the aboriginal Australian
The in vivo relevance of this surge in thyroxine release in
fevers is affirmed by what had been a perplexing finding,
the presence of two linked mutations in the TBG of aboriginal
Australians [24–26]. Surveys in West Australia had shown the
presence of this variant TBG in some 40% of the aboriginal population in association with lowered levels of
total-thyroxine and total-TBG. The clue as to the functional
significance of the mutations, a replacement of alanine 191
by a threonine and of leucine 283 by a phenylalanine, came
from their placement on the periphery of the thyroxine-
binding site [4] (figure 1b). Alanine 191 is immediately
adjacent to the point of entry of the reactive loop into the
main beta-sheet of TBG and its replacement by a polar threo-
ine will predictably affect the H-bond network that links to
the bound thyroxine. The consequence of this replacement is
shown here with the change in thyroxine-binding affinity of the
recombinantly expressed Ala191Thr TBG. The replacement
critically results (figure 3a,b) in an abolition of the
accelerated release of thyroxine that takes place as the tem-
perature rises above 37°C, with the 23% increase in free
thyroxine that would otherwise occur at 39°C being reduced
by the mutation to a 10% increase.

An enigmatic accompanying finding strengthens the
deduction that the Ala191Thr mutation in aboriginal Austra-
lians is selectively advantageous. This is the presence of the
linked second mutation, with the replacement of the leucine
at 283 by a bulkier phenylalanine. The puzzle was that this
Leu283Phe mutation occurs not uncommonly in other
populations without any discernible functional consequen-
ces [26]. We confirm here (table 1 and figure 1d) that this
variant does, by itself, precisely retain the affinity and temp-
erature response of the wild-type TBG. However, when the
Leu283Phe replacement is co-expressed with that of
Ala191Thr there is clearly an advantageous interaction. The
thyroxine affinity of the double mutant moves closer to the
physiological Kd at 37°C, with little loss of the thermal pro-
tection provided by the Ala191Thr replacement. In doing
so, the linked mutation maintains the percentage saturation
of TBG closer to the norm. With just the single Ala191Thr
mutation, the saturation of TBG would need, by the law of
mass action, to decrease from 20 to 15% in order to provide
a physiological free thyroxine of 20 pM at 37°C. But with
the addition of the second Leu283Phe mutation, this decrease
changes to a more adequate 17% saturation.

The findings fit well with the quantitation by Takamatsu
et al. in 1987 [27] of ancillary changes in an aboriginal
hemizygote for the A191T/L283F TBG, giving a TBG concen-
tration 74% of that of the normal and a much-reduced total
thyroxine at 58% of a normal pool. The decreased overall
level to 74% is in keeping with studies of other serpins,
which show that functional mutations consistently result in
a comparable decrease in the efficiency of expression. The
further reduction in the saturation of this diminished level of
TBG, as determined here with the recombinant variant
from 20 to 17% (figure 3b), would result in an overall
reduction in the total thyroxine to 62%, compatible with
the 58% observed in the 1987 study. Further support for the
relevance of the findings reported here with recombinant
TBG to those occurring in vivo [27] is provided by the identical
denaturation temperature of the recombinant double mutant
at 52°C (figure 3c) to that measured in an earlier study of the

Figure 2. Variation in free thyroxine with temperature from Kd/Kd37°C ratios (hatched bars), 20 pM at 37°C rising to 25 pM at 39°C with wt-TBG, but
dampened to 22 pM in the 191/283 TBG (Aus) variant (upper normal limit, dashed line). The open (non-hatched) bars show values from indepen-
dent determinations by others using plasma-derived thyroxine [18] and direct
assays of plasma-free thyroxine [19,20]. The comparative bars are based on a
defined Kd of 80 pM, free thyroxine of 20 pM and a TBG saturation of 20%,
at 37°C.
plasma variant [28] and, as in that study, being only slightly decreased from the denaturation temperature of the wild-type plasma TBG at 55°C [8].

Thus, the paired polymorphisms provide the aboriginal Australian with a TBG that maintains its properties as a storage and carrier protein while having the additional local advantage of providing a reduced metabolic response to increased body temperatures. In a temperate climate, the boost to thyroxine release and increased metabolism that accompanies the rise in body temperatures in fevers will be an advantageous response to infection. But the same accelerated increase in metabolism could affect the survival of a population historically exposed to the arid environment of central Australia, with ambient temperatures of 45°C or above. There the life threatening risk is not so much the infection itself, but rather the dehydration and heat exhaustion that accompany dysentery and other common illnesses in infancy and childhood.

(d) Wider implications

The recognition of this environmental adaptation in the aboriginal Australian has much wider significance in affirming the physiological relevance of the temperature-regulated release of thyroxine in blood. Although the central secretion of TSH controls the concentration of thyroxine in the longer term, the variations with temperature of free-thyroxine concentrations in the circulation will be rapid and reversible. This temperature responsive adjustment of the concentration of free thyroxine has been largely overlooked in the past, as it had been assumed that the thyroxine–TBG binding affinity remains constant. Moreover, thyroxine assays have customarily been carried out at room temperature. Measured retrospectively in this way, blood samples taken in hypothermia, in heatstroke, or from an infant with fever, will all be reported as having an unchanged free thyroxine.

The demonstration that TBG, as with CBG [6,7], acts as a protein thermocouple, has direct physiological implications. Such temperature-sensitive regulation of hormone release will affect everyday lives. For example, the accelerated release that will take place as the body core temperature rises to 39°C in a hot-bath or sauna will contribute to an enhancement of the metabolism of body and mind—euphoria and eureka! A similar boost in hormone release also opens a contributory explanation for the common occurrence of febrile convulsions in infancy [29]. The brain is sensitive to changes in free thyroxine and raised levels in thyrotoxicosis in adults can result in convulsive seizures that cease, with no after-effects, when the thyroxine level returns to normal [30,31]. Comparable seizures also commonly occur in infants in conjunction with the spiking increases in temperature that accompany incidental infections at that age. The surge in thyroxine release that will occur in response to the increases in the temperature of the brain in fevers [32] poses a potential exacerbating factor in the childhood seizures—a conclusion reinforced by the prompt cessation of the convulsions as the infant’s body temperature is cooled.

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