# Formation of titanium(IV) transferrin by reaction of human serum apotransferrin with titanium complexes

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**Abstract The reaction of human serum apotransferrin with titanium(IV) citrate under physiological conditions results in the formation of a specific bis-titanium(IV) transferrin adduct ($Ti_2Tf$ hereafter) with two titanium(IV) ions loaded at the iron binding sites. The same specific $Ti_2Tf$ complex is formed by reacting apotransferrin with titanium(III) chloride and exposing the sample to air. The derivative thus obtained was characterized by spectroscopic techniques, including absorption, UV difference, circular dichroism and $^{13}C$ NMR spectroscopies, and shown to be stable within the pH range 5.5–9.0. Surprisingly, the reaction of apoTf with titanium(IV) nitrilotriacetate (NTA) does not lead to formation of appreciable amounts of $Ti_2Tf$, even after long incubation times, although some weak interactions of Ti(IV)-NTA with apoTf are spectroscopically detected. Implications of the present results for a role of transferrin in the uptake, transport and delivery of soluble titanium(IV) compounds under physiological conditions are discussed.**



*Key words:* Titanium; Transferrin; UV difference spectroscopy; Circular dichroism

## 1. Introduction

Transferrin is the principal iron transport protein in higher organisms [1–3]. Owing to the high stability of the iron complex, transferrin prevents hydrolysis of iron(III) at physiological pH and allows delivery of the essential iron(III) ions to the various compartments of the body through a sophisticated receptor-mediated mechanism [3]. Under normal conditions, transferrin is only 30% iron-saturated, and therefore may act as a specific carrier for several other tripositive, tetrapositive and bipositive metal ions. The ability of transferrin to bind to a variety of metal ions besides iron, preventing their precipitation at physiological pH, is well-documented [4]. Thus, transferrin may have a significant impact on the metabolism and the biodistribution of trace elements but may also potentiate the deleterious effects of toxic metal ions such as actinides, that otherwise would not be bioavailable [5]. Extensive studies now exist on this topic [6].

The demonstrated ability of transferrin to interact with several metal ions prompted us to study the interaction of human serum apotransferrin with titanium(IV) complexes. Titanium is, indeed, a relatively abundant element with a number of industrial and biomedical applications. Titanium compounds are generally poorly soluble and poorly bioavailable; thus toxicology of titanium essentially deals with the local effects of orthopedic titanium implants [7] and with lung damage caused by titanium dioxide particles [8]. Very limited data exist on the biological effects and the toxicity of soluble titanium(IV) complexes. In our opinion, however, this topic deserves particular attention, given the growing use of titanium implants in medicine and the possible release of soluble titanium species in vivo. In the latter case it is likely that transferrin may act as a specific carrier of this metal ion and may play a central role in the transport and biodistribution of soluble titanium species throughout the organism [9].

Thus, we selected two representative, water-soluble titanium(IV) complexes – namely titanium(IV) citrate and titanium(IV) nitrilotriacetate [Ti(IV)NTA], both stable within a physiological environment – and monitored their reactions with human serum apotransferrin (apoTf hereafter) at pH 7.4. The formation of adducts with apoTf and their stability as a function of pH were investigated by a variety of spectroscopic methods. Elucidation of the reactions between titanium(IV) complexes and apotransferrin, the key protein for the transport of highly charged metal ions in the blood stream, will contribute to understanding the metabolism in vivo of this widespread element and shed light on the molecular mechanisms of its biological (or toxic) effects. By analogy with the case of aluminum(III) and gallium(III) complexes [10,11], it is likely that soluble titanium complexes may follow in vivo the biological route of iron(III) and may in this way exert their effects.

## 2. Materials and methods

### 2.1. Materials

Titanium(IV) citrate was prepared by mixing titanium(III) chloride with a 1.2-fold excess of sodium citrate at pH 3; exposure to air caused quantitative oxidation of titanium(III) citrate to colorless titanium(IV) citrate. A similar method was used to prepare titanium-(IV)NTA. Human serum apoTf was purchased from Sigma Chemical Company and used as such; its concentration was determined by measuring its UV absorbance at 280 nm ($\varepsilon_{280} = 91\,200\ M^{-1}\ cm^{-1}$). The other reagents were of analytical grade.

### 2.2. Circular dichroism (CD), UV difference and absorption spectra

CD spectra were carried out at room temperature with a Jasco J500 instrument interfaced with a PC. Samples for CD measurements contained $1\times10^{-4}$ M human serum apotransferrin, prepared in 50 mM HEPES buffer, 100 mM sodium bicarbonate, pH 7.4. Data analysis was performed with the standard JASCO software package. UV-visible absorption spectra were recorded with a Perkin Elmer Lambda 20 instrument. UV difference spectra were measured according to the previously reported method [12]; samples usually contained $\sim1\times10^{-5}$ M apotransferrin. The kinetics of metal uptake and release processes were followed through spectrophotometry and CD.

### 2.3. NMR spectra

The $^{13}C$ NMR spectra were acquired on a Bruker Avance 600

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instrument working at 150 MHz. Samples for $^{13}C$ NMR measurements contained 1.5 mM transferrin, 50 mM HEPES buffer, pH 7.4, and 10% $D_2O$, in a volume of about 0.5 ml. $^{13}C$-enriched sodium bicarbonate (6 mM final concentration) was added to the samples before addition of a twofold excess of titanium(IV) citrate. Typically $^{13}C$ NMR acquisition parameters used in this study were as follows: 70° pulse length, 3.0 s repetition time, 20 000 Hz sweep width. A line broadening of 10–30 Hz was applied to $^{13}C$ data prior to processing.

## 3. Results

### 3.1. Preparation of titanium(IV) transferrin starting from titanium(IV) citrate

The reaction of apoTf with titanium(IV) citrate was first followed through CD spectroscopy, which represents a very appropriate tool to detect metal binding to chiral macromolecules [13]. Addition of titanium(IV) citrate to apotransferrin at physiological pH (2:1 Ti/apoTf molar ratio) results in the appearance of an intense CD band at 310 nm, with a pronounced shoulder at 330 nm, suggesting the formation of a specific $Ti_2Tf$ derivative (Fig. 1A). These bands, tentatively assigned as ligand to metal charge transfer (LMCT) bands, slowly develop with time, reaching maximum intensity within about 6 h at 20°C; afterwards the spectrum is stable for days.

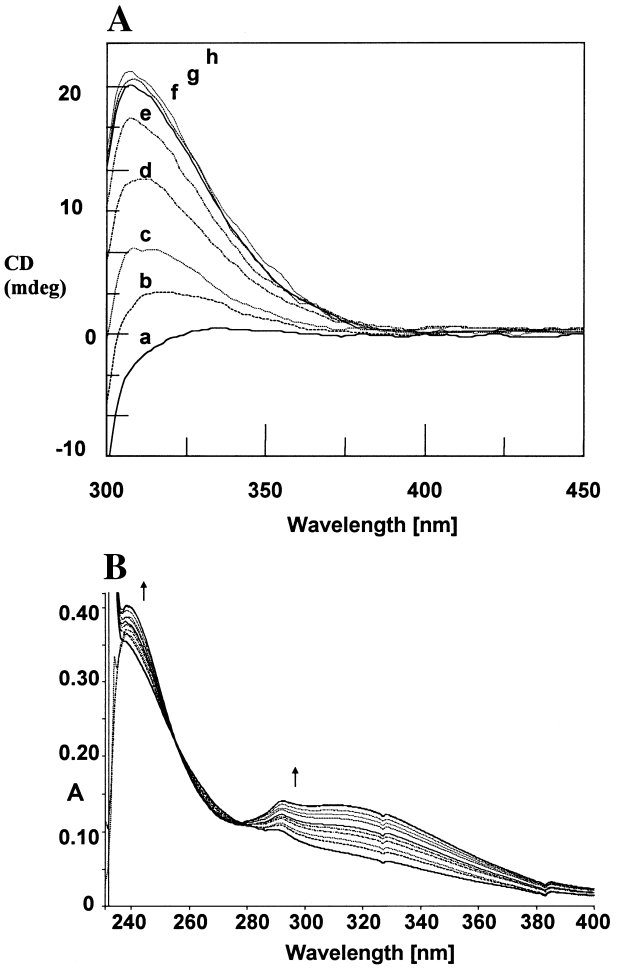


Fig. 1. A: CD spectra of apoTf at increasing times after addition of titanium(IV) citrate (2:1 Ti/apoTf molar ratio). Spectra were taken before addition (a) and 1 (b), 5 (c), 15 (d), 30 (e), 90 (f), 210 (g) and 420 (h) min after titanium(IV) citrate addition. Conditions were the following: apoTf $1\times10^{-4}$ M; 50 mM HEPES buffer, 100 mM sodium bicarbonate, pH 7.4. B: UV difference spectra of apoTf at increasing times after addition of titanium(IV) citrate (2:1 Ti/apoTf molar ratio). Spectra were collected at the following times after mixing: 10, 20, 30, 40, 60, 80, 120, 160, 210, 330, 390 and 420 min. ApoTf concentration was $1\times10^{-5}$ M; the other conditions as in A.

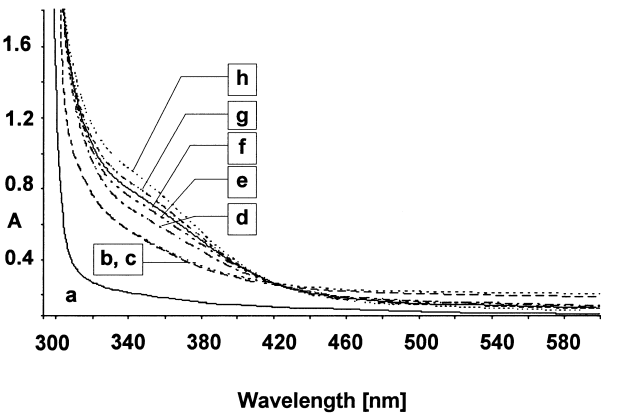


Fig. 2. Spectrophotometric monitoring of titanium(III) to titanium(IV) transferrin interconversion, measured in the 300–400 nm region. Spectrum a, apoTf; b and c, apoTf, 5 and 10 min after addition of Ti(III) citrate (2:1 Ti/apoTf molar ratio); spectra d, e, f, g, h were taken at increasing times after opening the cuvette and exposing the sample to air (80–260 min). Other conditions as in Fig. 1A.

The same reaction was monitored through UV difference spectroscopy. This revealed characteristic UV difference bands at 240 and 290 nm, diagnostic of phenolate deprotonation and titanium binding to the iron sites [12]. Again, a LMCT band is detected at 320 nm (Fig. 1B).

Titration studies (both CD and absorption) show that transferrin specifically binds ca. 2 mole titanium(IV) per mol protein, in good agreement with the hypothesis that titanium(IV) ions occupy the empty metal binding sites of transferrin (data not shown). Only weak binding is observed when titanium(IV) citrate is added in excess of a 2:1 stoichiometry. The profiles of CD and absorption titrations of apoTf with increasing amounts of titanium(IV) citrate at pH 7.4 are rather regular, suggesting that the two sites are nearly identical. Notably, the presence of the synergistic carbonate anion is required for binding.

### 3.2. Preparation of titanium transferrin starting from titanium(III) chloride

Remarkably, the same $Ti_2Tf$ derivative could be prepared by reacting apoTf with titanium(III) chloride anaerobically (2:1 Ti/apoTf ratio) and oxidizing the sample in the air. Such an experiment leads first to formation of titanium(III) transferrin, which then oxidizes to titanium(IV) transferrin when exposed to air. Conversion from Ti(III) to Ti(IV) transferrin is directly monitored by measuring the spectral changes in the 300–400 nm region (Fig. 2). Notably, the final species is spectroscopically identical to that obtained starting from titanium(IV) citrate.

### 3.3. pH-dependent properties of Ti(IV) transferrin

We next investigated the stability of the obtained titanium(IV) transferrin derivative ($Ti_2Tf$) as a function of pH. Both CD and spectrophotometric results indicate that $Ti_2Tf$ is quite

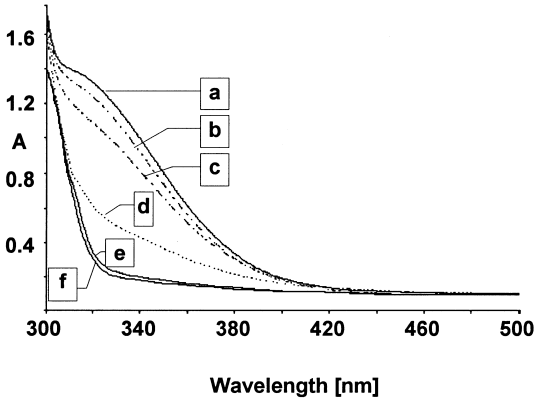


Fig. 3. pH dependence of the electronic spectra of bis-titanium(IV) transferrin in the high pH region. Spectra were taken at the following pH values: 7.40 (a); 7.93 (b); 8.50 (c); 9.70 (d); 10.50 (e); 11.50 (f). Other conditions as in Fig. 1A.

stable within the pH range 5.5–9.0. So $Ti_2Tf$ is more stable than other metallotransferrins at low pH, breaking down only for pH values lower than 5.0. In contrast, $Ti_2Tf$ is relatively less stable at high pH; indeed, at pH 9.7 more than 60% of the original spectral intensity is lost without evidence of conformational changes (Fig. 3), whereas other metallotransferrins (e.g. cobalt(III), iron(III), aluminum(III) transferrin, etc.) were previously reported to be stable up to pH $\approx$ 11 [14]. The behavior of titanium(IV) transferrin at high pH reproduces that of thallium(III) transferrin [15] suggesting that in both cases for pH values $\geq$9.0 hydroxide precipitation is favored over formation of the respective transferrin derivative.

### *3.4. Reaction of apoTf with Ti(IV)NTA*

Surprisingly, reaction of apoTf with Ti(IV)NTA gives rise to substantially different CD and UV difference spectra as compared to titanium(IV) citrate (Fig. 4). The absence of the characteristic tyrosine deprotonation bands at 240 and 290 nm suggests that specific binding of titanium(IV) at the iron sites does not take place in this case. Only residual effects at 240 and 290 nm are observed in the UV difference spectra, possibly indicating formation of a very limited amount of $Ti_2Tf$ (ca. 10–15% as compared to the case of titanium(IV) citrate). Failure to form $Ti_2Tf$ starting from Ti(IV)NTA is ascribed to the fact that Ti(IV)NTA is far more stable than titanium(IV) citrate, so that transfer of titanium(IV) ions from NTA to apoTf is disfavored. This hypothesis was confirmed through a simple CD experiment demonstrating that a slight excess of the NTA anion is able to scavenge more than 70% of the bound titanium from titanium transferrin within a few hours. Conversely, observation of CD effects following addition of Ti(IV)NTA to apoTf indicates that some specific interactions indeed occur between this metal complex and the apoprotein.

### *3.5. $^{13}C$ NMR studies*

As previously reported for several other metallotransferrins [16,17], observation of the characteristic $^{13}C$ NMR signal of protein-bound, $^{13}C$-enriched synergistic anion provides evidence that the ternary protein-metal-anion adduct has formed. $^{13}C$ NMR spectra were recorded at 150 MHz on $Ti_2Tf$ samples prepared with $^{13}C$-enriched sodium bicarbonate. Indeed, a new $^{13}C$ NMR signal corresponding to protein-bound carbonate could be observed at 166.7 ppm (signal a in Fig. 5); the position of the signal agrees quite well with that observed for other metallotransferrins [16,17], although the present $^{13}C$ NMR signal of $Ti_2Tf$-bound carbonate is significantly broader and weaker than in previously reported cases.

## 4. Discussion

### *4.1. Formation of $Ti_2Tf$ by reaction with titanium(IV) citrate*

Transferrin is known to act as a general ligand for many metal ions of different charge and size besides the physiological iron(III) ions. Since in vivo transferrin is largely present as the apo-form, this protein is often involved in the biodistribution and metabolism of metal ions that are known to be either trace elements or pollutants. In the present study, we considered the reactions of apotransferrin with two representative titanium(IV) complexes, i.e. titanium citrate and titanium nitrilotriacetate, under physiological conditions, to establish whether such reactions lead to quantitative formation of the corresponding titanium(IV) transferrin adducts.

A rather stable $Ti_2Tf$ derivative does indeed form when apotransferrin is reacted with titanium(IV) citrate at physiological pH; UV difference spectra provide evidence that bind-

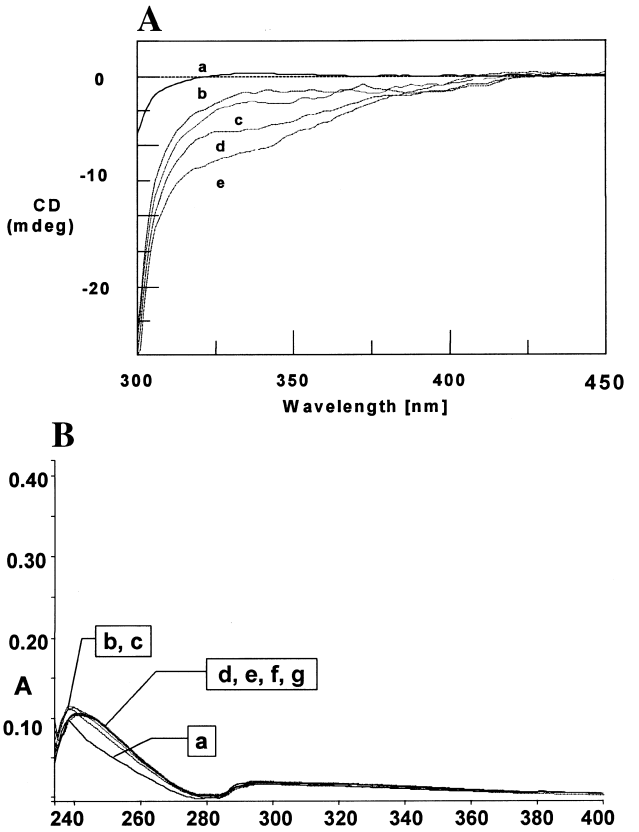


Fig. 4. Effects of the addition of titanium(IV) nitrilotriacetate (2:1 Ti/apoTf molar ratio) on the CD (A) and the UV difference spectra (B) of apoTf. CD spectra were taken before (a) and 1 min (b), 8 min (c), 30 min (d) and 480 min (e) after addition of Ti(IV)NTA. UV difference spectra were taken 1 min (a), 5 min (b), 10 min (c), 80 min (d), 140 min (e), 200 min (f) and 260 min (g) after Ti(IV)-NTA addition. Conditions for the CD spectra were as in Fig. 1A; those for UV difference spectra were as in Fig. 1B.

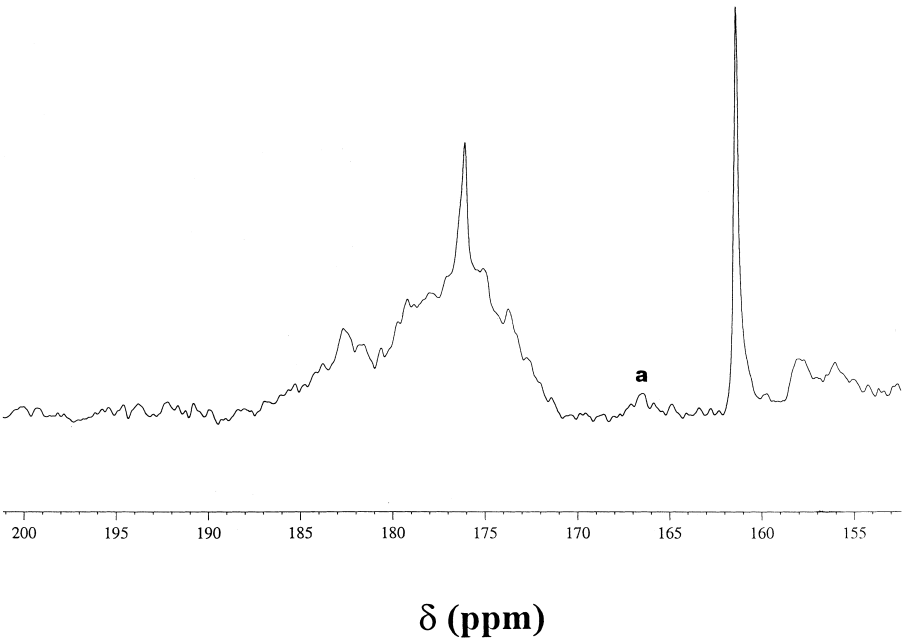


Fig. 5. 150 MHz $^{13}C$ NMR spectrum of $Ti_2Tf$ (1.5 mM, 10% $D_2O$, HEPES buffer 50 mM, pH* 7.4) in the presence of 6 mM $^{13}C$-enriched $NaH^{13}CO_3$. The signal labeled a is assigned to $Ti_2Tf$-bound synergistic anion.

ing of titanium occurs at both transferrin sites and causes tyrosine deprotonation. The reaction characteristically exhibits a stoichiometry of two titanium ions per protein molecule and reaches completion within a few hours after mixing. The characteristic signal of protein-bound carbonate revealed in the $^{13}C$ NMR spectra further supports our conclusions. The same $Ti_2Tf$ derivative may be prepared by addition of titanium(III) chloride to apoTf followed by oxidation of the sample in air; oxidation of titanium(III) transferrin to titanium(IV) transferrin can be directly monitored through spectrophotometric analysis of the 400–300 nm region.

Surprisingly, reaction of apotransferrin with Ti(IV)NTA does not result in formation of the corresponding titanium(IV) transferrin adduct as in the case of titanium(IV) citrate; only a very limited amount of titanium transferrin apparently forms. This fact is ascribed to the higher stability of titanium(IV) nitrilotriacetate compared to titanium(IV) citrate. In any case limited interactions of Ti(IV)NTA with the protein are detected, which likely correspond to formation of hydrogen bonds and/or electrostatic interactions of Ti(IV)NTA with amino acid residues situated in proximity of the empty sites of transferrin.

### 4.2. *pH-dependent properties*

$Ti_2Tf$ exhibits an unusual pH dependence profile; in fact, while this derivative is stable in the pH range 5.5–9.0, it rapidly breaks down above pH 9. Thus, while being considerably stable at low pH, it is relatively unstable at high pH. The lower stability of $Ti_2Tf$ at high pH compared to other metallotransferrins probably reflects the fact that transferrin is no longer able to prevent titanium(IV) (hydr)oxide formation, as previously observed in the case of thallium(III) transferrin.

### 4.3. *Implications for the in vivo metabolism of soluble titanium species*

Overall, the present study unambiguously demonstrates that a stable adduct between titanium(IV) and apotransferrin forms under physiological conditions, provided that titanium(IV) is presented to the protein as the appropriate titanium(IV) complex. Titanium(IV) citrate was shown to meet these requirements whereas Ti(IV)NTA was not able to transfer Ti(IV) ions to the metal binding sites of apotransferrin. Though preliminary, our results may be of interest for studies of the in vivo metabolism of soluble titanium(IV) complexes; for example, it might be hypothesized that, when injected into the blood stream, titanium(IV) citrate will form substantial amounts of titanium(IV) transferrin and will follow the typical route of iron ions.

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