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A Spectroscopic Study on the Binding of Plutonium(IV) and its Chemical Analogues to Transferrin*

JOHN R. DUFFIELD†

Department of Applied Chemistry, U.W.I.S.T., P.O. Box 13, Cardiff CF1 3XF, U.K.

and DAVID M. TAYLOR

Kernforschungszentrum Karlsruhe GmbH, I.G.T./T.O.X., Postfach 3640, D-7500 Karlsruhe 1, F.R.G.

Transferrin (TF) is the principal iron binding and transport protein found in the blood plasma of a variety of animal species, including man [1]. It has also been implicated in the binding and transport of a number of other metal ions, both endogenous and exogenous, including many of the actinide and actinide-like elements [2].

Recent experiments have shown, however, that although metal ions such as Pu^{4+} and Hf^{4+} are bound to TF in plasma and appear to be associated with the iron storage protein ferritin in the liver cell, they do not appear to be taken up into cells via the transferrin-receptor pathway as is the case for iron(III) [3]. In fact, TF seems to play an inhibitory rôle in that it prevents the uptake of Pu(IV) into certain cell types. In addition, one of the primary metabolic functions of TF is the transport of Fe(III) from sites of absorption and storage to sites of utilization, e.g., from the liver to the bone marrow for incorporation in haem. *In vivo*, however, no plutonium is found in the red blood cells of experimentally contaminated animals [2]. Thus, although Pu(IV) and its chemical analogues follow the iron metabolic pathways, they do so imperfectly.

The reasons for this apparently anomalous behaviour are, at present, unknown but are of obvious importance in gaining a clearer understanding of actinide element metabolism for radiation protection and chelation therapy purposes.

The postulated inhibitory rôle played by TF and its association with the TF-receptor found on the surface of nearly all mammalian derived cells will be central to a comprehension of the aforementioned processes. A variety of factors, or a combination thereof, may be involved in the decreased uptake of Pu(IV) and its analogues, such as the strength and stoichiometry of actinide binding to TF (thermodynamics), the kinetics of binding and changes in protein conformation affecting TF–receptor interactions during receptor-mediated endocytosis.

The work reported in this study has been designed to compare and contrast the binding by transferrin of a number of tetravalent cations with that of Fe(III) in an attempt to clarify some of these problems, *viz.* stoichiometry and mechanism of interaction.

Experimental

Chemicals

With the exception of plutonium and TF, all chemicals were obtained from Merck (Darmstadt, F.R.G.). $^{239}Pu(NO_3)_4$, specific activity 67 nmol μCi^{-1}, was purchased from Amersham–Buchler, Braunschweig, F.R.G., whilst human TF, approximately 98% pure and substantially iron-free, was obtained from Sigma, Munich, F.R.G.

Method

Metal–TF interactions were investigated by difference ultraviolet spectroscopy on a Varian DMS80 UV–Vis spectrophotometer. Solutions of apotransferrin (apo-TF, the metal-free protein) were thus used in the reference cell with addition of buffer, whilst apo-TF, with metal ion solutions, was placed in the sample cell.

Transferrin was dissolved in 20 mmol dm^{-3} Tris-HCl buffer, pH 7.4, containing 130 mmol dm^{-3} NaCl and 1 mmol dm^{-3} $NaHCO_3$ to give a concentration of 12.5 mmol TF cm^{-3}.

All dilute metal solutions were made up from metal stock solutions (5 mmol dm^{-3} metal in 20 mmol dm^{-3} HCl) by the addition of nitrilotriacetate (NTA) ([NTA]:[metal] = 4) and subsequent dilution with Tris-HCl buffer, pH 7.4, as above, to give solutions of metal concentration 325 μmol dm^{-3}. The NTA served both to prevent metal ion hydrolysis and to 'deliver' the metal ion to the transferrin–metal binding sites [4]. TF (in the sample cuvette of the Varian DMS80) was saturated to varying extents by addition of 10 μl aliquots of metal–NTA solution and spectra (against apo-TF with appropriate amounts of buffer) obtained in the UV from 350–200 nm.

Results and Discussion

The TF molecule contains two specific metal ion binding sites, one near the C-terminal end, and one near the N-terminal end of the protein [5]. *In vivo* binding of a metal ion to TF requires the concomitant binding of carbonate or bicarbonate to form a

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†Author to whom correspondence should be addressed.

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ternary metal–protein–ligand complex [6]. The carbonate is effectively behaving as a synergistic ligand which stabilizes the metal–protein complex. *In vitro* a number of other ligands will substitute for carbonate and fulfill this synergistic rôle. Almost all such ligands contain carboxylate groups.

The binding of metal ions within the TF molecule may be observed spectrophotometrically in the visible and ultraviolet regions of the electromagnetic spectrum. The absorption is generally specific and dependent on the degree of saturation of the binding sites as shown in Fig. 1 for Fe(III) and Fig. 2 for Pu(IV). As can be seen, the interaction of Fe(III) with TF gives rise to spectra containing two distinct absorption peaks. Pu(IV), however, gives rise to only one absorption peak at ≈240 nm, the shape of these spectra being typical also of Th(IV)– and Hf(IV)–TF interactions.

In this case (Fig. 2), the spectra are complicated by the presence of nitrate from the Pu(IV) stock solution which interacts in a non-specific fashion with the TF giving rise to an absorption peak at a wavelength of 230–240 nm which interferes with the metal–TF absorption peak. However, by plotting the molar absorption coefficient ($\lambda \simeq 240$ nm) against molar ratio of metal-to-transferrin it is possible to determine both the stoichiometry of binding and the long-term stability of the metal–TF complexes.

For Th(IV) and Hf(IV), these saturation curves (Fig. 3) are relatively straightforward, showing a levelling-off at metal:TF ratios of 2, thus indicating that one mole of transferrin will bind, in a specific fashion, two moles of either Th(IV) or Hf(IV). In addition, binding appears to be rapid and the resulting complex stable for up to 2 days following preparation.

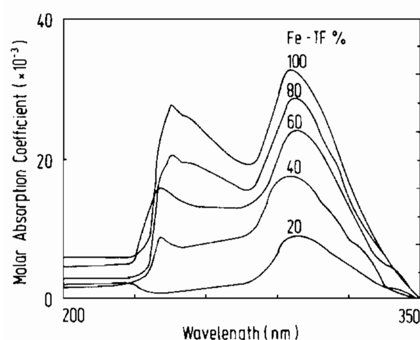


Fig. 1. Iron–transferrin difference spectra.

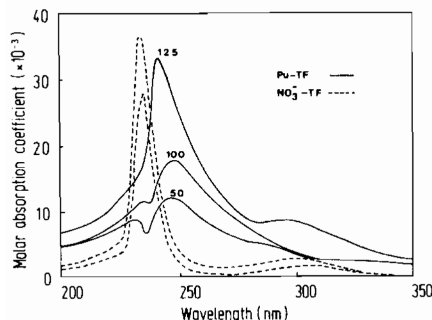


Fig. 2. Plutonium–transferrin difference spectra.

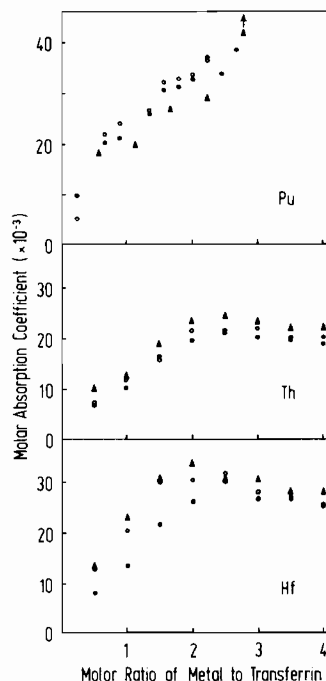


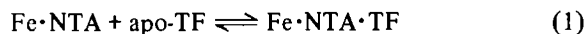
Fig. 3. Metal–TF saturation curves (●, 1h; ○, 1d; ▲, 2d).

For Pu(IV), the saturation curves are rather more complicated in appearance due to the non-specific nitrate binding mentioned above. However, the curves do show two inflection points at molar ratios of Pu(IV) to TF of 1 and 2, respectively, with absorbances being 'swamped' at ratios >2. It thus seems reasonable to ascribe the inflection points in the curves to binding of Pu(IV) at the two sites on TF. Furthermore, this binding is specific and the resulting complexes appear stable with time.

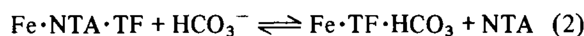
From the aforegoing, it can be seen that although the UV spectra of Fe(III)–TF differ in detail from those of Pu(IV)–, Th(IV)– and Hf(IV)–TF, binding of these three exogenous metal ions gives rise to similar stoichiometric characteristics in that 1 mole of TF binds 2 moles of metal ion in a specific fashion and, presumably, at the iron-binding sites.

Following this, the mechanism of Pu(IV) binding to TF was investigated as differences in the formation of Pu(IV)–TF and Fe(III)–TF could give rise to the anomalous *in vivo* behaviour of Pu(IV) described in the introduction.

It is known that the interaction of Fe(III)–NTA with apo-TF is biphasic and occurs via an intermediate state as shown below [7], where NTA is behaving synergistically.



$$\mathrm{Fe{\cdot}NTA + apo\text{-}TF \rightleftharpoons Fe{\cdot}NTA{\cdot}TF} \quad (1)$$

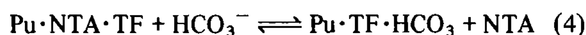


$$\mathrm{Fe{\cdot}NTA{\cdot}TF + HCO_3^- \rightleftharpoons Fe{\cdot}TF{\cdot}HCO_3 + NTA} \quad (2)$$

It was thus decided to investigate reactions 1 and 2 for PuNTA. The results are shown in Fig. 4. As can be seen, in the absence of bicarbonate a spectral peak is produced at a wavelength of ≈240 nm which is of relatively low absorbance and which shifts to lower wavelengths over a period of time up to 5 days post-preparation.

Incubation of a similar solution with added bicarbonate (to both sample and reference cells) leads to a spectrum with a much more pronounced absorbance which is stabilized with respect to time. These spectra can be explained in a similar fashion to the proposed mechanism of Fe(III)–NTA interaction with TF in that the reaction is biphasic:

$$\mathrm{Pu{\cdot}NTA + apo\text{-}TF \rightleftharpoons Pu{\cdot}NTA{\cdot}TF} \quad (3)$$



$$\mathrm{Pu{\cdot}NTA{\cdot}TF + HCO_3^- \rightleftharpoons Pu{\cdot}TF{\cdot}HCO_3 + NTA} \quad (4)$$

In conclusion, these preliminary investigations have shown that binding of Pu(IV) and its analogues to TF is similar both stoichiometrically and mechanistically to the binding of Fe(III).

The differences in metabolic behaviour between Fe(III) and the exogenous elements may thus be due to changes in protein conformation upon binding, an effect which has been previously noted for Fe–TF [8, 9]. Alternatively, the strength of binding to TF may be the critical factor. The influence of these parameters, and others likely to be of importance in the biological milieu, is currently under investigation.

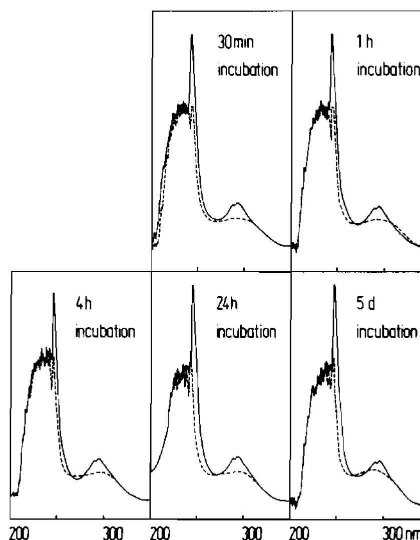


Fig. 4. Time-dependent spectra for the interaction of plutonium with TF. Legend: ———, plutonium + TF + NTA + bicarbonate; – – –, plutonium + TF + NTA.

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