Spectroscopic Studies on Titanium Ion Binding to the Apolactoferrin

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ABSTRACT

Titanium (Ti) is a relatively abundant element that has found growing applications in medical science and recently some of Ti compounds are introduced as anticancer drugs. In spite of very limited data which exist on the Ti metabolism, some proteins might be involved in the mechanism of action of Ti. Up to our knowledge, there is not any report in the literature concerning binding of Ti to apolactoferrin. Binding of apolactoferrin with Ti(IV)-citrate was studied by spectroflourimeterey and spectrophotometery techniques under physiological conditions. The spectroflourimeteric studies revealed a significant fluorescence quenching, that indicated binding of apolactoferrin with Ti(IV). The same reaction was monitored through spectrophotometry technique; this represents a characteristic UV difference band at 267 nm, which is different from lac-Fe (III). Titration studies show that lactoferrin specifically binds two moles Ti(IV) as complex with citrate per mol protein. Spectroflourimeterey and spectrophotometery techniques indicated that Ti(IV) ions cause a reduction (13%-14%) in binding of Fe(III) to lactoferrin. In overall, we may come to this conclusion that this element might be involved in the iron metabolism. *Iran. Biomed. J. 10 (2): 93-98, 2006*

Keywords: Titanium (Ti), Lactoferrin, Iron

INTRODUCTION

Titanium (Ti) is the second most abundant transition metal (ninth of all elements) on earth [1]. Consumption of this metal and the number of biochemical applications of Ti have been increased. Pure Ti and Ti alloy are widely used for orthopedic implants and dental implants [2]. Also, a series of Ti(IV) complexes have been shown to exhibit high anti-tumor activity against a wide range of murine and human tumors [3-5].

Lactoferrin is a bilobal glycoprotein that contains two homologous metal binding sites [6-8]. Lactoferrin is originally isolated from bovine milk, but since it has also been found at lower concentration in variety of physiological fluids such as seminal fluids, cervical mucus, nasal secretion and tears [8, 9]. Chemical modification, titration and spectroscopy studies on lactoferrin have shown that the amino acid involved in binding the Fe^{3+} are two tyrosine (Tyr), one histidine and one aspartic acid [6, 9-11]. Like serum transferrin, lactoferrin binds two equivalent of ferric ion with the concomitant binding of bicarbonate as synergistic anion. However, lactoferrin has a significantly greater binding affinity for ferric iron, which has led to the speculation that it may function as bacteriostatic agent by restricting the availability of this essential nutrient [9, 10, 12].

Since it has shown that some of Ti(IV) complexes (titanocene dichloride and budotitane) are anticancer drugs [3], so far it seems that transferrin group including lactoferrin may have an important role in carrying and storing Ti(IV) complexes and TI anticancer activity. In the other hand, lactoferrin as well as Fe^{3+}, binds to other metals such as Cu^{2+}, Co^{3+}, Cr^{3+}, Mn^{2+} and Al^{3+} [8, 9, 13, 14]. This ability of lactoferrin prompted us to study the binding of human apo-lactoferrin (apo-lac) with Ti(IV) complex using spectrophotometeric and spectroflourometric techniques. The interaction of these metals ions with each other on the binding to apo-lac was also investigated.

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MATERIALS AND METHODS

General. To avoid contamination by extraneous metal ions, all glassware were soaked in a solution of concentrated nitric acid and rinsed with distilled and deionized water before use. Plasticware was pre-washed with 10 mM EDTA and then washed with distilled and deionized water. Ti(IV)citrate was prepared by mixing Ti(III) chloride with a 1.2-fold excess of sodium citrate at pH 3 and then exposed to the air. This caused quantitative oxidation of Ti(III)citrate to colorless Ti(IV) citrate [15]. Human lactoferrin was purchased from Sigma Chemical Co. (Germany). The concentrations of apo-lac were determined by using its molecular weight (80,000 gmol $^{-1}$) and also by measuring the absorbance at 278 nm with the absorbance coefficient $\epsilon278\sim 107000M^{-1} cm^{-1}$ [9, 16]. All other chemical used in this project were analytical grade and purchased from Sigma Chemical Co. and also Merck (Germany).

All UV experiments were performed with a 1-cm cuvette on a computer controlled Shimadzu multispec-1501. The fluorescence intensity (F.I) of sample tubes was carried out with a Perkin-Elmer luminescence spectrophotometer LS -3B.

Spectroscopy studies. Apo-lac solutions were prepared by diluting aliquots of stock apo-lac solutions to ca. $1 \times 10^{-5}M$ with 50 mM Tris buffer pH 7.4. Immediately before the titration, sufficient NaHCo3 was added to produce a 20 mM $HCo3^{-}$ concentration [17]. For both spectrophotometric and

spectroflourometric experiments, 200 µl of an apo-lac solution (100 µM) was added to both sample and reference tube. Then, aliquots of 1.2 mM Ti(IV) citrate or Fe(III) citrate complex were the added. To achieve titration experiments aliquots of Ti or iron ions (5-50 µl) were added to sample tube, while equal volumes of distilled and deionized water were added to the reference tube. The solutions were then mixed and then the tubes were capped and left for up to 120 min at room temperature.

Metal ion interactions with each other for binding to apo-lac. The binding of iron to apo-lac and the effect of Ti(IV) on iron binding activity and vice versa were also investigated. Firstly, a solution of apo-lac (10 µM) in 50 mM Tris-HCl containing 20 mM $NaHCo_3$ at pH 7.4 was equilibrated with 2 equivalent of Ti(IV) citrate and then Fe^{3+} solution was added in 5-60 µl aliquots to the sample tube and the equal volumes of distilled and deionized water were added to the reference tube. Spectra were taken 2 h after each addition. Secondly, a similar titration was carried out for Fe-lactoferrin (2:1 molar ratio) using Ti(IV) citrate solution as a titrant.

RESULTS

Absorption studies. The spectra characterization of Ti(IV)-lactoferrin and iron-lactoferrin complexes was carried out by scanning in the range of 200-600 nm (Figs. 1 and 2). As shown in Figure 1,
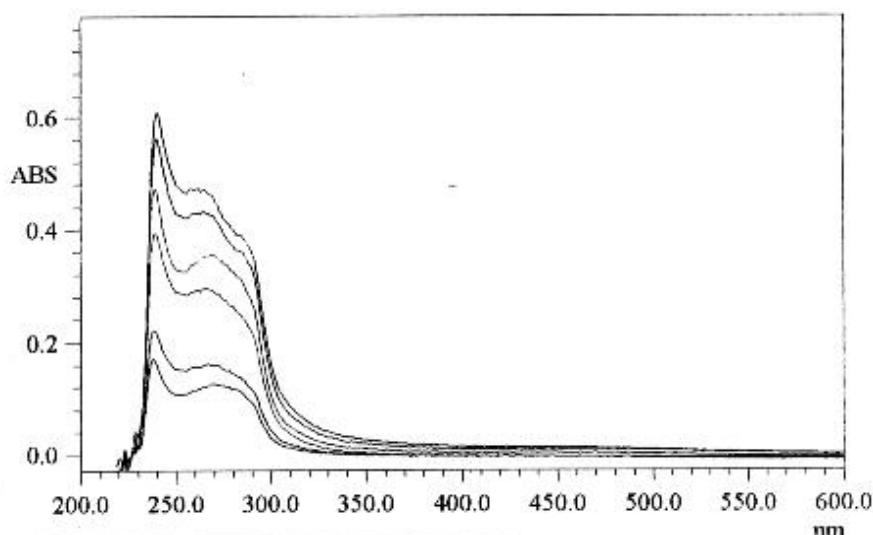

Fig. 1. Different spectra for titration of apolactoferrin (1×10^5 M) with Ti(IV)-citrate in 50 mM Tris-HCl buffer, 20 mM $NaHCo_3$, pH 7.4 at 298K. The molar ratios of Ti to lactoferrin from bottom to top are: 0.5, 1, 1.6, 2.13, 2.7 and 3.2.
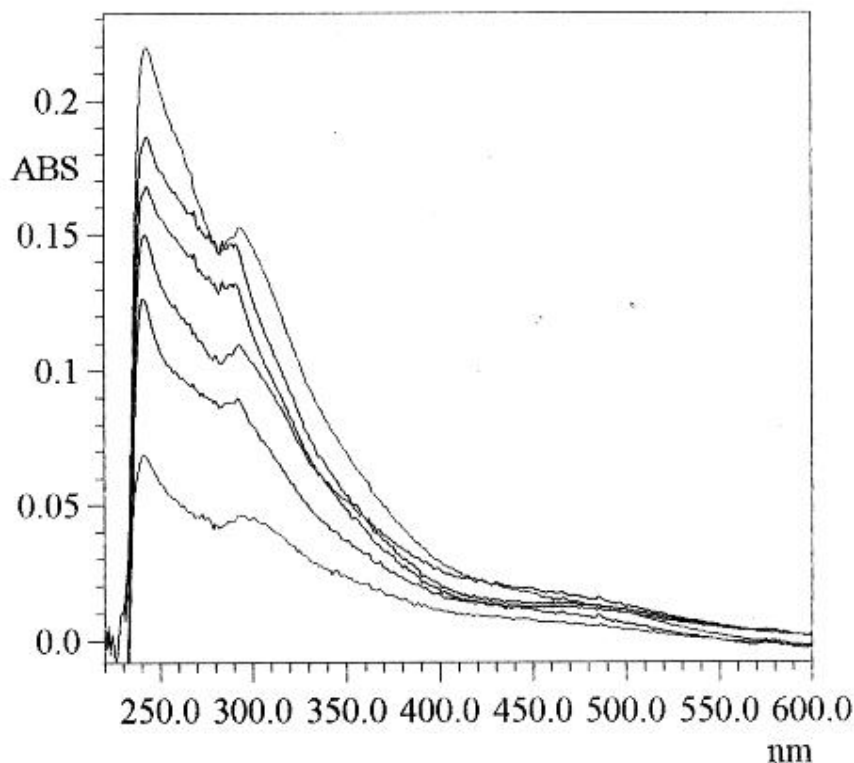
Fig. 2. A series of difference spectra for Fe(III)-citrate binding to apolactoferrin. The molar ratios of Iron to lactoferrin from bottom to top are: 0.5, 1, 1.6, 2.13, 2.7 and 3.2. Other conditions as in Figure 1.

after addition of Ti^{4+} to apo-lac, two absorbance bands appeared at 240 nm and 267 nm.

Ti(IV) titration.   Apo-lac was titrated with a solution of Ti(IV) citrate and difference UV spectra recorded after the addition of each aliquots of Ti (Fig. 1). The absorbencies data have been converted to absorptivities, $\Delta\varepsilon$, by dividing the absorbance maximum at the 240 nm by the total lactoferrin concentration. Titration curve is prepared by plotting $\Delta\varepsilon$ versus, r, the ratio of the Ti(IV) concentration to the total lactoferrin concentration as shown in Figure 3. The shape of the curve indicates that lactoferrin binds two moles Ti(IV) per mol protein, in good agreement with hypothesis that Ti(IV) ions occupy the empty metal binding sites of lactoferrin.

Fe(III) titration.   A similar titration was performed for Fe (III) citrate (Fig. 2). Values of $\Delta\varepsilon$ were calculated from the absorbance maximum at

465 nm and plotted as a function of r the ratio of total iron concentration to lactoferrin concentration as shown in Figure 4. Using Figure 4, it was found that each mole of apo-lac was saturated with 2 mole of Fe(III).
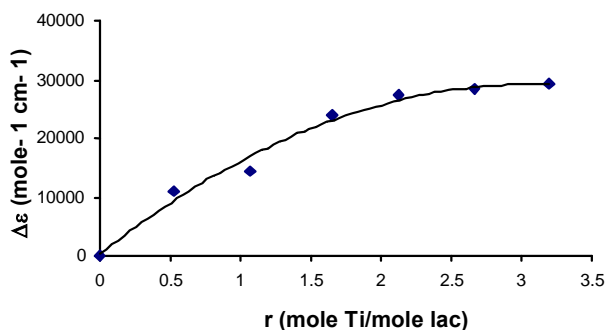

Fig. 3.   Titration curve for additions of Ti(IV)-citrate to apolactoferrin ; $\Delta\varepsilon$ equals the absorbance at 240 nm dividing by the apolactoferrin concentration, other conditions as in Figure 1.
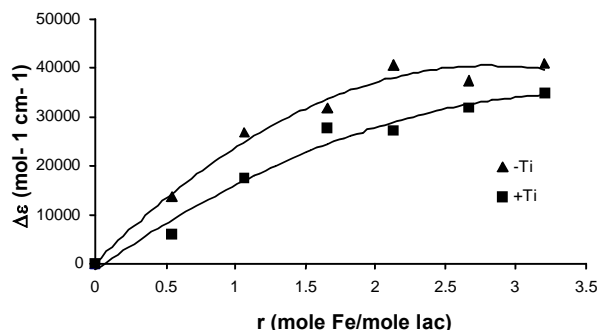
Fig. 4. Titration curve for addition of different concentrations of Fe(III)-citrate(0-36 μm) to apolactoferrin in the absence (▲) and presence (■) of titanium (24 μm). [Apo-lac] = 10 μm, in 50 mM Tris-HCl buffer, 20 mM $NaHCo_3$, pH 7.4 at 298 K.

Metal interaction. The lac-Fe(III) absorption band in the visible region at 465 nm was used to monitor the competitive binding of Fe(III) and Ti(IV) to apo-lac. According to the previously reported, the UV region was not used because the phenolic groups of Tyr side chains and therefore similar absorbance changes occur in this region for both ions [18]. When Ti(IV) ion was added to lac-Fe(III) solution , the absorbance of lac-Fe(III) at 465nm was reduced by approximately 13-14% (Fig. 4). In another experiment, the presence of the Fe(III)ion in the titration of apo-lac by Ti(IV), the change of absorbencies of lac-Ti(IV) solutions at 465 nm were very slightly (Fig. 5). Therefore both sets of results indicate that Ti(IV) can not displace Fe(III) completely but Fe(III) can easily replace Ti(IV).
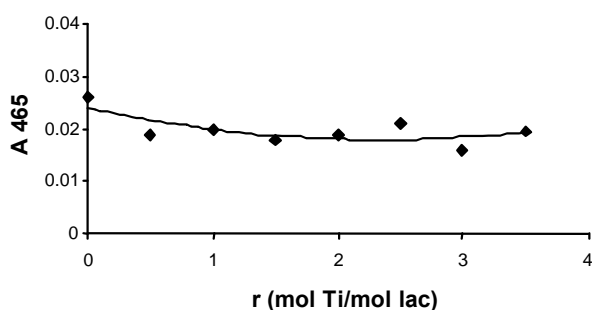

Fig. 5. Titration of apolactoferrin with addition of Ti(IV)-citrate in the presence of Fe(III), conditions as in Figure 1.

Fluorescence spectra. Figure 6 presents the Fluorescence emission spectra of apo-lac in the absence and present of iron or Ti(IV) complex in 50 mM Tris-HCl buffer containing 20 mM $NaHCO_3$ at pH 7.4 and excited at 290 nm.

The intensity of fluorescence was decreased by 65% when apo-lac was conjugated with iron citrate. The addition of Ti citrate to apo-lac caused a reduced of λ emission by approximately 30%.
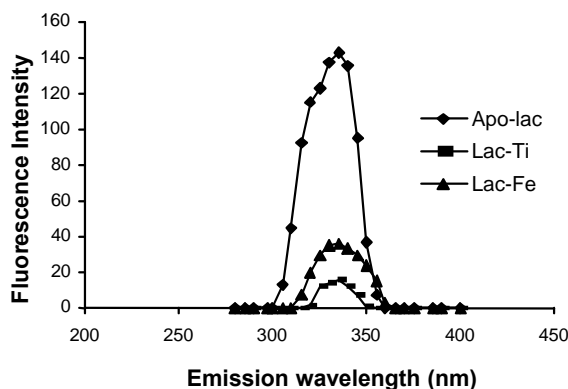

Fig. 6. Fluorescence emission spectra of apo-lac in the absence (♦) and in the presence of iron (■) and titanium (▲). Samples were excited at 290 nm. [Apo-lac] = 10μm, [Fe³⁺] = [Ti⁴⁺] = 25 μm in 50 mM Tris-HCl buffer, 20 mM $NaHCo_3$, pH 7.4 at 298K.

Intrinsic protein fluorescence is mainly due to Tyr and tryptophane (Trp) residues. Excitation of protein at 280 nm stimulates fluorescence of both Tyr and Trp residues. Since non-energy transfer occurs from Tyr residues to Trp residues the transferrins fluorescence peak around 330 nm attributed to Trp residue [19]. This F.I quenched upon addition of iron and/or Ti to apo-lac. Fluorescence titration of apo-lac by addition of iron or Ti was accomplished. In the plots, F.I at 335 nm vs.r (Figs. 7 and 8) was observed that a non-linear decrease in the protein fluorescence. This means that both Ti and iron bind to the same site.
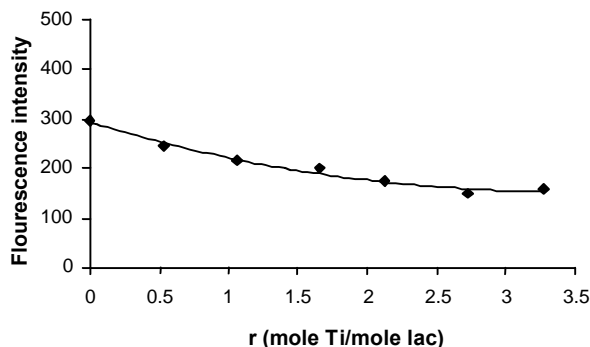

Fig. 7. Fluorescence titration for Ti(IV)-citrate binding to apolactoferrin. The decrease in fluorescence at 335 nm is plotted vs. the [Ti(IV)/apo-lac] ratio, under the same conditions as in Figure 1.
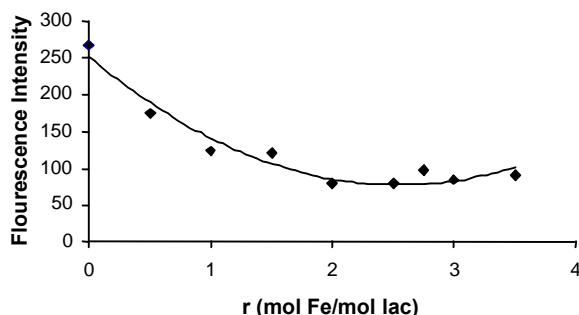
Fig. 8. Fluorescence titration of apo-lac with different concentrations of iron(0-36 μm) [Apo-lac] = 10 μm, in 50 mM Tris-HCl buffer, 20 mM NaHCO$_3$, pH 7.4 at 298K, λ_{ex} = 280 nm, λ_{em} = 335 nm.

DISCUSSION

Proteins of the transferrin family including serum transferrin, lactoferrin, ovotransferrin and melanotransferrin have the characteristic ability to bind tightly but reversibly two Fe^{3+} ions. Many other metal ions can be bound in place of Fe^{3+} not only transition metals, such as Cr, Mn, Co, and Cu, but also Al, Ga and large cations such as Lanthanides and actinides [20-22]. This binding ability is of potential importance for medical purposes and for metabolic fate of heavy metals ingested from environment. While there is a little report that Ti ions bind to the serum protein transferrin [3, 15], there is not any report about binding of Ti to apo-lac.

In the present study, we considered the reaction of apo-lac with Ti(IV) complex under physiologic conditions. Thus by spectrophotometery and spectrofluorometry, we have shown that Ti(IV) is taken up into the two specific iron sites of apo-lac. UV difference spectra obtained from lac-Ti(IV) complex revealed characteristic UV difference at 240 nm and 267 nm. Binding of metal ions to phenolic oxygen of the Tyr residue in the specific metal-binding sites of apo-lac perturbs the π-π* transition of aromatic rings and leads to the production of two new absorption near 241 and 295 nm [3, 23, 24].

These new bands are rapidly in different UV spectra of metal-lac specific binding by some metal ions. This provides a convenient way to detect specific metal- lac or other transferring binding and release by uv-vis spectroscopy [23, 24].

The titration curve obtained by monitoring the absorbance change at 240 nm (Fig. 3) show a break

near the ratio of Ti/lac of 2:1 that suggest Ti(IV) binds to specific metal-binding sites in both the N-lobe and C-lobe. Moreover the displacement of Ti(IV) from lactoferrin by Fe(III) provides further evidence for specific binding of Ti(IV) to the two lobes of protein [18].

As shown in Figure 7, the F.I of apo-lac at 335 nm, decreased with the addition of aliquots of iron (III)-citrate. The concomitant quenching of intrinsic protein fluorescence of transferrins upon binding iron was first reported by Lehrer [25] and further investigated by Evans and Holbrook [26]. The decrease of F.I of lactoferrin-metal complex is due to the displacement of protons of Tyr residues in binding sites or conformational change of protein after metal binding [19].

Figure 7 shows that the binding of iron to apo-lac quenches the fluorescence of protein. The data on the fluorescence quenching intensity obtained by us for lac-Ti(IV) provides for the interaction between these components, but as shown in Figure 8 Ti(IV)lac has lower florescence quenching when compared with Fe(III)-citrate. Therefore it seems that Ti(IV)-citrate bind to apo-lac, but binding of iron to apo-lac is more tightly than Ti.

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